

**TARGETING BONE MARROW MICROENVIRONMENT TO
ERADICATE THERAPY-RESISTANT STEM CELLS**

ESTHER SATHYA BAMA B

PhD THESIS

2023



**SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL
SCIENCE AND TECHNOLOGY, THIRUVANANTHAPURAM**

An Institution of National Importance established by an Act of the
Indian Parliament (Act No.52 of 1980)

Dept. of Science and Technology, Govt. of India
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DECLARATION BY THE STUDENT

CERTIFICATE

I, **Esther Sathya Bama B** hereby certify that I had personally carried out the work depicted in the thesis titled, “**Targeting bone marrow microenvironment to eradicate therapy-resistant stem cells**”. No part of this thesis has been submitted for the award of any other degree or diploma prior to this date.

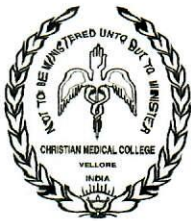
A handwritten signature in blue ink, appearing to read 'Esther B', is enclosed within a rectangular box.

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The thesis entitled, "**Targeting bone marrow microenvironment to eradicate therapy-resistant stem cells**" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

*Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

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ERADICATE THERAPY-RESISTANT STEM CELLS**

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DEDICATION

With heartfelt gratitude, I dedicate this doctoral thesis to my beloved Appu. Your lessons in dreaming without bounds have illuminated my path and emboldened my aspirations.

To my dearest Ammu, you have been the unwavering pillar by my side, offering relentless support and guidance. Your strength has lifted me through challenges, and your presence has shaped me into the person I stand as today.

And to the God almighty, you remained a steadfast shadow during moments of uncertainty, making the seemingly impossible not only possible but a reality beyond my dreams.

ACKNOWLEDGEMENT

“You make beautiful things out of the dust.”

I want to thank my entire family for their unwavering support, patience, and prayers as they made this accomplishment possible. The Divine Power has been a guiding force throughout my life, and I am grateful for it.

I cannot express my gratitude to my supervisor, **Dr. Poonkuzhali Balasubramanian**, who constantly guided, encouraged, and assisted me. Her mentorship has greatly benefited me and has played a vital role in my academic growth and development. I am grateful to her for providing systematic guidance, putting in great effort to train me in scientific research and publications, and preparing this thesis. She always assisted and helped me with her advice and suggestions. Her continuous motivation and remarks improved my scientific work and documentation skills. I thank her for the extensive amount of time dedicated to engaging in discussions, evaluating drafts, and offering valuable feedback, all of which have greatly enhanced the standard of this thesis.

I express my gratitude to the members of my Doctoral Advisory Committee for providing practical advice regarding the study. **Dr. Shaji Velayudhan** offered invaluable support and guidance in improving the quality of research work and publications. He has motivated me during the hard times with his commendable knowledge and enthusiasm for research.

I would like to extend my sincere appreciation to **Dr. Vikram Mathews** for his invaluable contributions as a member of my DAC (Dissertation Advisory Committee). **Dr. Mathew's**

thought-provoking questions and insightful comments during our meetings have been instrumental in shaping the direction and quality of my research. I acknowledge the privilege of pursuing my doctoral studies at the Department of Haematology, Christian Medical College and the Sree Chitra Tirunal Institute for Medical Sciences and Technology in Thiruvananthapuram, India. I thank **Dr.Santhosh Kumar**, Registrar and the academic section and administration staff for their help and support throughout the course. Specifically,I would like to extend my thanks to **Dr.Santhosh Kumar**, Registrar for his opportunity.

I would like to express my profound gratitude to **Dr. Aby Abraham**, the Head of the Department, for his unwavering support throughout my PhD journey. His guidance, mentorship, and encouragement have been invaluable in shaping my academic and research experiences.

I wish to extend my profound gratitude to **Dr. Elizabeth Vinod**, whose unwavering guidance and support have illuminated our path in the realm of Mesenchymal Stem Cells (MSCs). Her contributions have been invaluable, and I am deeply appreciative of her continuous support in advancing our MSCs research endeavors.ge have greatly enriched my understanding of this complex area of study.

I extend my heartfelt thanks to **Dr. Bharathi Rajamani** and **Mr. Stallon Illangeeshwaran** for their unwavering support and guidance throughout my doctoral journey.

I convey my sincere love and appreciation to **Dr Nithya Balasundaram** for being my most inspiring and good companion. Her presence in my life has been a true blessing, and I am sincerely thankful for the incredible journey we have shared together.

My heartfelt thanks to my friends, **Mr Aswin Anand Pai** and **Mr Gaurav Joshi** whose presence, support and friendship made the journey smoother and enjoyable. I express my sincere gratitude for their support and motivation during my most challenging time, both in life and experimental work. Their presence and encouraging words have been a driving force of strength and kept my passion alive. There is a saying when the going is tough, the tough gets going. It has proven in my case, as they stood by my side when things got tough. I acknowledge them for the criticism and invaluable contributions made along the journey.

My heartfelt gratitude goes to my good friends **Sreenithi** and **Aruna** for their continuous support, friendship, and companionship during my journey. They have been a pillar of strength in both good and bad times, lending a listening ear, a helping hand, and words of encouragement when I needed them the most. Their generosity, commitment, and understanding have enhanced my life.

I would like to convey my deepest gratitude to **Sangeetha**, our exceptionally skilled and dedicated technician. I am truly fortunate to have Sangeetha as a part of our research endeavor. Her contributions have been invaluable, and I am sincerely thankful for her unwavering support and exceptional work ethic.

I would like to express my heartfelt thanks to CSCR flow cytometry and imaging facility. Specifically, **Saranya, Abdul,** and **Dr. Sandhya** for their invaluable support and expertise in the field of flow and confocal imaging. Their contributions have been instrumental and I am sincerely appreciative of their assistance with flow and confocal imaging.

I am deeply appreciative of the support and expertise that **Ganesh** and **Dr. Soosai** have provided in our MSCs research.

I would like to extend my sincere thanks to **Ashok** and **Esther** for their invaluable contributions to the animal facility.

I want to express my heartfelt gratitude to my esteemed colleagues, **Dr. Balaji Balakrishnan, Dr. Hamenth, Arvind, Rosy, Ranita De, Reeshma, Swathi, Abirami, Shruti Aparna, Sweety, Rakshini, and Jothi.** Their collective support and contributions have been instrumental in making our work environment not only productive but also enjoyable.

I extend my sincere thanks to **Senthamarai Selvi** for her invaluable support in the realm of real-time PCR.

I thank **Shareen, Sathya Amritha, Bhuvana,** and **Manoj** for their invaluable contributions and support.

I extend my sincere thanks to **Mr Sam Prabakar, Mrs Chudar Kodi, Mr. Radhakrishnan** and **Ms Rekha** for their tireless efforts in facilitating the reagent ordering and acquisition process. Their dedication and attention to detail greatly

contributed to the smooth progression of our experiments. I extend my appreciation to **Mr Christopher** for his pivotal role in patient enrollment and providing us with the apheresis samples. Lastly, I would like to express my gratitude to **Ms Revathi** for her invaluable assistance with administrative work.

I am truly fortunate to have had the opportunity to work alongside such dedicated and supportive individuals. I would like to express my gratitude to the CMC transport facility and Security for their invaluable assistance in supporting my research work.

I thank housekeeping staff team for their extraordinary dedication and hard work.

I am grateful to my acquaintances and teachers on and off campus, whose assistance has been invaluable and deserves recognition. I would like to extend my heartfelt appreciation to my fellow students from other departments within our campus for their valuable support and cooperation. I thank the administrative department, accounts department, stores and purchase division, and library personnel for their invaluable assistance.

Lastly, I extend my heartfelt appreciation to the participants of my study who selflessly dedicated their time and shared their valuable experiences with me. Their contribution has been instrumental in shaping the outcome of my research. Without their willingness to participate, this study would not have been possible, and I am forever indebted to them.

I thank **Anand** and **Chitra** for making me feel at home. I also convey my thankfulness to **Mrs Rani, Mr Santhakumar, Geetha and Kishore** for their support and kindness. I'm more privileged to have you **Dr. Issac Nelson** as my source of support and guidance. No

words can express how much I'm grateful to have you as my brother. I can't thank you but feeling wonderful to have your unwavering support, companionship and source of motivation whenever it needs the most.

I would like to express my deepest gratitude to beloved brothers **Pinku** and **Chweetu** for their unwavering support and steadfast companionship. Their presence has been a source of strength and encouragement throughout my journey, has been a motivating force, pushing me to strive for excellence. I am truly fortunate to have Mrs Soushana Immanuel as a sister-in-law, and I sincerely appreciate her support and guidance have made a significant impact, and I am deeply grateful for her presence in my life.

Finally, It is my deepest gratitude and heartwarming acknowledgement to my beloved **Mother** (Ammu) and **Father** (Appu) for orchestrating my life and educational journey. Their love, guidance, and sacrifices have paved the way for my success. Their unwavering belief in me and their relentless support have been the cornerstones of my achievements. Their contributions have been immeasurable, and I am sincerely thankful for their pivotal roles in shaping my life and educational endeavors. It made me what I'm today.

In conclusion, I wholeheartedly thank everyone who has contributed to my academic and personal growth. If any names are missed, please accept my heartfelt gratitude and appreciation. Your support and encouragement have been invaluable, and I will always cherish it.

ESTHER SATHYA BAMA

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LIST OF ABBREVIATIONS

ACAN	Aggrecan
ACTB	Actin Beta
ADAR1	Adenosine Deaminase RNA Specific
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AML	Acute Myeloid Leukemia
AMPK	adenosine monophosphate-Activated Protein Kinase
AP	Accelerated Phase
APC	Allophycocyanin
ATP	Adenosine Triphosphate
AZU1	Azurocidin 1
BC	Blast Crisis
BFP	Blue Fluorescent Protein
BM	Bone Marrow
BMM	Bone Marrow Microenvironment
BMP	Bone Morphogenetic Protein
BMP	Bone Morphogenetic Protein
CBP	CREB-Binding Protein
CDH2	Cadherin 2
CDH4	Cadherin 4
cDNA	Complementary DNA
CHR	Complete Hematological Response
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CML	Chronic Myeloid Leukemia
CP	Chronic Phase
CREB	cAMP Response Element-Binding Protein
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
CTSG	Cathepsin G
CXCL12	C-X-C Motif Chemokine Ligand 12
CXCL12-CXCR4 axis	Chemokine CXCL12-CXC Chemokine Receptor 4 axis
CXCL13	C-X-C Ligand 13
CXCR4	C-X-C Chemokine Receptor Type 4
ddPCR	Droplet Digital Polymerase Chain Reaction
DYRK2 XX	Dual Specificity Tyrosine Phosphorylation Regulated Kinase 2
DZMS	German Collection of Microorganisms and Cell Cultures

ECs	Endothelial Cells
EED	Embryonic Ectoderm Development
ELN	European Leukemia Net
ELOVL15	ELOVL Fatty Acid Elongase 15
EPAS1	Endothelial PAS Domain Protein 1
ESCs	Embryonic Stem Cells
ETC	Electron Transport Chain
ETC	Electron Transport Chain
EZH2	Enhancer of Zeste Homolog 2
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FDR	False Discovery Rate
FISH	Fluorescence In-situ Hybridization
FOXO3A	Forkhead Box O3A
Gal-3	Galectin-3
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
Gas6	Growth Arrest-Specific Gene 6
G-CSF	Granulocyte-Colony Stimulating Factor
GEO ID	Gene Expression Omnibus Identifier
GO	Gene Ontology
GSTM1	Glutathione S-transferase mu 1
GUS	β -Glucuronidase
GUSB	Beta-Glucuronidase
HIF-1 α	Hypoxia-Inducible Factor 1 Alpha
HISAT2	Hierarchical Indexing for Spliced Alignment of Transcripts 2
HIST14C	Histone proteins
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic Stem Cells
HSPCs	Hematopoietic Stem and Progenitor Cells
hTERT	Human Telomerase Reverse Transcriptase
huKO+	Human Kusabira Orange
iCML MSC	Immortalized Chronic Myeloid Leukemia Mesenchymal Stromal Cells
IGFBP6	Insulin-Like Growth Factor-Binding Protein 6
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-2RA	Interleukin-2 Receptor Alpha Chain
IL-3	Interleukin-3
IL-6	Interleukin-6
IM	Imatinib Mesylate

iPSC	Induced Pluripotent Stem Cell
iPSCs	Induced Pluripotent Stem Cells
IRB	Institutional Review Board
ITGA3	Integrin Subunit Alpha 3
JAK	Janus Kinase
KIR	Killer Cell Immunoglobulin-Like Receptor
LSCs	Leukemia Stem Cells
LTF	Lactotransferrin
MHC	Major Histocompatibility Complex
miRNA MicroRNA	
MMR	Major Molecular Response
MNCs	Mononuclear Cells
mPB CD34+	Mobilized Peripheral Blood CD34+
MSC	Mesenchymal Stromal Cells
MSTNpp	Myostatin Propeptide
MTDH	Metadherin
MYC	MYC Proto-Oncogene
NCCN	National Comprehensive Cancer Network
NFAT	Nuclear Factor of Activated T Cells
NGS	Next-Generation Sequencing
NK cells	Natural Killer Cells
NSG	NOD scid gamma
OXPHOS	Oxidative Phosphorylation
OxPHOS	Oxidative Phosphorylation
P3	Proteinase 3
PB	Peripheral Blood
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pDCs	Plasmacytoid Dendritic Cells
PE	Phycoerythrin
PGC-1 α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
Ph	Philadelphia Chromosome
PI3K/AKT	Phosphatidylinositol 3-Kinase/Protein Kinase B
PIGF	Placental Growth Factor
PKM2	Pyruvate Kinase M2
PML	Promyelocytic Leukemia Protein
PPARG	Peroxisome Proliferator-Activated Receptor Gamma
PPARGC1B	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Beta
PRC2	Polycomb Repressive Complex 2

PRMD16	Protein Arginine Methyltransferase 16
PTN P	Pleiotrophin
QPCR	Quantitative Reverse-Transcription PCR
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPKM	Reads Per Kilobase Million
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RUNX2	Runt-Related Transcription Factor 2
SIRT1	Sirtuin 1
SIRT1	Sirtuin 1
STAT3/5	Signal Transducer and Activator of Transcription 3/5
STAT5	Signal Transducer and Activator of Transcription 5
TCA	Tricarboxylic Acid
TET2	Ten-Eleven Translocation 2
TFR	Treatment-Free Remission
TIMP1	Tissue Inhibitor of Metalloproteinase 1
TIMP-2	Tissue Inhibitor of Metalloproteinase 2
TKI	Tyrosine Kinase Inhibitor
TNF α	Tumor Necrosis Factor Alpha
TP53	Tumor Protein 53
TRA-1-60	T cell receptor alpha locus-1-60
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen 4

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1. INTRODUCTION

In 1960, Nowell and Hungerford made a groundbreaking revelation in blood samples obtained from individuals affected with chronic myeloid leukemia (CML), uncovering a tiny chromosome known as a minute chromosome (Peter C. Nowell and David A. Hungerford, 1960). This pivotal discovery marked a fresh era in hematology and triggered the current emphasis on molecular medicine. This genetic anomaly was subsequently identified as a balanced translocation of chromosomes 9 and 22 $t(9;22)(q34;q11)$, resulting in the fusion of Abelson tyrosine-protein kinase 1 (ABL1) and breakpoint cluster region protein (BCR). This fusion triggered constitutive tyrosine kinase activity, leading to the abnormal accumulation of granulocytes (Deininger et al., 2000; Nowicki et al., 2003).

Efforts to improve the quality of life for CML patients began with arsenicals in the late 1800s and treatments like radiation therapy, anti-leukocyte sera, and benzene in the early 1900s (Hehlmann et al., 1993). In 1953, Busulfan was introduced and showed promising results, which was then replaced by hydroxycarbamide due to safety concerns in the 1960s (Galton, 1953). Interferon alpha (IFN- α) gained popularity in the mid-1980s, with studies in the 1990s demonstrating its survival benefits (Allan et al., 1995). Imatinib replaced IFN- α as the preferred treatment in 2001, followed by the development of next-generation tyrosine kinase inhibitors (TKIs) (Druker et al., 1996, 2001). TKIs have radically changed the treatment paradigm of CML for most patients, turning it from a fatal to a chronic disorder (Druker et al., 2001). Currently, CML patients who receive proper TKI treatment, undergo adequate monitoring and display favorable treatment responses can anticipate an existence that closely resembles normal life expectancy.

Despite their promising results, TKI-based therapies do not eradicate leukemic stem cells (LSC) and have significant drug toxicities (Soverini et al., 2019).

Risks of prolonged TKI treatment include noncompliance, associated with the risk of relapse and progression, toxicities including serious vascular complications and teratogenic effects, precluding pregnancy while on treatment (Kumar and Krause, 2021a). A significant area of research in CML is focused on targeting the quiescent and self-renewing LSCs implicated in refractoriness and disease progression. These LSCs can persist even when no measurable residual disease is present, and upon changes in the bone marrow (BM) microenvironment, they can exit quiescence and drive relapse, representing a bottleneck to cure. Some survival mechanisms of LSCs involve altering the BM microenvironment, transitioning from the quiescent state to the proliferative state through intracellular signaling changes and the secretion of cytokines (Camacho et al., 2017; Jin et al., 2008; Manshouri et al., 2011; Zhang et al., 2013, 2019; Zhao et al., 2009, 2022). These changes can disrupt metabolic processes, creating an anti-apoptotic microenvironment and dysregulated immune system (Brück et al., 2018; Kuntz et al., 2017; Zhao et al., 2022).

Various strategies for combinatorial therapies to target and eradicate CML stem cells are being tested. These strategies include (a) targeting specific LSC surface markers (CD26, CD25, IL1-RAP), (Herrmann et al., 2014; Landberg et al., 2016; Sadovnik et al., 2017) (b) modulation of signaling pathways (i.e., Wnt or Hh) (Zhang et al., 2013; Zhao et al., 2009) to reverse LSC quiescence by interfering with the activity of transcription factors (i.e., FoxO, BCL6, or PML) (Ito et al., 2008; Lagadinou et al., 2013), (c) interaction with the bone marrow microenvironment, (d) inhibition of drug-efflux pumps

(e.g., ABC transporters), (e) targeting epigenetic factors distinguishing normal and LSCs (Abraham et al., 2019; Li et al., 2012). Despite an improved understanding of LSC biology, they remain elusive and difficult to target. Not all LSCs can be characterized by surface markers, and the LSCs are heterogeneous in comprehending the mechanisms and pathways that lead to the survival and resistance of CML LSCs, necessitating further research for new drugs and treatment options.

The interplay between CML LSCs and the BMM plays a significant role in malignant transformation (Colmone et al., 2008; Zhang et al., 2012). The specific mechanisms and molecules involved in this communication are not yet fully understood. Although potentially druggable targets have been identified in CML to eradicate LSCs, drugs against these targets have not yet been implemented in the clinic as a standard of care. These limitations highlight the need for further research to deepen our understanding of CML LSCs and develop more effective treatments for CML aiming toward treatment-free remission.

1.1 Rationale and Hypothesis

1. Genome-wide transcriptome analysis of CML and normal stem cells will enable us to identify novel targets for eradicating CML LSCs.
2. Hypoxic microenvironment-associated miRNA induced in CML stem cells could provide a survival advantage for CML LSCs
3. Mesenchymal stromal cells derived from CML patients will differ from those of healthy donors. The generation of a CML MSC line will be of great advantage in studying the leukemia-stromal cell interaction.

4. CML LSCs are rare, making it difficult to study the survival mechanisms. CML patient-derived iPSCs will be an ideal model for drug and CRISPR library screening, as it can retain the patient's pathophysiology without altering the genetic makeup.

1.2 Objectives

1. To elucidate the survival mechanisms of CML-LSCs by
 - Transcriptomic profiling of CML CD34+ cells to study the differentially expressed genes between CML LSCs and normal hematopoietic stem cells.
 - To study the role of hypoxia-associated miRNAs in CML LSCs.
2. Models to study LSCs by
 - Generation and characterization of immortalized CML MSC lines and assessing their roles in maintaining LSCs
 - Generation and characterization of iPSCs from primary CML cells for modeling CML LSCs

1.3 Brief overview of the thesis chapters

1.3.1. Literature review

Chapter 2 focuses on literature review, which elaborates on the CML, its pathology and molecular mechanisms, and the treatment options. The literature review highlights the evolving understanding of LSC biology, including genetic and epigenetic alterations, signaling pathways, and microenvironment interactions. It emphasizes the significance of LSC-targeted therapies, especially tyrosine kinase inhibitors, in managing CML. Despite progress, challenges in LSC isolation and persistence remain. Ultimately, the

review underscores the need for continued research to advance personalized treatments targeting CML LSCs to enhance patient outcomes and eradicate the disease.

1.3.2. Materials and Methods

This section describes the various techniques and methods used in the experiments and analyzing data. The study design and methods utilized for each experiment, methods used to assess molecular modulation by knockdown and knockout assays, and protein and mRNA expression techniques are described here. Detailed protocols describing the establishment of an immortalized MSC line and the generation of CML iPSCs are also described in this section.

1.3.3. Results and Discussion

The results section is divided each objective-wise, as listed above. The first part focuses on the differential gene expression between CML CD34⁺ cells and normal CD34⁺ cells. It also highlights the significance of CTSG in maintaining CML LSCs and its impact on LSC survival. In the second part, the emphasis is on the role of hypoxia-associated miRNAs and their effects on stemness. The third part highlights the importance of CML MSCs and the creation of an immortalized MSC line for studying BMM interaction. Lastly, the fourth part describes the generation of CML iPSCs for drug screening.

1.3.4. Summary, Conclusion, and Bibliography

This chapter comprehensively summarises the observations from this doctoral research and outlines the ongoing research and future directions to expand understanding of this disease. The references cited are listed in the Bibliography

2. LITERATURE REVIEW

2.1. Hematopoiesis

The hematopoiesis process involves the precise regulation of hematopoietic stem cells (HSCs) to generate fully developed blood cells, categorized into either myeloid or lymphoid lineages. This intricate differentiation of HSCs into specific lineages is a continuous journey encompassing a sequence of progressively committed progenitor stages (**Figure 2.1**). These distinct progenitors traverse separate pathways, culminating in the production of diverse mature blood cells. The instructive model postulates that HSCs react to external cues, particularly cytokines, which actively prompt them to embark on a differentiation pathway of a particular lineage. This model implies a directed and responsive process where specific signals dictate the fate of HSCs, guiding them toward becoming specialized blood cells. The stochastic model presents an alternative perspective. According to this theory, the commitment of HSCs arises from spontaneous stochastic variations in cell phenotype. These intrinsic fluctuations are then followed by a selective step driven by cytokines. In this scenario, the selection process refines the initial stochastic variations, determining the eventual lineage of the differentiated blood cell (Dussiau et al., 2022; Olson et al., 2020).

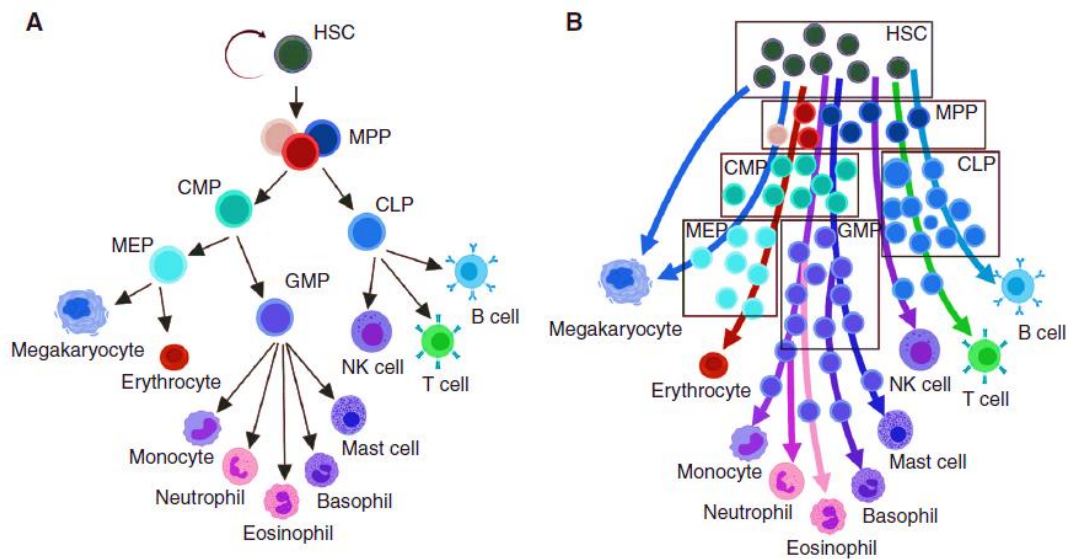


Figure 2.1: Models of hematopoietic stem cell (HSC) lineage commitment. (A) Classical tree-like model (B) The continuous differentiation model. Boxes represent progenitor states based on phenotypic markers and illustrate the heterogeneity within each population. MPP-multipotent progenitor; CMP-common myeloid progenitor; CLP-common lymphoid progenitor; MEP-megakaryocyte-erythroid progenitor; GMP-granulocyte-macrophage progenitor (Olson et al., 2020).

HSCs are well regulated by intrinsic and extrinsic mechanisms. Disruptions to this regulatory mechanism, either through single or multiple driver mutations, can lead to malignant transformation.

2.2. Origins of Leukemia

A hierarchical organization exists in normal, self-renewing tissues, featuring a specific group of stem cells responsible for maintaining tissue integrity throughout an individual's lifetime. Studies have identified cells with stem cell properties in various types of cancer, known as cancer stem cells (CSCs) (Cho and Clarke, 2008; Dick, 2008; Reya et al., 2001; Trumpp and Wiestler, 2008).

The initial evidence supporting cancer stem cells originated from investigations in leukemia research. Earlier studies showed a subset of leukemic cells displayed

clonogenic activity *in vitro* and demonstrated the ability to initiate the disease in mice upon transplantation (Bonnet and Dick, 1997; Lapidot et al., 1994; Wang and Dick, 2005). Subsequently, these cells were termed leukemic stem cells (LSCs). Interestingly, the cell surface marker phenotype of these LSCs was identical to that of HSCs.

LSCs are leukemia-initiating cells and, through serial transplantation, can propagate leukemia, recapitulating the primary tumor and its heterogeneity in the recipient mouse. Additionally, LSCs can arise from transformed HSCs/progenitor cells that regain the self-renewal capacity (Eppert et al., 2011; Goardon et al., 2011; Jamieson et al., 2004; Wang et al., 2010). Leukemic cells can remodel the bone marrow microenvironment to support their maintenance and activity (Hou et al., 2020; Roecklein and Torok-Storb, 1995; Zhang et al., 2013)

LSCs have displayed significant resistance to the majority of existing treatment approaches. Traditional therapies such as irradiation and cytotoxic drugs primarily target rapidly dividing cells, rendering them ineffective against the mostly quiescent LSC population. Furthermore, LSCs have exhibited resistance not only to irradiation and chemotherapy but also to targeted treatments like immunotherapy and tyrosine kinase inhibitors (Corbin et al., 2011a; Goff et al., 2013; Lagadinou et al., 2013; Sykes et al., 2011). Consequently, LSCs are believed to be the main contributors to a significant proportion of treatment failures and instances of relapse. As a result, focusing on targeting LSCs holds paramount importance from a clinical perspective.

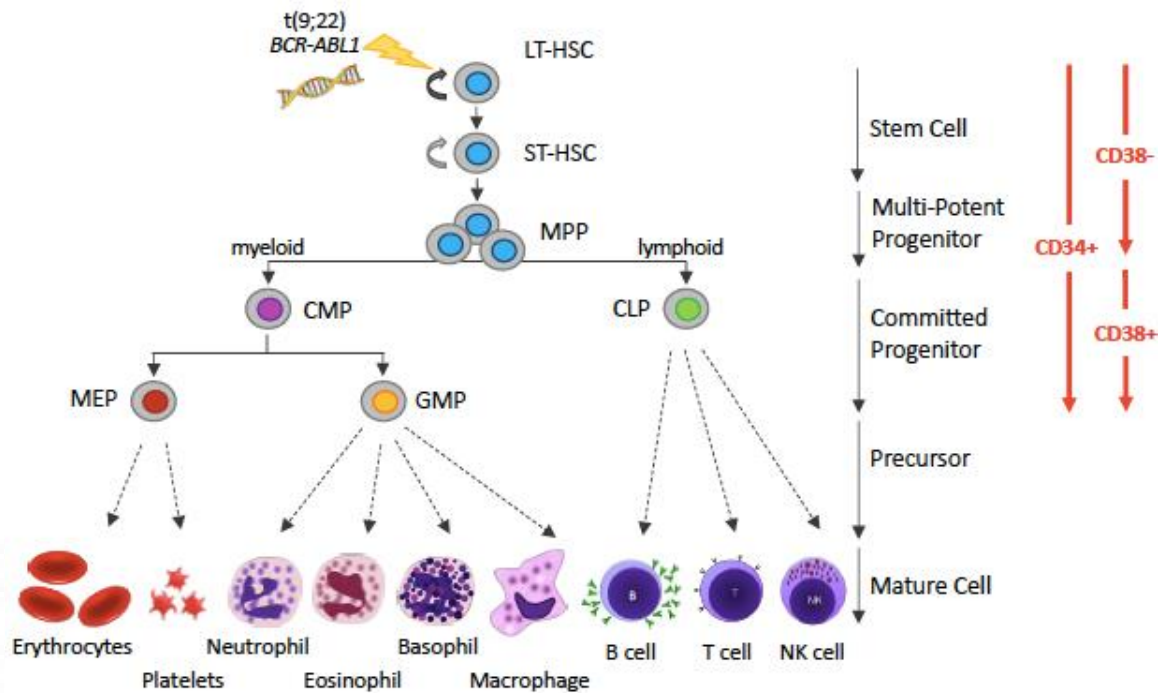


Figure 2.2: Simplified representation of the hematopoietic hierarchy. The driver lesion of CML, the t(9;22) chromosomal translocation giving rise to the BCR-ABL1 fusion gene, is gained at the HSC level. Abbreviations: LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; MPP, multi-potent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor (Soverini.S et al., 2021).

2.3.Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a stem cell malignancy characterized by t(9;22) resulting in uncontrolled proliferation of myeloid cells (Kantarjian et al., 2019)(Figure 2.2). It is a relatively rare disease, with 1-2 individuals per 100,000 people being diagnosed with CML annually, accounting for 15% of all newly diagnosed cases of leukemia in adults (Elias Jabbour, Hagop Kantarjian 2020). Despite its rarity, CML is one of the most extensively studied malignancies and was among the first to be linked to

LSCs. Consequently, it now serves as a model disease for stem cell-based malignancies. The development of CML is associated with a reciprocal translocation between chromosomes 9 and 22, resulting in "Philadelphia chromosome" (Ph) and the BCR::ABL1 fusion protein (Clarke and Holyoake, 2017; Deininger et al., 2020; Hochhaus et al., 2020) (Figure 2.3).

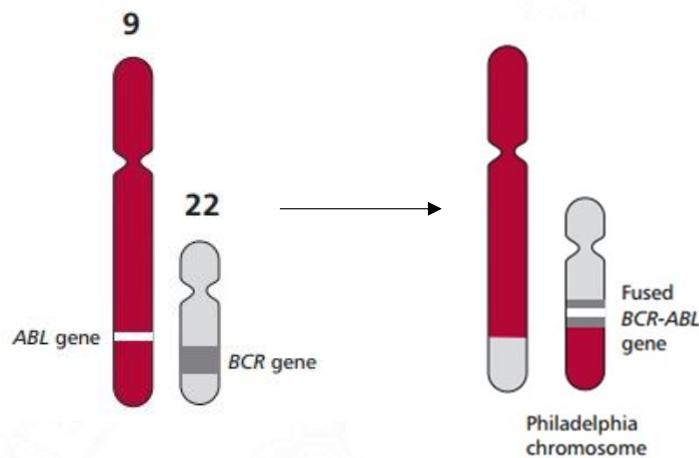


Figure 2.3: The origin of the Philadelphia chromosome. The Philadelphia chromosome results from the translocation between the Abelson murine leukemia (ABL1) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22.

(Adapted from https://www.brainkart.com/article/Causes-of-Cancer_16408/)

2.3.1 Epidemiology

CML typically manifests at a median age of 60 in Caucasians while appearing at a younger age (35–45 years) in Asians. It is uncommon in children, accounting for only 2–3% of all pediatric leukemia cases (Jabbour and Kantarjian, 2014a). The global incidence rate of CML is 15 cases per 1,000,000 people annually, with a male-to-female ratio of 1.34 (Suttorp et al., 2021). The age-adjusted incidence rate for individuals under 18 is 1.0 cases per 1,000,000 (Suttorp et al., 2021). The introduction of imatinib in the year 2000

significantly decreased annual mortality rates, reducing them from 10%-20% to 1%-2%(Goldman, 2010).

2.3.2 Disease phase

CML is mostly diagnosed in the chronic phase (CP), but if left untreated, it can progress to an accelerated phase (AP) and eventually advance to a terminal phase known as blast crisis (BC), which resembles acute leukemia (Clarke and Holyoake, 2017; Sawyers, 1999)(Figure 2.4). The initial comprehensive data on the development of the disease originated from the study of atomic bomb survivors (Preston et al., 1994). It was observed that following an extended latent period, most patients (85%) initially presented with the CP, yet within a 5-year timeframe, they transform from accelerated phase to a blast crisis. However, the duration it takes for patients to progress to the blast crisis stage varies significantly among individuals, with some remaining in the CP for up to 20 years, while others advance to blast crisis within a few months (Cortes and O'Dwyer, 2004). The reason for disease progression in some patients is worth exploring (Branford et al., 2018). Recent NGS-based studies show that additional gene mutation may drive this progression (Branford et al., 2019).

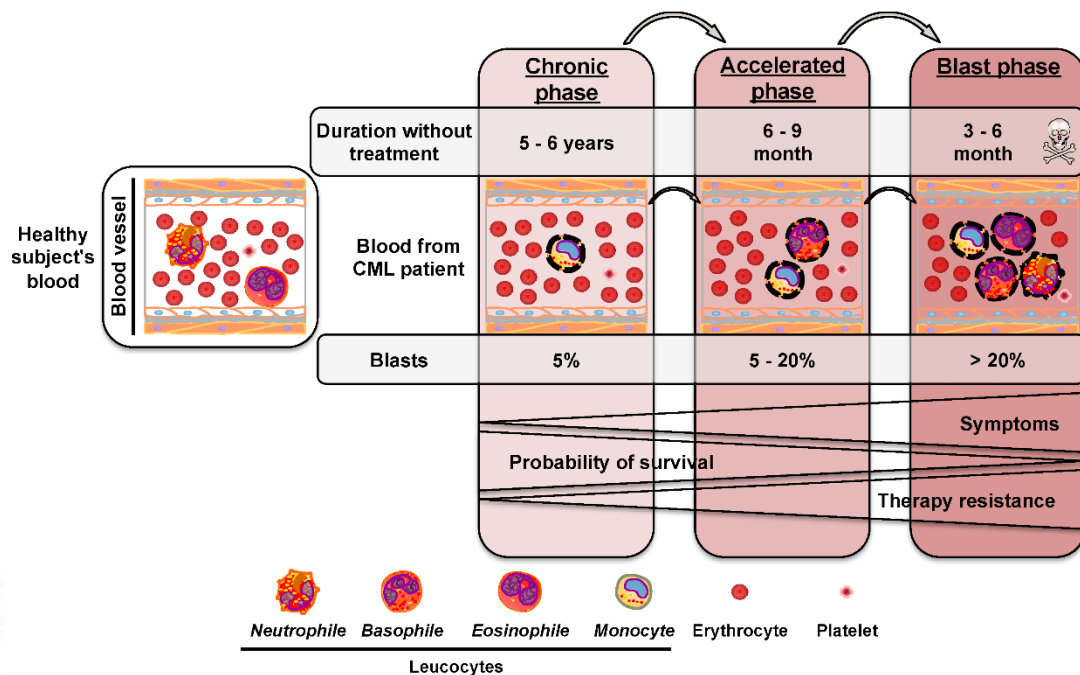


Figure 2.4. Progression of chronic myeloid leukemia. CML is characterized by an excessive proliferation of non-functional leukocytes (dotted lines) or blasts in the bone marrow and then in the blood (Losson et al., 2020).

2.3.3 Symptoms and signs

Most patients are diagnosed with CML during the CP of the disease, often through routine physical examinations or blood tests. Interestingly, approximately 30-50% of newly diagnosed patients display no symptoms at diagnosis. Common presenting symptoms for the remaining CP patients include fatigue, weight loss, malaise, early satiety, and discomfort or pain in the left upper quadrant. Medical examinations usually reveal elevated white blood cell (WBC) counts and an enlarged spleen (splenomegaly).

The definitive diagnosis of CML typically involves confirming the presence of the Philadelphia chromosome (Ph) through conventional cytogenetics or fluorescent in situ hybridization (FISH). *BCR::ABL1* fusion transcript is also detected using highly

sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) (Jabbour and Kantarjian, 2014b, 2020).

2.3.4. The role of BCR::ABL1 as the driver of chronic myeloid leukemia

The constitutively active BCR::ABL1 tyrosine kinase disrupts a wide range of signal transduction pathways, resulting in the promotion of leukemic cell growth, proliferation, and survival (Figure 2.5) (Cortes et al., 2021; Deininger et al., 2000; Dell'aversana et al., 2022; Michor et al., 2005). Some key dysregulated pathways include PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT. Dysregulated BCR::ABL1 pathways not only suppress apoptosis and stimulate cell proliferation but also elevate an reactive oxygen species (ROS) within the mitochondria (Bolton-Gillespie et al., 2013; Nieborowska-Skorska et al., 2013). Subsequent elevation in ROS levels results in oxidative DNA damage, characterized by an increased occurrence of DNA point mutations and double-stranded breaks. Due to inefficient and erroneous DNA repair mechanisms, these changes ultimately lead to abnormal alterations in chromosomal structure (Bolton-Gillespie et al., 2013).

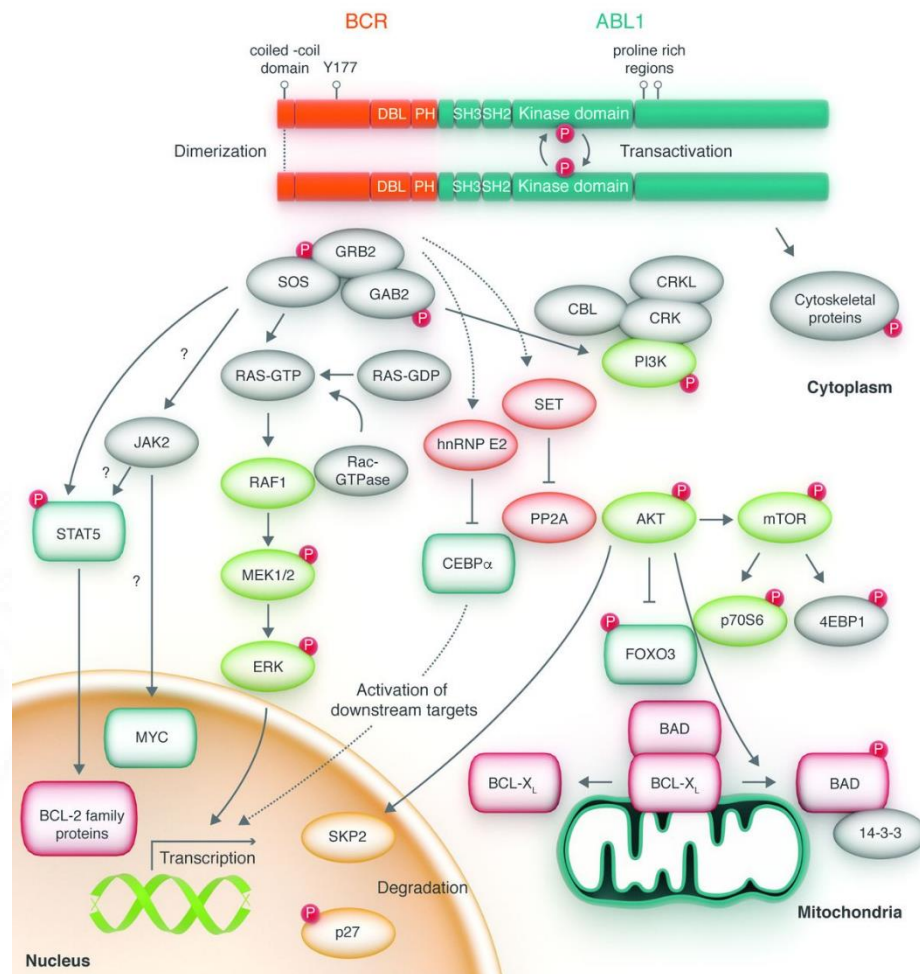


Figure 2.5: BCR::ABL1-dependent signaling complexes: Several signal transduction pathways are deregulated by the constitutively active BCR::ABL1 tyrosine kinase, including PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT, ultimately resulting in leukemic cell survival, growth, and proliferation (Mughal et al., 2016).

2.4 Evolution of CML treatment

2.4.1 Initial treatment strategies

Until the end of the 20th century, CML therapy restricted to nonspecific agents, evolving from arsenic-containing compounds in the 19th century to the administration of busulfan, hydroxyurea and, followed by, interferon-alpha (IFN α) (Silver et al., 1999).

Interferon was used to treat CML patients in 1983 (Talpaz et al., 1983). In the initial trial, 5 out of 7 patients achieved a complete hematological response (CHR) following treatment with high doses of IFN α , and splenomegaly observed in 3 patients was reversed. From that point onward, IFN α emerged as the preferred medication for CML patients. The remission rate and survival were improved and the 5-year survival rates were improved from 42% to 57% (1997). However, IFN α was linked to a broad spectrum of significant side effects that impeded quality of life, making long-term usage impractical for many patients. Many studies in the early 1980s established the therapeutic potential of allogeneic stem cell transplant (allo-SCT) for CML, and allo-SCT the treatment of choice for young patients with CML and a human leukocyte antigen (HLA)-identical donor (Goldman et al., 1982; Thomas et al., 1982). Up to the early 1990s, CML CP was the most common indication for allo-SCT worldwide. It was also the most effective treatment for patients with advanced-stage CML, though the results in CML AP and CML BP were considerably worse than those in the chronic phase because of increased rates of recurrence and treatment-related mortality (TRM) (Crawley et al., 2005; Gratwohl et al., 2002; Thomas et al., 1982).

2.4.2 Imatinib targeted therapy

During the 1990s, a groundbreaking advancement in the management of CML emerged with the development of the first-generation tyrosine kinase inhibitor (TKI) targeting BCR::ABL1, known as imatinib (STI571, Gleevec®, Glivec®, formerly CGP 57148B), by biochemist Nicholas Lyndon. Imatinib, a 2-phenylamino pyrimidine, inhibits the enzymatic action of the constitutive active BCR::ABL1 tyrosine kinase by occupying the ATP-binding pocket of the BCR::ABL1 oncoprotein, thereby inhibiting the activation

SCTIMST, Trivandrum

downstream effector molecules that cause the leukemic phenotype (Figure 2.6) (Druker et al., 2001). With the advent of imatinib, the treatment and management of CML patients underwent a revolutionary transformation, resulting in significantly improved prognosis, response rate, overall survival, and patient outcomes when compared to previous therapeutic approaches (Druker et al., 2006; Hochhaus et al., 2009; O'Brien et al., 2003). The 8-year overall survival rate saw remarkable improvement, rising from approximately 20% to 80-90% (Jabbour and Kantarjian, 2014b).

About 60% of CML patients stayed on the standard dosages of imatinib after six years, and about 40% necessitates alternative treatment or higher doses of imatinib (de Lavallade et al., 2008). The main reason for this is secondary resistance in most cases results from the point mutations in the BCR::ABL1 kinase domain (KD). This situation often results in disease relapse upon cessation of treatment (Goldman, 2010; Michor et al., 2005). As a result, most CML patients require ongoing TKI therapy, which brings with it potential risks of toxicity, challenges with adherence, development of drug resistance, recurrence of the disease, and the accompanying financial burden (Senapati et al., 2023).

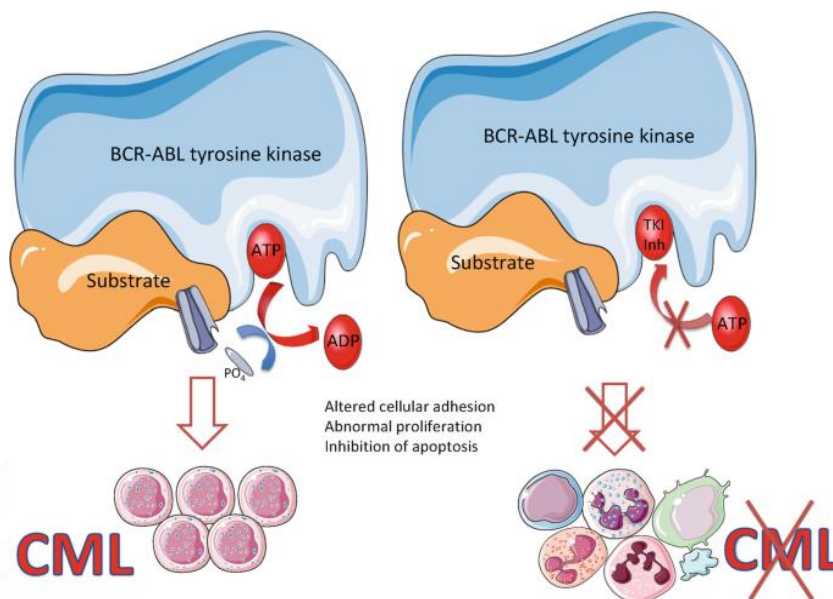


Figure 2.6: Schematic representation of the mechanism of action of TKI (Lussana et al., 2018).

2.4.3 Second- and third-generation tyrosine kinase inhibitors

The identification of mutations in the BCR::ABL1 kinase domain confer resistance to imatinib and associated with drug resistance led to the invention of the second-generation TKIs, dasatinib, nilotinib, and bosutinib (Golas et al., 2003; Lombardo et al., 2004; Weisberg et al., 2006).

Dasatinib is 350 times more efficacious than imatinib *in vitro* (Tokarski et al., 2006) and known to inhibit the Src kinase family (Shah et al., 2004). **Nilotinib** is a structural analog of imatinib with a 50 times higher affinity to the BCR::ABL1 ATP binding site *in vitro* (Weisberg et al., 2006). The second-generation TKIs were active against most imatinib-resistant single-mutants, except for the T315I mutation (Weisberg et al., 2007), leading to the development of the third-generation TKI, ponatinib (O'Hare et al., 2009) with

activity against the T315I mutation (Cortes et al., 2013). However, the acquisition of multiple mutations lead to BCR::ABL1 resistant to **ponatinib** (Zabriskie et al., 2014).

Four BCR::ABL1 TKIs are approved for frontline therapy: imatinib, dasatinib, bosutinib, and nilotinib. All four of these options offer the potential for a nearly normal quality of life and life expectancy, provided that patients adhere to the treatment regimen, undergo optimal monitoring with minimal interruptions in therapy, and are managed appropriately at the earliest indication of genuine treatment resistance (Senapati et al., 2023). Furthermore, long-term toxicity events due to second-generation TKIs are raising concerns about their use, especially in specific groups of patients.

Asciminib was approved in 2021 for the third-line therapy of CML and the treatment of T315I-mutated CML. It works through a novel mechanism that involves binding to the myristoyl pocket of the ABL1 and allosterically inhibiting the TK activity (Figure 2.7). Though all grade and $\geq$ grade 3 AEs were lower with asciminib than bosutinib in the ASCEMBL trial when studied at the higher 200 mg BID dose for T315I-mutated CML, asciminib showed $\geq$ grade 3 adverse events (AEs) in (56.4% vs. 68.4%) of patients and arterio-occlusive events (AOE) in 8% of patients. However, longer follow-ups in larger patient numbers will better define its safety (Hughes et al., 2019).

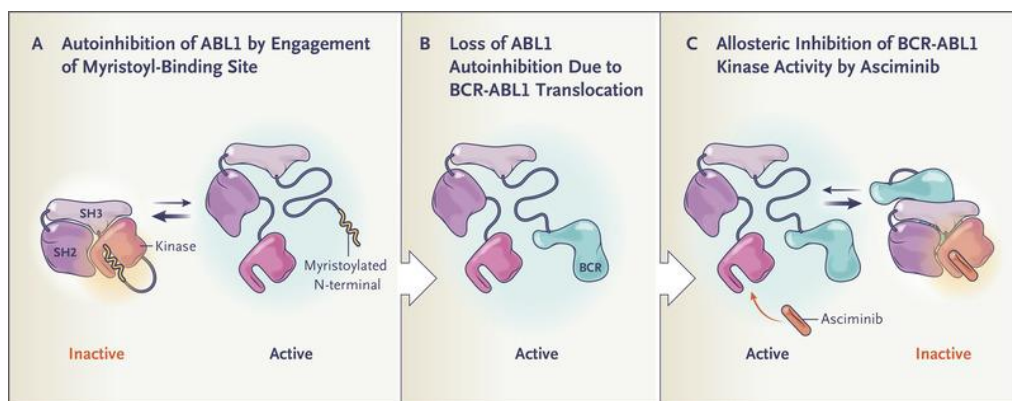


Figure 4.7: Binding of the Myristoyl Site of the BCR::ABL1 Protein by Asciminib (Hughes et al., 2019)

Olverembatinib (HQP1351) is a new third-generation TKI that could inhibit both wild-type and T315I-mutated BCR::ABL1 in cell lines. The drug is approved in China for the treatment of adults with TKI-resistant CML in CP or AP harboring the T315I mutation (Jiang et al., 2022).

2.5 Response to Therapy

Response to TKI therapy is highly predictive of long-term outcomes, and milestones of response by National Comprehensive Cancer Network (NCCN) and European Leukemia Net (ELN) consensus panels. The response to treatment with TKI is monitored through hematologic response (HR), cytogenetic response (CR), and molecular response (MR). HR is evaluated by observing peripheral blood (PB) blood counts, and CR is assessed by determining the percentage of BM cells that test positive for the Ph chromosome using Fluorescence in-situ Hybridization (FISH). MR is gauged using Quantitative reverse-transcription PCR (QPCR), which measures the reduction in BCR::ABL1 mRNA compared to a standard baseline or the International Scale (Table 2.1 and 2.2).

Table 2.1: Definition of response (Mughal et al., 2016)

Type of response	Definition
CHR	Complete hematologic response
MCyR	Major cytogenetic response
CCyR	Complete cytogenetic response
MMR	Major molecular response
MR ^{4.0}	BCR-ABL1/ABL1 ratio $\leq 0.01\%$ (international scale): this is a 4-log reduction
MR ^{4.5}	BCR-ABL1/ABL1 ratio $\leq 0.003\%$ (international scale): this is a 4.5-log reduction
CMR	Complete molecular response

WBC: white blood cells.

Table 2: European LeukemiaNet and National Comprehensive Cancer Network treatment response milestones for chronic myeloid leukemia expressed as BCR::ABL1 on the International Scale. Bold text indicates where the recommendations differ (Branford and Apperley, 2022)

Milestones	ELN 2020 Optimal	NCCN V3.2022 TKI-sensitive disease	ELN 2020 Warning	NCCN V3.2022 Possible TKI resistance	ELN 2020 Failure	NCCN V3.2022 TKI-resistant disease
Baseline	NA	NA	High-risk ACA, High-risk ELTS score	NA	NA	NA
3 months	$\leq 10\%$	$\leq 10\%$	$> 10\%$	$> 10\%$	$> 10\%$ if confirmed within 1-3 months	NA
6 months	$\leq 1\%$	$\leq 10\%$	$> 1-10\%$	NA	$> 10\%$	$> 10\%$
12 months	$\leq 0.1\%$	$\leq 0.1\%$ or $> 0.1-1\%$	$> 0.1-1\%$	$> 1-10\%$	$> 1\%$	$> 10\%$
Any time	$\leq 0.1\%$ or $\leq 0.01\%$ for patients with the aim to achieve TFR		$> 0.1-1\%$, loss of MMR indicates failure after TFR		$> 1\%$, resistance mutations and high-risk ACA	

NA: not applicable; ACA: additional chromosome abnormalities in Philadelphia-positive cells; ELTS: EUTOS long-term survival; MMR: major molecular response ($\leq 0.1\%$); ELN: European LeukemiaNet; NCCN: National Comprehensive Cancer Network; TKI: tyrosine kinase inhibitor; TFR: treatment-free remission. *NCCN guidelines: $\leq 0.1\%$ is optimal if the treatment goal is treatment-free remission and $\leq 1\%$ is optimal if the treatment goal is long-term survival.

2.5.1. Treatment-free remission

Despite the efficacy of TKI in improving the survival of CML patients, it results in certain off-target toxicities. Vascular events and pleural effusions are among the potential adverse effects. Additionally, interactions with other drugs can occur, and discontinuation of TKI therapy is recommended for young women planning to become pregnant. The financial burden and lifelong therapy can also be substantial (Efficace et al., 2013; Force et al., 2007; Heiblig et al., 2018).

The Stop Imatinib (STIM) trial focused on discontinuing imatinib treatment in CML patients who had achieved complete molecular response (CMR) for at least two years. Over half (58%) of these patients experienced CML relapse within six months of stopping imatinib. However, the positive outcome was that all relapsed patients responded well to the reintroduction of TKI therapy, suggesting a similarity between the relapsed disease and the initial CML (Bordeaux et al., 2010).

The Australian TWISTER study, with selection criteria similar to STIM, demonstrated that less than half (47.1%) of the patients remained relapse-free at 24 months after discontinuation of TKI therapy. No patients with molecular relapse after discontinuation have progressed or developed *BCR-ABL* mutations (median follow-up, 42 months). Patients relapsed remained sensitive to imatinib re-treatment (Ross et al., 2013) (Figure 2.8) (Table 2.3).

The factors contributing to sustained Treatment-Free Remission (TFR) in some patients have often been attributed to the immune system's anti-leukemic role, particularly within the bone marrow microenvironment (BMM), implying that immune response plays a critical role in preventing CML relapse after discontinuation of TKI therapy (Ilander et al., 2017). Further research is needed to better understand the complex interplay between TKIs, the immune system, and the persistence of leukemia stem cells to improve treatment strategies and outcomes for CML patients.

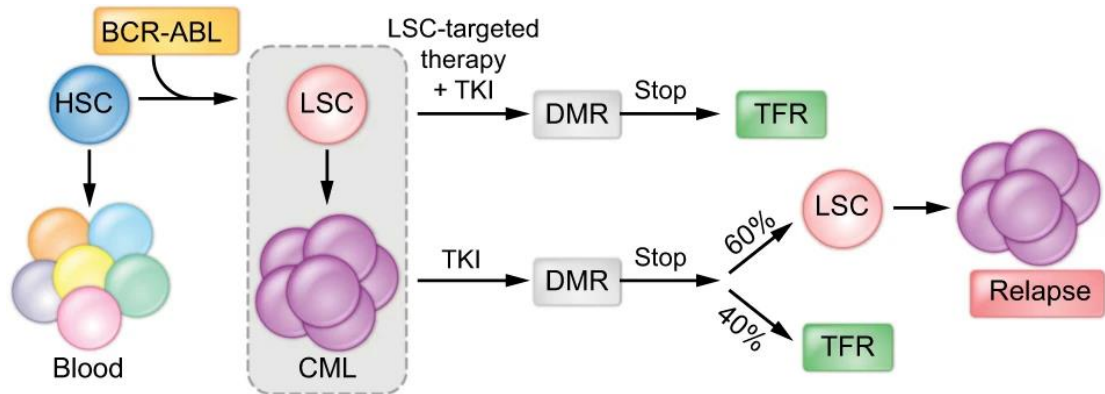


Figure 2.8: Pathobiology and treatment of chronic myeloid leukemia (Park, C.S., and Lacorazza, H.D., 2020).

Table 2.3. Key characteristics of selected studies of discontinuation of tyrosine kinase inhibitor therapy.

Reference	N. of patients	TKI(s)	Eligibility criteria			Median time in MR, months (range)	Median duration on TKI, months (range)	Trigger to restart TKI
			Duration of TKI (y)	Depth of MR	Duration of MR (y)			
ENESTfreedom ^a	222	Nilotinib	2	MR4.5	1	NR	43.5 (32.9–88.7)	Loss of MMR
STIM1 ^b	100	Imatinib [#]	3	MR5.0	2	36.4 (24–107)	58.8 (35–112)	At least two positive RT-PCR results showing an increase of one log or confirmed loss of MMR
TWISTER ^a	40	Imatinib [#]	3	MR4.5	2	30*	70*	Loss of MMR or two consecutive positive samples at any value
EURO-SKI ^a	368	Imatinib or nilotinib or dasatinib	3	MR4.0	1	56.4 (34.8–82.8)	90 (60–118.8)	Loss of MMR
DasFree ^c	79	Dasatinib	2	MR4.5	1	28 (13–116)	9 (29–244)	Loss of MMR
LAST ^d	172	Imatinib or dasatinib or nilotinib or bosutinib	3	MR4.0	2	56.6 (25.1–176.6)	82.7 (36.1–199)	Loss of MMR

MR: molecular response (with log reduction in *BCR::ABL1* transcripts); TKI: tyrosine kinase inhibitor; y: years; NR: not reported; RT-PCR: reverse transcriptase polymerase chain reaction; MMR: major molecular response. ^aPatient on imatinib only; range not reported. ^bPrior interferon therapy allowed.

2.6 CML LSCs

Although TKIs have revolutionized CML treatment, transforming it from a fatal to a manageable disease, they cannot eliminate the quiescent LSC population, which remains a potential source of relapse (Figure 2.9).

CML LSCs are not addicted to BCR::ABL1 activity and, therefore, are not eliminated by TKI therapy. Corbin et al. reported initially that CML LSCs survive TKI therapy despite inhibition of BCR::ABL1 activity in both differentiated and primitive cells.

Primitive CML cells survive imatinib at a concentration 10 more than the inhibitory SCTIMST, Trivandrum

concentration of differentiated cells (Corbin et al., 2011). Hamilton et al. showed that prolonged exposure with the dasatinib, combined with the absence of growth factors, is insufficient to target primitive CML LSCs despite complete inhibition of BCR::ABL1 tyrosine kinase activity (Hamilton et al., 2012).

These studies demonstrated that CML LSCs are insensitive to TKIs, and thus, new strategies targeting BCR::ABL1 independent signaling pathways may be needed to eliminate them effectively.

2.6.1 Origin and Evolution of CML LSCs

In 1999, Tessa Holyoake presented the first direct evidence of a primitive, CD34⁺lin⁻ quiescent subset of leukemic cells in CML patients with in vitro and in vivo self-renewal properties (Holyoake et al., 1999). Later, it was found that these cells were unaffected by TKI. Moreover, BCR::ABL1-positive LSCs were identified in CML patients who achieved long-term remission after imatinib treatment. The BCR::ABL1-positive CD34⁺CD38⁻ cells isolated from these patients demonstrated long-term repopulation capacity in immunocompromised mice (Hamilton et al., 2012). Further investigations revealed that these cells are not vulnerable to TKIs since their survival is independent of BCR::ABL1 kinase activity. BCR::ABL1 is either inactivated or expressed at very low levels in these cells (Kumari et al., 2012).

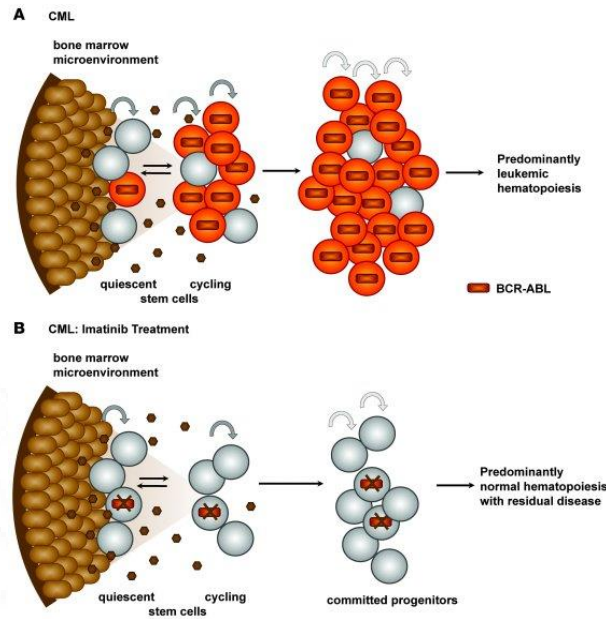


Figure 2.9: Model of CML disease persistence with imatinib treatment (Corbin et al., 2011).

6.2 Surface Markers in CML LSCs

Although CML LSCs share many features with their normal counterparts, like self-renewal capacity, multilineage differentiation ability, and the expression of similar surface markers (they are $CD34^+CD38^-Lin^-$ cells) there are some differences that have allowed their in vitro and in vivo characterization.

Extensive screening in CML LSC has been performed to find surface molecules that distinguish between LSC $BCR::ABL^+$ and normal HSC.

2.6.2.1 IL-1RAP

The first molecule proposed for the identification of $BCR::ABL1^+$ LSC was Interleukin 1 Receptor Accessory Protein (IL-1RAP), which is selectively up-regulated in CML LSC. It has been shown that IL-1RAP is a promising biomarker activated by

Interleukin 1 (IL-1), which plays an essential role in maintaining LSCs (Landberg et al., 2016; Warda et al., 2019).

2.6.2.2 Dipeptidyl peptidase IV (DPP4; CD26)

CD26 is selectively upregulated in CML LSC, becoming a marker of LSC BCR::ABL1. CD26 disrupts the CXCL12-CXCR4 axis by cleaving the CXCL12 molecule and is implicated in releasing CML LSCs from the BMM into the blood (Bocchia et al., 2018, 2020; Raspadori et al., 2019; Sharma et al., 2022).

2.6.2.3 IL-2 receptor (CD25)

BCR::ABL1⁺ LSC expresses Interleukin 2 receptor alpha chain (IL-2RA; CD25), a molecule that depends on STAT5 activity. CD25 serves as a negative regulator of the growth of CML LSC, acting as a tumor suppressor in CML (Sadovnik et al., 2017).

2.6.2.4 CD93

CD93 is expressed on a CD34⁺CD38⁻CD90⁺ subpopulation that engrafts patient-derived cells in an immunocompromised model (in contrast to the CD93⁻ counterpart that fails to engraft) and displays a stem cell-like signature of gene expression. CD93⁻ expressing cells were not eliminated by TKIs and persisted in patients even after prolonged treatment. Comparison between patients in TFR who did or did not experience molecular recurrence showed that the former harbored detectable CD93⁺ cells within the CD34⁺CD38⁻CD90⁺ fraction, whereas the latter did not. CD93 is expressed in CML LSCs and other cell types, it is highly unlikely that CD93 may serve as a therapeutic target. However, further studies are warranted to investigate whether CD93 may be used as a predictive biomarker to distinguish those CML patients at high risk of molecular recurrence after discontinuation (Kinstrie et al., 2015, 2020).

2.6.2.5 CD33

the sialic acid receptor CD33 is expressed in CML CP LSC but only variably in BP LSC (Herrmann et al., 2012).

2.5.2.6 CD36

Primitive CML cells expressing CD36 showed decreased imatinib sensitivity on BC LSC in proximity to adipose tissue, whereby it mediates fatty acid uptake and oxidation (Landberg et al., 2018).

Over the last decade, researchers have successfully validated additional aberrant surface markers that offer improved discrimination between residual normal HSCs and CML LSCs. The discovery of highly specific, robust, and reproducible biomarkers holds significant potential for enhancing diagnosis and facilitating the isolation and biomolecular/functional characterization of LSCs.

Moreover, precise estimation of LSCs may have multiple implications beyond predicting treatment response. It could enable the tracking and quantification of residual LSCs during and after therapy as a predictor of TFR success. Additionally, identifying specific targets exclusively expressed on the surface of CML LSCs but not on normal HSCs or other cell types could pave the way for highly selective therapeutic targeting of LSCs.

2.7 Factors influencing CML LSC survival

CML LSC has shown several intrinsic mechanisms of resistance to therapy, including those dependent or independent of the presence of BCR::ABL1 fusion protein. In addition, several reports have suggested that LSC could remain in specific protective

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niches within the BMM, where they have an optimal microenvironment conducive to developing resistance to therapy and inducing relapse (Zhang et al., 2012).

2.7.1 Intrinsic mechanism of resistance

Several pathways specific to LSCs have been recognized, primarily transcription factors such as Signal Transducer and Activator of Transcription 3/5 (STAT3/5), Forkhead transcription factors of the class O 3A (FOXO3A), β -catenin, p53, c-MYC, NOTCH, BMI-1, and others, these master regulators govern crucial cellular processes like apoptosis, self-renewal, determination of cell fate, and senescence (Abraham et al., 2016; Kuepper et al., 2019).

2.7.1.1 *Sonic Hedgehog (Hh) pathway*

Hh is a crucial regulator of both HSCs and LSCs by controlling self-renewal processes. When the Hh ligand binds to the Patched (PTCH) receptor, it relieves inhibition of the G protein-coupled receptor Smoothened (SMO). As a result, the transcription factor GLI1 is activated, translocating into the nucleus. Within the nucleus, GLI1 influences the transcription of various target genes involved in cell cycle regulation and apoptosis (CCND1, MYC, etc.) while also promoting MDM2-dependent degradation of p53. In CML LSCs, this pathway becomes hyperactivated due to the increased expression of SMO (Dierks et al., 2008; Zhao et al., 2009).

2.7.1.2 *Wnt/ β -catenin*

Nuclear β -catenin is crucial for the self-renewal and survival of both normal HSCs and LSCs. It activates essential genes like NOTCH and is key to LSC survival. Both canonical and noncanonical Wnt pathways influence β -catenin localization and activity. Noncanonical Wnt signaling activates NFAT, leading to increased autocrine

cytokine production that provides compensatory signals to LSCs during TKI exposure (Gregory et al., 2010; Wang et al., 2010).

2.7.1.3 PI3K/AKT

The PI3K/AKT pathway mediates proliferation and becomes activated by binding growth factors to receptor tyrosine kinases. In the context of CML LSCs, BCR::ABL1 activates the PI3K/AKT signaling, contributing to the maintenance of these cells. This activation leads to the phosphorylation and cytosolic retention of FOXO by AKT, which subsequently hinders the transcription of FOXO target genes involved in apoptosis (Sykes et al., 2011).

In addition to other pathways, the signaling of transforming growth factor β (TGF- β) also plays a role in influencing FOXO nuclear translocation through the PI3K/AKT signaling pathway (Naka et al., 2010a).

2.7.1.4 Janus kinases (JAK)

In CML, JAK2 signaling has been observed to converge on activating STAT3 and STAT5. Additionally, STAT3 signaling has been linked to the survival of CML LSCs. JAK2 and JAK1 have been implicated in activating STAT3 (Eiring et al., 2015).

2.7.1.5 Protein phosphatase 2A (PP2A)

The tumor suppressor PP2A gene encodes a multimeric serine/threonine phosphatase. In the context of CML, PP2A becomes inactivated due to the overexpression of its endogenous inhibitors (SET Nuclear Proto-Oncogene) SET and CIP2A (Cellular Inhibitor of PP2A). The loss of PP2A, resulting from BCR::ABL1-dependent expression of SET, has been linked to the maintenance of quiescent LSCs.

Moreover, it has been observed that BCR::ABL1 expression plays a vital role in the recruitment of JAK2 and the activation of a JAK2/ β -catenin pathway, which is crucial for LSC survival and self-renewal (Neviani et al., 2013).

2.7.1.6 Promyelocytic leukemia (PML)

PML gene is caused due to t(15;17) chromosomal translocation of acute promyelocytic leukemia, encodes a protein localizing to nuclear bodies that act as a tumor suppressor, controlling apoptosis, cellular proliferation, senescence, and negatively regulating *mTOR*. PML has been shown to play an indispensable role in maintaining LSC quiescence in CML (Bernardi et al., 2006; Ito et al., 2008).

2.7.1.7 Dual Specificity Tyrosine Phosphorylation Regulated Kinase 2 (DYRK2)

KLF4 (Krüppel family of transcription factors) transcription factor has been shown to sustain CML LSCs via downregulation of the dual-specificity kinase *DYRK2*, which mediates the stabilization of *p53* and the proteasomal degradation of *c-MYC* (*MYC proto-oncogene*). This results in reduced apoptosis and increased self-renewal. The central role of *p53* and *c-MYC* in LSC maintenance had already been elegantly demonstrated by a seminal proteomic study conducted by Tessa Holyoake's group (Park et al., 2019).

2.7.2 Epigenetic mechanisms

Enhancer of Zeste Homolog 2 (*EZH2*) is Polycomb Repressive Complex 2 (*PRC2*) that trimethylates histone H3 at lysine 27 but may also directly regulate gene expression. It is highly expressed in CML LSCs and contributes to their survival (Scott et al., 2016). Targeting histone deacetylases was also explored against CML LSCs in light of the reported efficacy of such a strategy in inducing apoptosis in non-proliferating

cells. It gave promising results when tested in combination with TKIs in cellular and mouse models. Sirtuin 1 (*SIRT1*) deacetylase is a multifunctional protein implicated in many cellular pathways, including energy metabolism and stress response, and *TP53* and *FOXOs* are its targets (Abraham et al., 2019). *SIRT1* is overexpressed in CML LSCs and contributes to LSC resistance to TKIs. Later, *SIRT1* was found to mediate increased mitochondrial oxidative phosphorylation in CML, a vital survival mechanism of LSCs. The histone acetyltransferase CREB binding protein (*CBP*) has also been implicated in LSC self-renewal, as opposed to p300, which promotes differentiation and senescence. Both can be recruited by b-catenin upon activation of the Wnt pathway. Inhibition of *CBP* favored the formation of b-catenin/p300 complexes, switching the balance from cell proliferation to differentiation (Li et al., 2012).

2.7.3 Micro RNA

microRNAs play a significant role in CML LSCs' self-renewal, maintenance, and potentially developing resistance to TKIs. The regulatory function of miR-126 in the dormancy of HSCs and CML LSCs has been well-documented. The BCR::ABL1 protein kinase triggers the phosphorylation of the SPRED1 protein, leading to the suppression of miR-126. This molecular event results in a decrease in the stemness state of the cells. In contrast, BM endosteal Sca-1⁺ endothelial cells have been observed to release miR-126 through extracellular vesicles. Treatment with TKIs can counteract the inhibitory impact of miR-126 by raising its levels via TKI therapy. The sensitization of CML LSCs to TKIs becomes evident in reduced miR-126 levels (B Zhang et al., 2018).

Enhanced JAK-STAT signaling, facilitated by BCR::ABL1, promotes an increase in ADAR adenosine deaminase RNA specific (*ADAR1*) expression, thereby enhancing the

conversion of adenosine to inosine. This enzymatic process plays a crucial role in miRNA stability control. The ADAR1 protein has been seen to impede the generation of mir-let7, a precursor molecule for miRNA, ultimately boosting the self-renewal capacity of CML LSCs (Zipeto et al., 2016).

Upon exposure to imatinib, CML LSCs show elevated miR-21 expression, leading to resistance against TKIs. Depletion of miR-21 in CML LSCs can be achieved by disrupting the PI3K/AKT signaling pathway, which results in the upregulation of programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN), thereby effectively restoring sensitivity to TKIs in CML LSCs. The transcription factor E2F Transcription Factor 1(E2F1) drives the proliferation of CML LSCs by upregulating miR-183. Furthermore, imatinib therapy reduces miR-30a levels by involving Beclin1 and Autophagy-related gene 5 (ATG5), promoting LSC resistance to TKIs (Wang et al., 2015).

CML CD34⁺CD38⁻ subset displays an increase in miR-29a-3p and miR-660-5p expression, independent of BCR::ABL1 kinase activity. This upregulation correlates with the downregulation of their respective targets, ten-eleven translocation (TET2) and endothelial PAS domain protein 1 (EPAS1). This molecular mechanism has been shown to confer resistance to TKI treatment *in vitro* (Salati et al., 2017).

Pellicano et al. have demonstrated that BCR::ABL1 kinase-dependent upregulation of hsa-mir-183 leads to the downregulation of its direct target, Early Growth Response 1 (EGR1) transcription factor. This, in turn, results in the upregulation of the cell cycle regulator E2F1, governing cell proliferation and p53-dependent/independent apoptosis. Inhibiting E2F1 activity reduces the ability of LSCs to form colonies, halts their cell

cycle progression, and initiates programmed cell death through the p53 pathway, affecting LSC survival and proliferation while sparing HSCs (Pellicano et al., 2018).

Recent findings indicate increased expression of miR-196a-5p in the CD26⁺CD34⁺CD38⁻ subset compared to the CD26⁻CD34⁺CD38⁻ subset. However, the precise functions and implications of this specific microRNA in the context of CML LSCs are not fully understood (Ruiz et al., 2021).

2.7.4 Autocrine factors

BCR::ABL1-induced transformation relies on various mechanisms, including the autocrine production of cytokines like Interleukin (IL)-3 and Granulocyte-Colony Stimulating Factor (G-CSF) by LSCs, leading to STAT5 activation and growth factor-independent signaling. Additionally, recent reports have highlighted kinase-independent autocrine loops that contribute to LSC persistence. Notably, the BCR::ABL1 kinase-independent autocrine production of Tumor Necrosis Factor-alpha (TNF α) supports CML LSC survival through the nuclear factor κ B (NF κ B)/p65 pathway, resulting in the expression of the IL-3 and granulocyte/macrophage-colony stimulating factor common β -chain receptor (Gallipoli et al., 2013).

Moreover, an autocrine loop has been identified, which increases the concentration of bone morphogenetic proteins (BMP) 2 and 4 in the BMM. These BMPs, typically produced by mesenchymal cells and involved in regulating the proliferation and fate of normal HSCs, have been implicated in LSC resistance to TKIs (Laperrousaz et al., 2013). Recent findings have also revealed that CML LSCs upregulate pleiotrophin (PTN), a heparin-binding growth factor usually produced by BM stromal cells and endothelial cells (ECs). PTN has been shown to promote CML LSC survival and confer TKI

resistance. These autocrine loops play a critical role in supporting the survival and persistence of CML LSCs and can potentially serve as targets for therapeutic intervention (Himburg et al., 2019).

2.7.5 Autophagy

Autophagy, an evolutionarily conserved catabolic process, involves the lysosomal degradation and recycling of unnecessary cellular components to generate adenosine triphosphate (ATP) and essential building blocks during nutrient and oxygen deprivation (Mortensen et al., 2011; Rubinsztein et al., 2012). While autophagy plays a role in maintaining HSCs, it also functions as a pro-survival pathway that enables tumor cells to withstand metabolic stress and evade apoptosis triggered by anti-cancer agents. In the context of CML, Autophagy is a crucial mechanism LSCs employ to enhance their survival against therapeutic challenges (Baquero et al., 2019). Treatment with TKIs promotes autophagy, and inhibiting autophagy intensifies the effectiveness of TKIs in targeting and eliminating both stem and progenitor cells (Bellodi et al., 2009; Mortensen et al., 2011). In summary, autophagy serves dual roles, essential for normal HSC maintenance but also exploited by both tumor cells and CML LSCs to bolster survival and counteract the effects of therapeutic interventions. The intricate regulation of autophagy, as influenced by BCR::ABL1 and therapeutic agents like TKIs, underscores its potential as a therapeutic target in leukemia treatment.

2.7.6 Energy metabolism

Since energy metabolism is essential in regulating HSCs, investigating its role in LSCs becomes particularly pertinent. Kuntz et al. demonstrated that CML CD34⁺CD38⁻ cells exhibit heightened TCA cycle flux and mitochondrial respiration compared to their

CD34⁻ counterparts. Interestingly, the more primitive CD34⁺CD38⁻ LSCs depend considerably more on OXPHOS than non-leukemic HSCs, aligning with the glycolytic metabolic profile typical of HSCs (Kuntz et al., 2017). An intriguing prospect emerges as integrin-linked kinase, a protein situated in focal adhesions with primarily a scaffolding function in cell-matrix interactions. It appears to influence mitochondrial metabolism and holds promise as a therapeutic target for quiescent CML LSCs that remain unresponsive to TKI treatment (Rothe et al., 2020).

Utilizing single-cell RNA sequencing, Giustacchini et al. elegantly revealed that BCR::ABL1⁺ LSCs within the same patient-derived CML sample exhibit overexpressed genes associated with OXPHOS in contrast to non-malignant HSCs (Giustacchini et al., 2017). This observation supports the previous global mRNA microarray analyses, where CP-CML LSCs showed enhanced oxidative phenotype marked by elevated expression of mitochondrial respiratory chain (MRC) genes compared to non-leukemic HSCs. Furthermore, transcriptome profiling identified deregulated miRNAs in TKI-nonresponsive CML LSCs compared to TKI-responsive ones, with dysregulation of miR-185. Intriguingly, the restoration of miR-185 function impeded drug-resistant CML LSCs. Notably, the target gene of miR-185, PAK6, was found to enhance drug resistance in TKI-nonresponsive LSCs. Coupled with the detection of increased OXPHOS and ROS expression in TKI-nonresponsive LSCs, these findings suggest that miRNAs and their target genes play pivotal roles in mitochondrial metabolism regulation within CML, including drug-resistant LSCs (Lin et al., 2020).

Abraham et al. uncovered a partial regulation of increased OXPHOS in CML LSCs through the NAD-dependent deacetylase sirtuin-1 (SIRT1). Their work demonstrated

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that SIRT1 expression activates the transcription factor PGC-1 α , either directly or in an AMPK-dependent manner. Subsequently, PGC-1 α stimulates the expression of mitochondrially encoded genes associated with mitochondrial respiration and enhances electron transport chain (ETC) activity. Direct inhibition of PGC-1 α only partially curbed CML pathogenesis *in vivo*, unlike SIRT1 inhibition. A prior study by the same group underscored the significance of SIRT1 in CML LSCs by inhibiting p53, highlighting the multifaceted role of this enzyme (Abraham et al., 2019; Li et al., 2012).

2.7.7 Immune response

Several studies have focused on the role of natural killer (NK) cell populations in TFR. In the EURO-SKI trial, longer relapse-free survival was associated with increased NK cells. Patients with more immature mature NK cell phenotype (CD56^{bright}) were less likely to sustain TFR (Burchert et al., 2015). The levels of tumor necrosis factor α and interferon γ secretion were associated with the success of discontinuation of TKIs (Ilander et al., 2017). Another study revealed that patients who stopped IM treatment displayed an increased number of NK cells and a reduction in CD3⁺CD8⁺CD62L⁺ cells compared to those still receiving IM. Among patients who maintained TFR, a higher proportion of activating NK cell receptors like NKG2D were present in NK cells. However, there was reduced expression of inhibitory NK cell receptors like KIR2DL2/DL3/DS236 and a lower percentage of regulatory T cells marked by FoxP3 expression and monocytic myeloid-derived suppressor cells (Irani et al., 2020; Ohyashiki et al., 2012). These observations suggest the potential utility of these factors in forming a predictive scoring system for TFR. In another prospective analysis, the expression of CD86 (B7.2), a ligand for the inhibitory receptor CTLA-4 on plasmacytoid dendritic SCTIMST, Trivandrum

cells (pDCs), was identified as predictive of TFR. A higher count (>95) of $CD86^+$ pDCs per 105 lymphocytes correlated with a reduced rate of relapse-free survival (30%), while a lower count (<95) of $CD86^+$ pDCs per 105 lymphocytes was associated with an increased TFR rate (70%) (Schütz et al., 2017). A more comprehensive study on the immune system and potential inflammatory influences, as well as their interactions with medications, holds promise for enhancing TFR rates in the future (Chen et al., 2021).

It's worth considering that many of these studies primarily assessed peripheral blood (PB) NK cells, potentially missing distinctions present in BM-derived NK cells. In the BM of CML patients, a mature $CD57^+$ subset was found to be significantly higher than in healthy controls, suggesting an influence of the BMM on the differentiation status of NK cells in CML. However, the specific importance of $CD57^+$ NK cells in TFR remains uncertain (Yao et al., 2020). Expanding NK cell immunotherapy is being explored to eliminate minimal residual disease (MRD) in CML, potentially serving as adjuvant therapy for patients attempting TFR. Other innovative NK-based treatments include anti-leukemic monoclonal antibodies that trigger targeted NK antibody-mediated responses and CAR-NK cell therapy, although these have not yet been described for CML but rather for other malignancies (Allison et al., 2022).

Below is the list of studies on TKIs with other agents targeting survival pathways of CML LSCs.

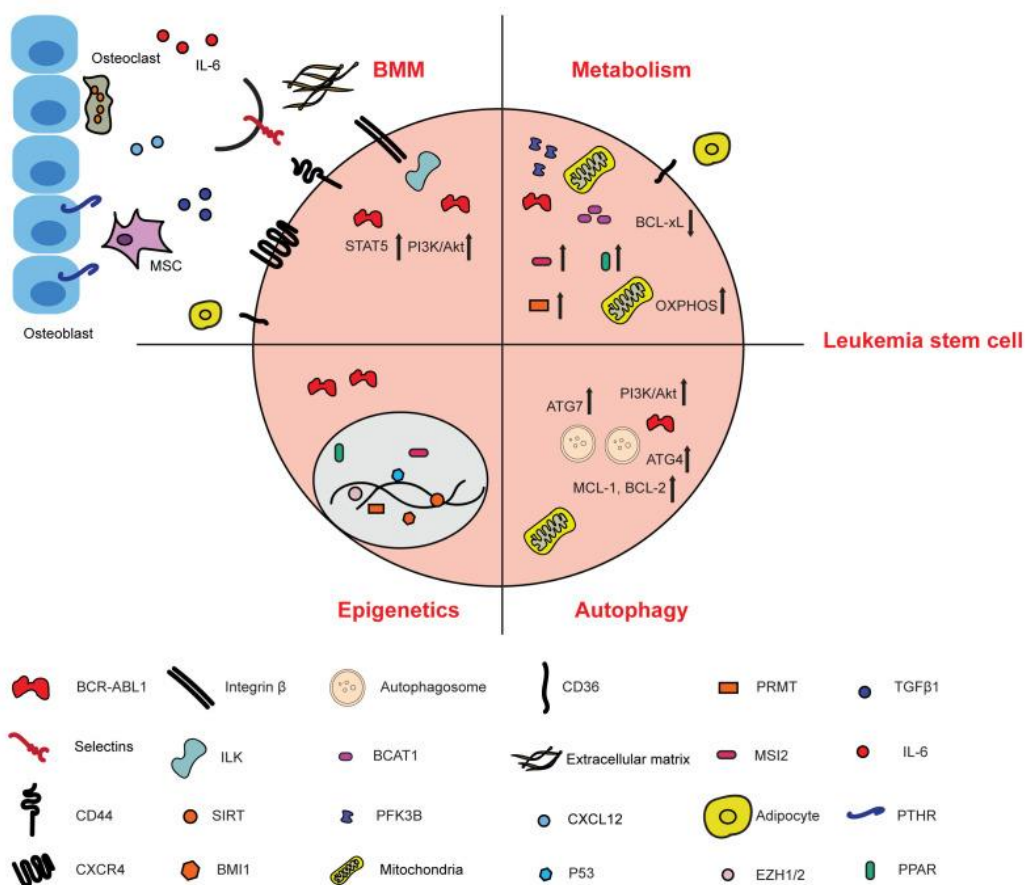
Zhang B et al. exhibited that by combining imatinib with inhibitors of histone deacetylases, they could effectively target quiescent CML cells in vitro and in vivo humanized mice models (Zhang et al., 2010)

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The inhibition of ALOX5, an enzyme responsible for converting fatty acids into leukotrienes, prolonged the lifespan of mice with leukemia, suggesting that it played a crucial role in sustaining the survival of CML LSCs (Chen et al., 2009).

Disrupting the Hedgehog pathway through the loss of Smoothened (SMO) was proven to eradicate CML LSCs. Consequently, a phase I clinical trial was launched to assess the viability and safety of combining imatinib with an SMO inhibitor alongside dasatinib in patients with chronic phase CML (CP-CML). However, the adverse effects reported with the SMO inhibitor and its limited clinical effectiveness discouraged further investigation and its application in CP-CML (Irvine et al., 2016).

CML LSCs demonstrated sensitivity to pioglitazone-induced activation of PPAR- α . Interestingly, the administration of pioglitazone in conjunction with imatinib resulted in sustained Complete Molecular Response (CMR) in some CML patients, though this was observed in a small subset. Notably, among a group of twenty-four CP-CML patients treated with a combination of pioglitazone and imatinib, 56% achieved MR (4.5), compared to fewer than half (21%) who were projected to achieve the same outcome with imatinib alone. More recently, Holyoake and colleagues showcased the potential of eliminating CML LSCs through the activation of Tumour Protein 53 (TP53) combined with the inhibition of MYC. However, as this treatment regimen lacks a TKI, which is the established standard care for managing CML, this innovative approach might not find application in CP-CML patients who respond well to TKIs (Pagnano et al., 2020; Rousselot et al., 2017).



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While numerous in vitro and preclinical investigations have uncovered novel approaches for addressing CML LSCs, their transition to clinical applications has been limited. The imperative need of the moment is to develop a successful strategy for eradicating CML LSCs.

2.8 The BM Microenvironment in chronic myeloid leukemia

Like their normal hematopoietic stem cell counterparts, LSCs inhabit a distinct microenvironment that governs their activities (Figure 2.10).

LSCs are known to coopt or hijack BMM that protect and nurture them at the expense of normal hematopoiesis. In light of this complex interplay, it is believed that the study of the BMM is at least as important as the study of LSCs, as a deep understanding of the role of BMM may provide essential clues on how to eliminate LSCs selectively (Magalhães-Gama et al., 2022).

The crosstalk between LSCs and BMM is mediated by

- i) Physiological factor- Hypoxic microenvironment
- (ii) cell-cell direct interactions via surface molecules and involves a broad array of cell types on the BM side, like mesenchymal stromal, endothelial, perivascular, osteolineage, and immune cells.
- (iii) soluble or exosome-incapsulated factors and

2.8.1 Hypoxic microenvironment

Low oxygen tension of BMM profoundly influences LSC features, most importantly, their metabolism. Quiescent and self-renewing HSCs rely on glycolysis for energy production, which is an adaptation to the hypoxic environment of the BMM and reflects

the low energetic demands of HSCs. A metabolic rewiring to oxidative phosphorylation (OxPHOS) occurs upon differentiation and lineage commitment. Cancer stem cells are dependent on OxPHOS or glycolysis, depending on the cancer type, for growth and maintenance. In CML LSCs, OxPHOS is crucial for producing energy and anabolic precursors, thus providing an attractive metabolic vulnerability that might be exploited for their selective targeting. Moreover, hypoxia stabilizes Hypoxia-Inducible Factors 1 (HIF1), a transcription factor that plays a crucial role in regulating the proliferation, maintenance, and survival of LSCs (Ng et al., 2014).

In CML, physiologic hypoxia has been shown to promote the maintenance of CML LSCs despite effective inhibition of the BCR::ABL1 protein. Hypoxia has been found to potentiate the survival of quiescent CML leukemia-initiating cells (Ng et al., 2014).

Several miRNAs have been reported to be differentially expressed in response to hypoxia in cancer. For instance, a 14-miRNA hypoxia signature was derived as a prognostic marker for poorer overall survival in invasive bladder cancer (Khan et al., 2021). These hypoxia-responsive miRNAs (HRMs) are capable of simultaneously regulating numerous genes and influencing multiple components of a signaling pathway (Licausi et al., 2011). Future studies on HRMs will aid in understanding their function and regulatory basis in response to hypoxia.

There are no studies exploring the hypoxia-regulated miRNA in mediating the survival of LSCs.

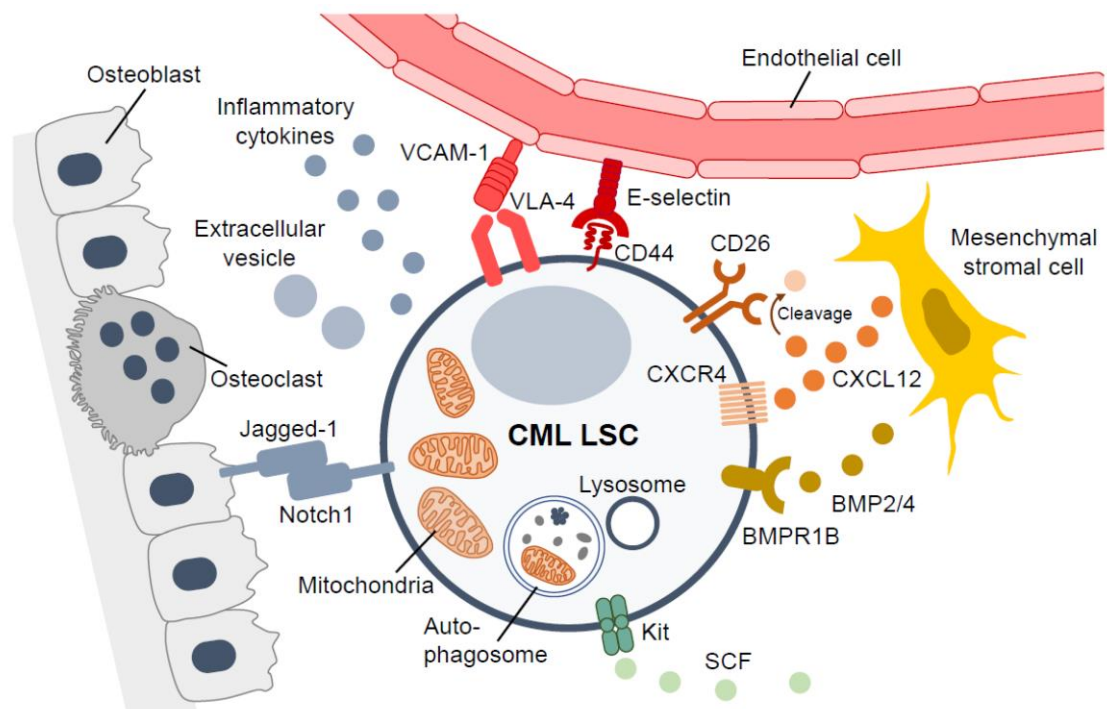


Figure 2.11. CML stem cells and the bone marrow microenvironment for CML LSCs.

CD26 disrupts the CXCL12/CXCR4 axis by cleaving CXCL12 to promote LSCs' mobilization into the blood. LSCs bind to VCAM-1 and Selectin on their bone marrow niche. Secretion of LSCs demands a tightly regulated metabolism. BMP2/4 and other chemokines/cytokines through auto- or paracrine mechanisms also support quiescence, cellular growth, and drug resistance of CML LSCs. LSC, leukemia stem cell; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen 4; CXCL12, C-X-C. (Ito et al., 2022)

2.8.2 Direct cell-cell contact interactions between CML LSCs and the BMM

2.8.2.1 Cell adhesion

One of the mechanisms controlling LSC survival is the interaction with the BMM (Schepers et al., 2015). The interaction of LSCs with the BMM is pivotal for the growth of leukemic cells, as it can confer resistance to treatment (Houshmand et al., 2019). This phenomenon has been elucidated through in vitro studies, where the growth inhibition of malignant cells by either JAK2 or ALOX5 inhibitors is observed solely in the absence of

stromal cells (Manshouri et al., 2011) and does not manifest the same effect in primary CML LSCs co-cultured with stromal cells (Dolinska et al., 2017) (Figure 4.11).

CD44: Elevation in the expression of the cell surface glycoprotein CD44 was observed in CML cells, facilitating their homing and engraftment through binding to extracellular matrix proteins and osteopontin. Furthermore, treatment with a CD44 antibody inhibits CML development in wild-type recipients in the *in-vivo* model (Krause et al., 2006).

E-Selectin: Various studies highlight the significance of E-selectins, crucial for the engraftment of CML cells (Krause et al., 2014), which are also prominently expressed on endothelial cells (ECs) within the AML BM environment, promoting chemoresistance within the vascular niche (Stucki et al., 2001)

Integrins: Integrins are essential in regulating leukemic cell functions both *in vitro* and *in vivo*. Integrin $\beta 1$, for instance, has been shown to modulate the proliferation of CML cells through adhesion to BM stromal cells and extracellular matrix components like fibronectin (Bhatia et al., 2001)

Fibronectin: The interaction of a leukemic cell line with fibronectin leads to treatment resistance of LSCs (Sengupta et al., 2009).

Galectin-3: Interaction with BM stromal cells induces the expression of Galectin-3 (Gal-3) expression in CML cell lines, promoting leukemic cell proliferation and consequent TKI resistance *in vitro*. Consistent with these findings, *in vivo* overexpression of Gal-3 encourages the lodgment of CML cells in the BMM (Yamamoto-Sugitani et al., 2011). Similarly, AML cells trigger the expression of a growth arrest-specific gene (Gas6) in the

BMM, further enhancing their proliferation. This same regulatory axis supports the self-renewal of CML LSCs (Jin et al., 2017).

N-cadherin: N-cadherin, responsible for mediating the adhesion of CML cells to the BMM, may induce TKI insensitivity in leukemic cells through the activation of β -catenin and heightened Wnt signaling (Zhang et al., 2013).

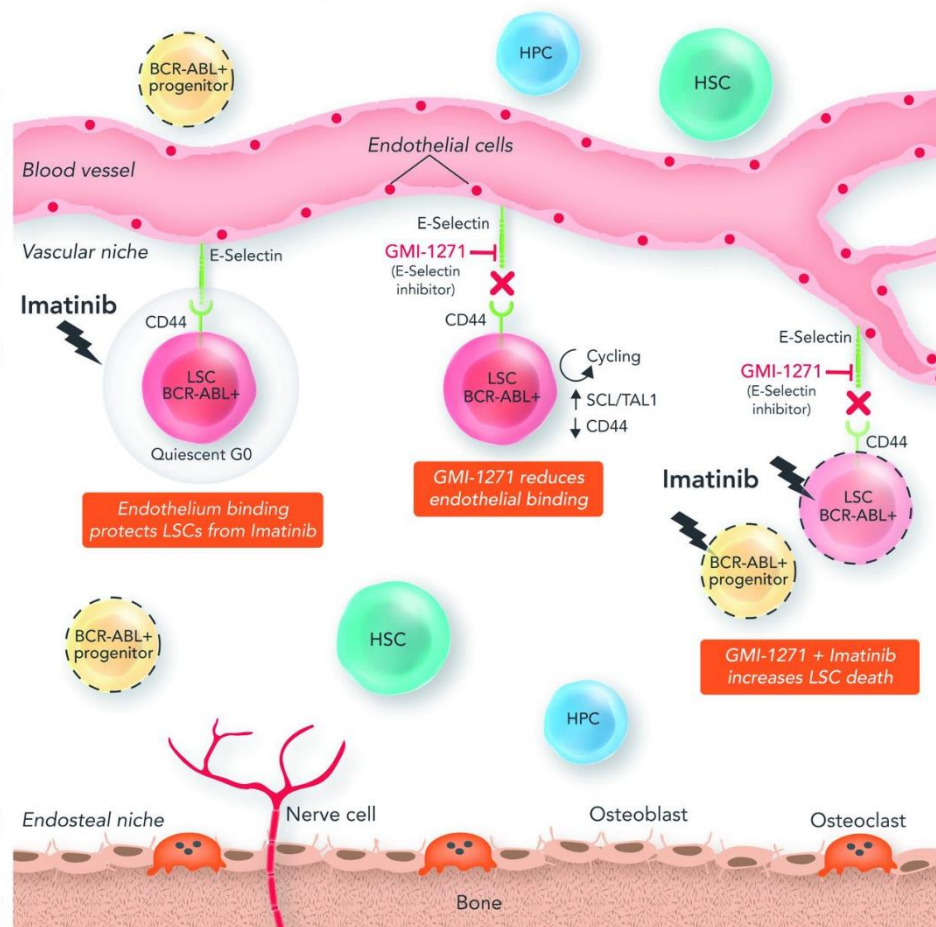


Figure 2.12. This schematic demonstrates the effects of E-selectin inhibition within the bone marrow microenvironment and the impact upon chronic myeloid leukemia stem cells and mechanistically how this is controlled by the SCL/TAL1 – CD44 axis. HPC: hematopoietic progenitor cell; HSC; hematopoietic stem cell; LSC: leukemic stem cell (Mitchell and Copland, 2020).

2.8.2.2 Cytokines and Chemokines:

The aberrant production of cytokines and chemokines within the BMM can drive the progression of the disease (Camacho et al., 2017).

An altered level of pro-inflammatory cytokines (including IL-1, IL-1b, IL 6, macrophage inflammatory protein-1 a (MIP-1a), MIP-1b, and TNF) or chemokines has been reported in CML BMM, and this was found not fully corrected upon TKI-induced remission. This differentially affects CML LSC and normal HSC in terms of maintenance and growth, providing a competitive advantage to LSCs (Zhang et al., 2012).

CXCL12: Levels of C-X-C motif chemokine ligand 12 (CXCL12), implicated in HSC maintenance, are reduced in CML BMM and are detrimental to HSCs but have been found to promote LSC expansion by increasing their self-renewing divisions (Agarwal et al., 2019).

CXCR4: In CML, CXCR4 is upregulated, leading to heightened survival of quiescent LSCs *in vitro* by inducing the migration of leukemic cells towards BM stromal cells . Consequently, inhibition of the CXCL12-CXCR4 axis restores the sensitivity of LSCs to TKIs *in vitro* (Jin et al., 2008).

Interlukin-1: Elevated expression of the IL-1 receptor in CML LSCs implies a contribution of IL-1 signaling to their maintenance. Correspondingly, treatment with IL-1 receptor antagonists curtails the growth of CML LSCs and exhibits a combinatorial effect when combined with TKI treatment (Zhang et al., 2016).

TNF α : tumor necrosis factor α (TNF α), an inflammatory cytokine often elevated in myeloid malignancies, fosters the survival of malignant cells (Gallipoli et al., 2013).

BMP: In CML BMM, there is an abnormal abundance of BMP2 and BMP4. Together with overexpression of their receptor BMPR1B, this leads to a strong upregulation of the BMP pathway in CML LSCs that has been implicated in their survival and expansion (Laperrousaz et al., 2013).

MSTNpp: High throughput screening of 313 human cytokines on the growth of CD34⁺CD38⁻ cells isolated from CML patients has confirmed the previously reported regulatory effects of IL-3, IL-1a/b, GM-CSF, and IFN γ but has also identified myostatin propeptide (MSTNpp) as a novel positive regulator. It was found that MSTNpp can indeed be detected in the plasma of CML patients and that it may be produced by mesenchymal stromal cells. By binding to a yet unknown receptor on the surface of CML cells, MSTNpp was confirmed to expand primitive cells via STAT5 and SMAD2/3 (von Palffy et al., 2020).

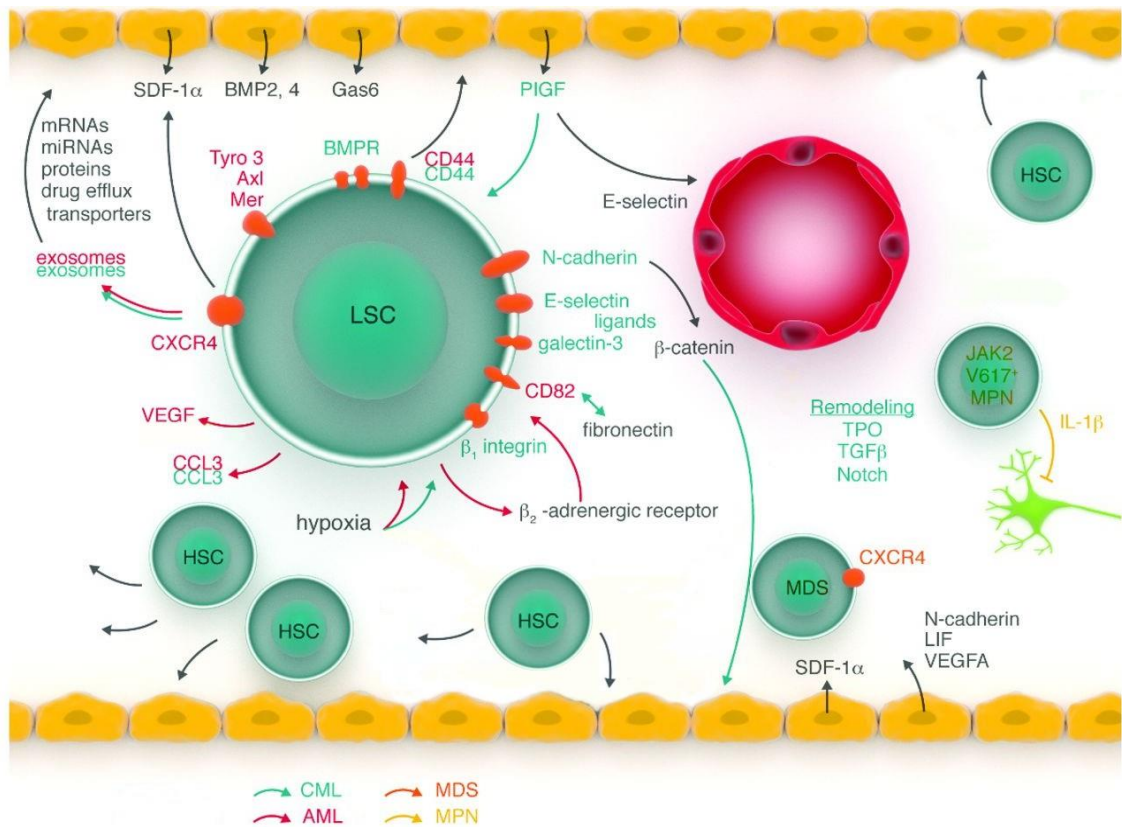


Figure 2.13: Malignant cells interact with their BMM through multiple pathways

2.8.2.3 Remodeling of BMM in CML

LSCs exhibit numerous shared biological characteristics with regular HSCs (Kreso and Dick, 2014). Considering the compromised nature of normal hematopoiesis in individuals with hematological malignancies, it has been hypothesized that LSCs possess the ability to outcompete HSCs and transform BMM into a self-reinforcing malignant environment (Hanahan and Coussens, 2012; Tabe and Konopleva, 2017). As a result, the BMM might play a pivotal role in both leukemic relapse and the progression of the disease (Figure 2.12)

In a transgenic mouse model of CML, the aberrant BMM produces a significantly higher quantity of cytokines that preferentially support the growth of malignant cells over SCTIMST, Trivandrum

healthy HSCs (Zhang et al., 2012). For instance, CML cells exhibit elevated production of G-CSF, which subsequently leads to decreased expression of CXCL12 by BMM cells. Furthermore, treating leukemic cells with imatinib partially restores the cytokine expression pattern, implying that leukemia induced the remodeling of the BMM (Zhang et al., 2012). Similarly, increased levels of GM-CSF, IL-6, and IL-1 α can be detected in the serum of CML mice (Welner et al., 2015).

The role of IL-6 is noteworthy, as exposing normal HSCs to CML cells heightens myeloid differentiation capacity, diminishes self-renewal potential, and induces altered gene expression in hematopoietic progenitor cells. Remarkably, treatment with an anti-IL-6 blocking antibody mitigates the onset of CML, indicating that BCR::ABL1⁺ leukemic cells facilitate the disease and influence normal hematopoiesis through IL-6 signaling. It is crucial to highlight that patient-derived CML cells evoke similar changes in normal HSCs and hematopoietic progenitors in culture, further substantiating the capacity of both mouse and human LSCs to modify the surrounding cells within the BMM (Welner et al., 2015).

Moreover, CML cells induce the secretion of PIGF by BM stromal cells during co-culture. This elevated PIGF level stimulates angiogenesis and proliferation of leukemic cells. Consequently, treatment with a PIGF antagonist prolongs the survival of CML mice and exhibits additional therapeutic efficacy when combined with imatinib, underscoring the ability of CML leukemic cells to reconfigure the BMM to promote disease progression (Schmidt et al., 2011) .

Using a similar CML mouse model, Schepers et al. demonstrate that CML cells remodel the BM endosteal niche, leading to an increased fraction of osteoblasts with an altered gene expression pattern and compromised ability to support normal HSCs (Schepers et al., 2013). Moreover, the removal of osteoblasts significantly accelerates leukemia development and diminishes the survival of CML mice. In this context, the CML BMM impacts the cycling of hematopoietic cells by upregulating the expression of the Notch ligand, Jagged-1 (Bowers et al., 2015).

2.8.2.4 Remodeling of BMM by exosomes.

Furthermore, the BMM can undergo reprogramming by malignant cells by releasing exosomes (Boyiadzis and Whiteside, 2015). Exosomes, the vesicles derived from the cell membrane containing protein and RNA, are potential mediators in intercellular communication (Mostafazadeh et al., 2021). The uptake of these exosomes induces changes in the secretion profile and modifies the proliferation and migration capabilities of BM stromal cells (Roccaro et al., 2013).

miR-126: In a CML blast crisis cell line transfers exosomes carrying miR-126 to endothelial cells (ECs) during co-culture. This transfer results in decreased expression of VCAM-1 and CXCL12 in ECs and a reduction in the adhesion capacity of the CML cell line (Taverna et al., 2014).

These investigations indicate that malignant cells establish a self-reinforcing niche by transmitting signals to neighboring BM cells. Consequently, the reshaped BMM becomes detrimental to HSCs while supporting leukemic cells, thereby accelerating

the progression of malignancy. There are no therapies targeting the altered CML BM microenvironment have been implemented in clinical practice.

2.9 iPSCs for disease modeling

2.9.1 Application of iPSCs in disease modeling

Reprogramming human somatic cells to induced pluripotent stem cells (iPSCs) that behave similarly to embryonic stem cells (ESCs), i.e., they are capable of self-renewal, expansion, and differentiation to all three germ layers, including blood (Choi et al., 2009; Park et al., 2008; Takahashi et al., 2007a; Yu et al., 2009). Because iPSCs recapitulate the entire genome of diseased cells, they are already used in modeling human diseases (Grskovic et al., 2011). iPSCs provide a unique opportunity to study human diseases in vitro, thus helping to unravel the complicated genotype-phenotype relationship in several monogenic disorders. iPSCs generated from patients with blood diseases can be used to generate blood cells carrying disease-specific genetic traits at the cellular level to recapitulate the disease phenotype under in vitro conditions and provide insights into the pathophysiology and emergence of the disease (Papapetrou, 2019a; Park et al., 2008).

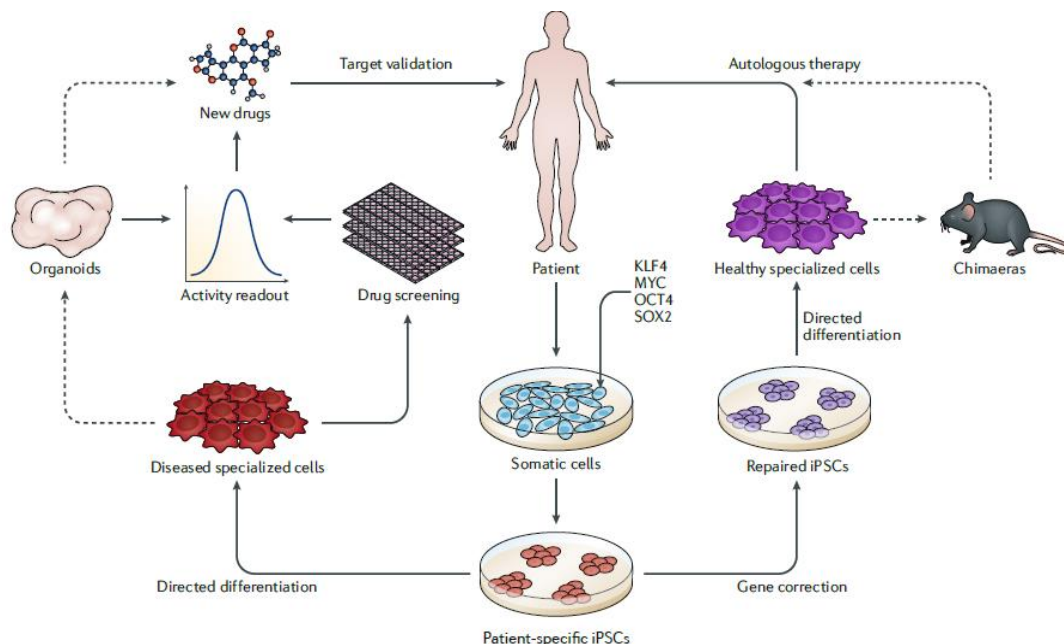


Figure 2.14: Progress in therapies based on iPSCs.

2.9.2 iPSCs to study LSCs

Due to the rarity of the LSC population, it is challenging to obtain sufficient cells for research to understand the mechanisms of TKI resistance of these cells and screen drugs to eradicate them. iPSCs derived from patients with genetic diseases or cells with genetic alterations can be differentiated into scalable quantities of disease-relevant differentiated cells (Hosoi et al., 2014; Kumano et al., 2012). Thus, they are a powerful tool for studying disease pathogenesis and drug screening. iPSCs generated from patients with leukemias help understand how oncogenes and patient-specific chromosomal abnormalities influence the development of leukemia-like phenotypes *in-vitro*.

Previous studies have demonstrated the successful generation of CML-iPSCs from peripheral blood mononuclear cells and CD34⁺ hematopoietic stem and progenitor cells (HSPCs) isolated from CML patients (Bedel et al., 2013; Charaf et al., 2017; Hosoi

et al., 2014; Imeri et al., 2023; Kumano et al., 2012; Miyauchi et al., 2018; Telliam et al., 2016; Turhan et al., 2019). CML-iPSCs exhibit resistance to IM like CML-LSCs, so they are considered suitable for investigating the cellular mechanisms underlying IM resistance. CML-iPSCs could be differentiated into hematopoietic progenitors that recapitulated some of the pathophysiologic features of the disease. ***However, it remains unclear how BCR::ABL1 expression and TKI treatment impact the efficiency and quality of iPSC generation from CML cells. CML-iPSCs generated from the same patient at various stages of the disease and from patients with different responses to TKI therapy are valuable resources for investigating molecular and epigenetic mechanisms of CML progression and drug resistance, leading to the identification of novel therapeutic targets and drug candidates for the treatment of CML. Moreover, iPSCs can be efficiently genetically manipulated by gene editing methods to identify specific genes involved in disease pathogenesis.***

2.10 Lacunae in research

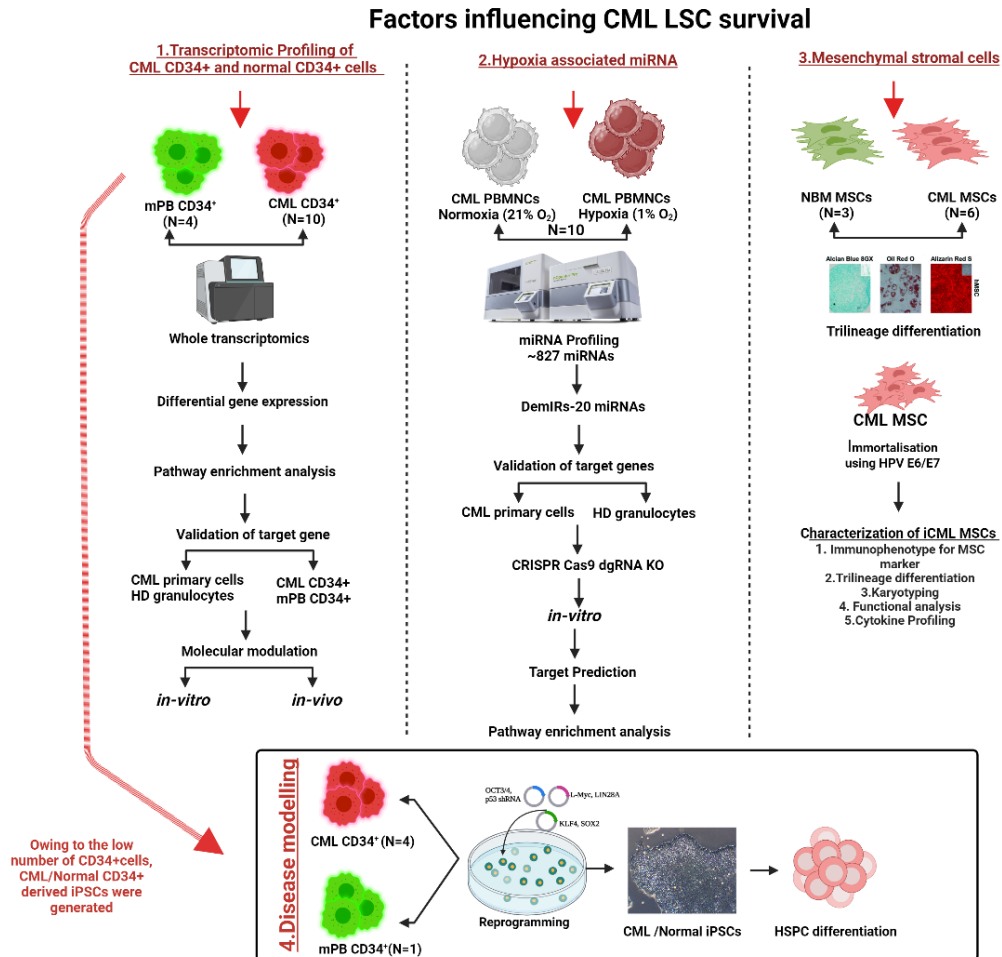
- ❖ Although several studies have compared transcriptomics in CML patients with good or poor responses to TKI, very few reports on transcriptomics using CML CD34+ cells; some of these studies have addressed only specific pathways in mediating CML LSC survival. There is no report on global transcriptome profiling in primary CML CD34+ cells and validation of identified targets in a large number of samples.
- ❖ Several studies have addressed the role of miRNAs in CML LSC maintenance, and modulating them could eliminate these LSCs. However, the role of

hypoxia-associated miRNA in regulating primary CML cell survival and maintenance is unknown.

- ❖ Primary CML MSCs have a limited lifespan and undergo senescence at the early passage. Currently, there is no CML patient-derived MSC line that can be used as a model to study the LSC-stromal interaction.
- ❖ As CML LSCs are a rare population of cells, several groups have generated induced pluripotent stem cells (iPSCs). However, it remains unclear how *BCR::ABL1* expression and TKI treatment could impact the efficiency and quality of iPSC generation from CML cells.

3. MATERIALS AND METHODS

3.1 Study design



3.2 Samples

3.2.1 Cell lines

EM-2, KU812, Lama84, KCL22, KYO, MEG01, JURL MK1, and K562 cell lines were procured from DZMS, German Collection of Microorganisms and Cell Cultures. Cell lines cultured in RPMI with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin and streptomycin (Gibco).

3.2.2 Primary cells

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After obtaining written informed consent, Peripheral blood / Bone marrow (BM) aspirates were collected from patients with chronic phase CML at diagnosis, post-treatment, and those with suspected lymphoma diagnosis (without bone marrow involvement- to be used as a source for normal MSCs). The IRB (Min No. 14754 dated 27.07.2022) approved this study.

3.3 Cell culture methods

3.3.1 Isolation of mononuclear cells

Briefly, Mononuclear cells (MNCs) were isolated using Lymphoprep (STEMCELL Technologies) by density gradient separation, and CD34⁺ cells were enriched immunomagnetically using an EasySep CD34 Positive Selection Kit (STEMCELL Technologies) following the manufacturer's protocol. Purity was verified by re-staining isolated cells with an allophycocyanin-labeled (APC) anti-CD34 antibody (Biolegend) and analyzing cells on a FACS Accuri C6 (BD) machine.

3.3.2 Culture and Expansion of CD34⁺ Cells

Cryopreserved CD34⁺ cells of the CML patients and the normal donor were thawed and cultured in CD34⁺ cell expansion medium constituting StemSpan™ SFEM II medium (Stem Cell Technologies) supplemented with CD34⁺ Expansion Supplement (Stem Cell Technologies) and UM729 (Stem Cell Technologies). The cells were cultured for 6-7 days with half medium change every alternative day to assess the cell proliferation.

3.4 RNA extraction

3.4.1 RNA Extraction from mononuclear cells

Total RNA was extracted from PB/BM Cells using TRI reagent (Sigma, St. Louis, USA) per the manufacturer's protocol. Briefly, 10×10^6 cells were lysed in 1ml of Trizol reagent, followed by adding 0.2ml of chloroform and incubated for 8 minutes. The mixture was centrifuged at 12000rpm for 15 minutes (4°C). The aqueous phase was transferred to equal volume of isopropanol and incubated for 10 minutes, centrifuged for 5 minutes at 12000rpm (4°C). The RNA pellet formed was gently washed with 75% ethanol (prepared using nuclease-free water) twice and centrifuged at 12000rpm (4°C). The pellet was air-dried for 2-3 minutes, resuspended in RNase-free water, and immediately stored at -80°C in aliquots.

3.4.2 RNA extraction protocol for low number of cells

Total RNA from the low number of cells was extracted using a Total RNA extraction purification plus kit (Norgen Biotek) per the manufacturer's protocol. Briefly, cells were lysed using Buffer RL buffer and then passed through the genomic DNA Removal column to remove the DNA contamination. Ethanol was added to the flowthrough and loaded onto an RNA purification column. The bound RNA is then washed with Wash Solution A, and the purified total RNA is eluted with the Elution Solution A. RNA concentrations were determined with a Nanodrop instrument (Thermo Fisher Scientific).

3.4.3 RNA Quantity and Quality

RNA concentration was determined by NanoDrop® ND-1000UV, which measures the absorbance at 260nm. Additionally, RNA quality was assessed in all the samples through electrophoresis in a 1% agarose gel in TAE buffer. High-quality, intact RNA appeared as two distinct bright bands showing the 28S and 18S ribosomal RNAs in the gel.

3.4.4 cDNA synthesis

Following manufacturer's instructions, 2µg total RNA transcribed to cDNA using a High-Capacity Reverse Transcription Kit (ThermoFisher Scientific). cDNA conversion was confirmed through RT-PCR by evaluating the housekeeping gene β glucuronidase (*GUS*) (Table 3.2).

3.5 Strand-specific RNA-sequencing

3.5.1 Samples

RNA was extracted from CML CD34⁺ (N=10) and mobilized peripheral blood (mPB) CD34⁺ (N=4) cells using Total RNA Purification Plus Kit (Norgen Biotek) as described earlier and then subjected to Strand-specific transcriptomics.

3.5.2 RNA QC

RNA QC was evaluated using Agilent Bioanalyzer 2100 and Qubit High sensitivity RNA assay.

3.5.3 RNA Sequencing

The transcriptome profile of CML CD34⁺ cells was compared to the mPB CD34⁺ cells, as well as publicly available datasets of normal CD34⁺ CD45RA⁻ and BM CD34⁺ (GEO ID: GSE48846 and GSM1937399). The raw reads were filtered using Trimmomatic for quality scores and adapters. The filtered reads aligned to the Human genome (hg19) using splice aware aligner like HISAT2 to quantify reads mapped to each transcript. The alignment percentage of the reads was in the range of 5.5-97.5% for all the samples. The total number of uniquely mapped reads was counted using feature counts. The uniquely

mapped reads were then subjected to differential gene expression using Deseq2 (Figure 1).

3.5.4 NanoString nCounter Plexset custom panel

With the differentially expressed gene list obtained from the transcriptomic data, we made a custom gene panel consisting of 21 target genes associated with cell surface markers, transporters, cell adhesion, and immune regulation based on previous publications. The expression of these 21 genes was then examined in an independent cohort of CML CD34⁺ (N=59) and mPB CD34⁺ (N=9) cells. Read counts were normalized using a positive control and three housekeeping genes (*ACTB*, *GAPDH*, and *GUSB*).

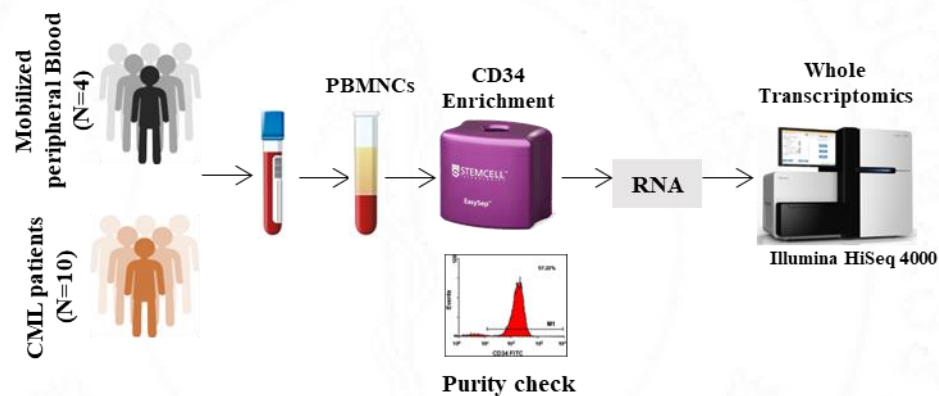


Figure 3.1: Schematic representation of the work outline of whole transcriptomics.

3.6 Establishment of hypoxic culture condition

3.6.1 Cell viability

CML cells were cultured under normoxic and hypoxic conditions. Cell viability were assessed with the trypan blue exclusion method. For cell lines, 0.5-1x10⁵ cells were

seeded in 24 well plates in 500 mL medium, and then cell viability was evaluated by culturing under normoxia and hypoxia inhibitors at 24, 48, 72, and 96h.

3.6.2 Cell proliferation

1×10^6 CML cells were stained with 10 mM CFSE (Carboxyfluorescein succinimidyl ester) for 15 minutes at 37°C in the dark. The cells were washed with a complete medium and maintained under normoxia and hypoxia conditions. Fluorescence intensity was measured using flow cytometry every 24 hours.

3.6.3 Cell cycle analysis

EM-2/ KU812 cells (1×10^6 cells/ml) were cultured in normoxia and hypoxic conditions for three days. Cell cycle status evaluated by using propidium iodide (PI) stain. The cells were collected appropriately and washed with PBS. The cells fixed with ice-cold 70% ethanol. Cells incubated at 4°C for 30 mins and followed by PBS wash. Initially, the cells were treated with RNase A (50ul of 100ug/ml) incubated at 37°C for 30 minutes, followed by PI (200ul of 50ug/ml) incubated on ice for 20 minutes. The cells were analyzed with FACS Calibur to detect PI fluorescence, which shows the cell cycle profile.

3.6.4 Immunofluorescence

1×10^5 cells were cytopsin and then fixed using 4% paraformaldehyde for 15 min, followed by permeabilization using 0.1% TritonX-100 (Sigma) in phosphate buffer saline (PBS) for 15 min and blocking using a buffer containing 1% bovine serum albumin (BSA) and 5% Fetal bovine serum (FBS) in PBS for 20 min at room temperature. The cells were incubated with anti-HIF1 α primary antibodies (Table 3.8) in the blocking buffer overnight (16 hours) at 4°C , followed by incubation with suitable labeled SCTIMST, Trivandrum

secondary antibodies for 2 hours at room temperature. For nuclear staining, the cells were incubated with 4',6-diamidino-2- phenylindole (DAPI) for 10 min at room temperature and visualized under a fluorescence microscope (DS6000, Leica Microsystems) with appropriate filters(Manian et al., 2018).

3.7 miRNA Profiling by Nano-string

3.7.1 Experimental design

CML primary cells were cultured under normoxic and hypoxic conditions for 48h, and then RNA was extracted using the Trizol Method (Sigma-Aldrich). CML cells showing increased *GLUT1* expression were subjected to miRNA profiling (Table 3.2). NanoString miRNA pre-designed panel simultaneously detects >800 human miRNAs, including five housekeeping transcripts, and was used to evaluate the dysregulated miRNA in hypoxic conditions(Jet et al., 2021).

3.7.2 Data analysis

All data analysis was performed using nSolver software. miRNA expression was assessed to identify differential expression between CML cells maintained under normoxia vs. hypoxia conditions.

3.8 Reverse transcriptase polymerase chain reaction

3.8.1 Analysis of BCR::ABL1 fusion transcripts

cDNA was subjected to 2 rounds of nested PCR as reported previously, and agarose gel electrophoresis was performed to identify the type of *BCR::ABL1* fusion transcript [e13a2 or b2a2/e14a2 or e1a2 or b3a2] (Table 3.2)(Markose et al., 2009).

3.8.2 BCR::ABL1 kinase domain mutation screening

As reported previously, mutations in the BCR::ABL1 kinase domain were screened using Sanger sequencing. The sequences were aligned using SeqScape (Applied Biosystems), and the type of the mutation was predicted using Mutation Taster (<http://www.mutationtaster.org/>)(Table 3.2)(Markose et al., 2009).

3.8.3 Real-time Quantitative PCR for mRNA

Quantitative RT-PCR was set up with SYBR Premix Ex Taq II (Takara Bio) using specific primers (Table 3.2) and analyzed with QuantStudio 6 Flex PCR systems (Applied Biosystems, Carlsbad, CA, USA).

3.8.4 Quantitative reverse transcription PCR for miRNA

RNA will be extracted and reverse transcribed using miR-X Single step CDNA Kit (Takara Bio). qRT-PCR will be performed in an Applied Biosystems 7500 instrument using the SYBR Green Advantage Real-Time Master Mix Kit (Takara Bio) as per manufacturer's instructions.

3.8.5 Droplet digital PCR

Droplet digital PCR was performed as described by Datari et al. Briefly, a duplex ddPCR reaction mix was prepared comprising of 1 X ddPCR Supermix for Probes (Bio-Rad, München, Germany), primers, probe, and template cDNA (2000 ng of reverse transcribed RNA). After sealing, the samples transferred to the QX200 Droplet Generator (Bio-Rad) and approximately 20,000 droplets per well was generated. The PCR was carried out at 95°C for 10 minutes, 94°C for 30 seconds, and annealing and extension at 60°C for 90 seconds, ending with 98°C for 10 minutes and holding at 12°C. Positive and negative droplets were counted using the QX200 Droplet Reader (Bio-Rad) and assessed

by Poisson statistics using the QuantaSoft software version 1.7.4.0917 (Bio-Rad, München, Germany).

3.9 Immunoblot

Protein lysates prepared using radioimmunoprecipitation assay buffer supplemented with a protease inhibitor mixture and phenylmethylsulfonyl fluoride. The cell lysate was added onto a 10% SDS-polyacrylamide gel and then PVDF membrane. The membrane was blocked using non-fat dry milk powder in tris-buffered saline containing Tween 20 and incubated with primary antibodies (Table 3.7). The proteins were detected using chemiluminescence kit and imaged using the FluorChem E system (Bedel et al., 2013).

3.10 Molecular inhibition

3.10.1. Preparation of lentiviruses

For the generation of lentiviruses, the gRNA and Cas9 expression plasmids were co-transfected with pMD2.G envelope (Addgene 12259) and psPAX2 packaging plasmid (Addgene 12260) (gifts from Didier Trono). The virus supernatants were collected after 48, 60, and 72 hours, pooled together, and concentrated 100 times using the Lenti-X Concentrator (Takara Bio) and stored at -80°C as aliquots.

3.10.2 Lentiviral-mediated CTSG knockdown in CML cells

For stable CTSG suppression, EM-2 cells were seeded at 2×10^5 cells/24-well and infected with 20ul of the concentrated lentiviral vector containing shRNA constructs targeting human CTSG or a non-targeting sequence (Tet-pLKO-puro (Addgene: 21915))(Figure 3.2) in the presence of polybrene for 24 hours. The expression of the

shRNA was induced with doxycycline (Dox) (1 μg/ml) for 5 days; subsequently, western blotting and *in vitro* assays were performed.

Figure 3.2: Vector map for Tet-pLKO-puro

3.10.3 Generation of CRISPR-based dual-guide RNA knockout of miR-30b

The dgRNAs were cloned in the pKLV2.2-h7SKgRNA5(SapI)-hU6gRNA5(BbsI)-PGKpuroBFP-W (Addgene plasmid # 72666; a gift from Kosuke Yusa)(Figure 3.3), to generate pKLV2.2 dgRNA expression lentiviral vectors. The plasmid was first digested with SapI for cloning the first gRNA and then with BbsI for cloning the second gRNA. The oligos for gRNA1 and gRNA2 were synthesized with specific 5' overhangs for cloning at the SapI and BbsI sites of the plasmid.

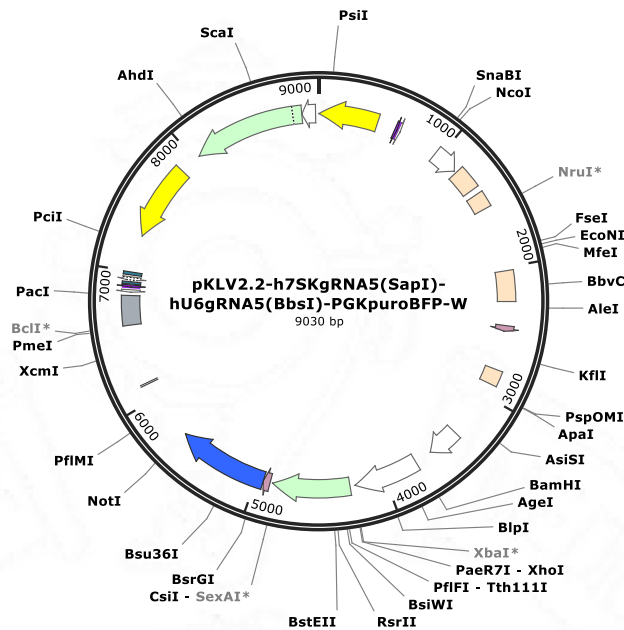


Figure 3.3: Vector map for PLKO2.2

3.10.3.2 Generation of Cas9 expressing cell lines

The K562 cell line was transduced with pCLIP-Cas9-Nuclease-EFS-Blasticidin (Transomic, Cat No. V085) lentiviral vector, and selected using 2 µg/mL of blasticidin starting five days after transduction, for ten days.

3.10.3.3. Transduction of dgRNA lentiviral vectors in K562 cell lines

K562-Cas9 cell lines were transduced with pKLV2.2 dgRNA viruses in the presence of 8 µg/mL polybrene by spinfection at 2250 rpm for 1.5 hours at room temperature. After six days, BFP⁺ cells were flow sorted and further cultured to estimate mutations caused by the dual gRNAs at the targeted genomic region.

3.10.3.4. Capillary Electrophoresis

The first round of PCR was carried out with target genomic locus-specific primers that flank the gRNA binding regions. The second round of PCRs was performed using a 5' SCTIMST, Trivandrum

FAM labeled forward primer and the same reverse primer used in the first round of PCR. The second round PCR products (~2µl) were denatured in deionized formaldehyde and analyzed by capillary electrophoresis using an ABI 3500 Genetic Analyzer (Thermo Fischer Scientific). The peak sizes and heights of the amplified products were determined using the Peak Scanner software (Thermo Fisher Scientific).

3.11 Colony forming unit – assay

Colony-forming cell assays were performed to investigate the proliferation and differentiation of hematopoietic and leukemic progenitor cells by their ability to form colonies of mature blood cells in a semisolid medium. Briefly, 1,000 cells were mixed in 1 mL MethoCult (STEMCELL Technologies), and then the number of colonies was scored on day 14 following the manufacturer's protocol.

3.12 Xenotransplantation model

EM-2 cells were transduced with lentivirus containing the Dox inducible shCTSG followed by Dox induction for 48h. These cells induced with and without dox were transplanted into sub-lethally irradiated (2 Gy) 8–10 weeks old NSG mice (The Jackson Laboratory, Bar Harbor, ME, USA) via tail vein. The median survival of mice was evaluated (Rothe et al., 2020).

3.13 Generation and characterization of CML MSC

3.13.1 Isolation and culture of bone marrow-derived MSC

BM-MNCs from healthy and CML patients resuspended in α -MEM containing 20% FBS and 1% penicillin-streptomycin and plated at an initial 5×10^5 cells/cm² density. After three days, the cells were washed with PBS (Media Tech Inc.), and the remaining

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adherent cells were cultured until the cells reached approximately 80% confluency. MSCs from passage one were cryopreserved and stored for RNA/DNA (Zhang et al., 2019).

3.13.2 Immunophenotyping of CML MSCs

Healthy donor and CML patient-derived MSCs were subjected to trypsinization and washed once with PBS. The cells incubated for 20 minutes with 1µl of fluorochrome-conjugated antibodies (all purchased from BioLegend) (Table 3.5). Flow cytometric data were analyzed using FlowJo software (BD Biosciences).

3.13.3 Generation of Immortalised CML MSC lines (iCML MSC)

Cryopreserved MSCs from the first passage were revived and used for immortalization. 0.2×10^6 cells were transduced with TRE-HEE-UbC-hKO1-rtTA lentiviruses encoding HPV16-E6/E7 (HEE) genes (a kind gift from Yukio Nakamura) by spinfection at 2250 rpm for 1.5 h (Figure 3.4). They were cultured in 20% MEM, with half-medium change on alternate days. The medium was supplemented with 1 µg/mL doxycycline (dox) (Sigma-Aldrich, St. Louis, MO, USA) from the fourth day. The cells were maintained throughout the culture at a seeding density of $5\text{--}6 \times 10^5$ cells/mL with medium changed on alternative days. Single cells were sorted and characterized immunophenotypically for the MSC surface markers (Bagchi et al., 2021).

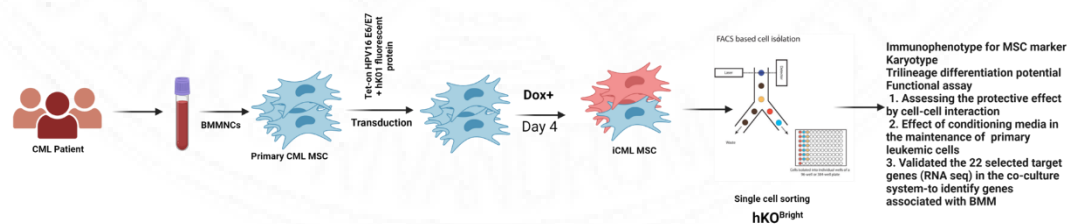


Figure 3.4 Experiment outline of immortalization of CML MSCs

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3.13.4 Multilineage differentiation

Trilineage differentiation for osteogenic, adipogenic, and chondrogenic lineage was evaluated in the primary CML MSCs, iCML MSC line, and HS-5 (Healthy donor iMSCs) as control. Cells seeded in 24 well culture at 5000 cells/cm² for adipogenic differentiation, expanded to sub-confluence, and replaced with the adipogenic differentiation media (Stem Cell Technologies). Undifferentiated MSCs cultured on a stromal medium were used as a control. After 21 days, cells fixed with 10% formalin, washed, and stained with Oil Red O. All images were acquired using the Olympus virtual slide system. The Oil Red O staining was eluted with 100% isopropanol for 15 minutes at room temperature, and dye concentration was quantified by absorbance measurement at 510nm (Vinod et al., 2023).

3.13.4.1 Adipogenic differentiation

Following adipogenic differentiation, the cells fixed with 10% formaldehyde, washed, and stained with Oil Red O (Sigma). Parallel controls cultured on a stromal medium were also stained, and all images were visualized using the Olympus virtual slide system. The Oil Red O staining was eluted with 100% isopropanol for 15 min at room temperature, and dye concentration was quantified by absorbance measurement at 510 nm.

3.13.4.2 Osteogenic differentiation

After 28 days of differentiation, the pellets were subjected to fixation using 4% paraformaldehyde, paraffin-embedded, sectioned (4µm).

Alizarin Red: After deparaffinization and hydration, the sections were stained with Alizarin red solution for 5 minutes, followed by dehydration, and mounted using a DPX mount.

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Von Kossa stain: Sections were incubated with 1% silver nitrate solution for 20 minutes under UV light. After distilled water rinse, the slides were treated with sodium thiosulphate and then counterstained with nuclear fast red each for 5 min. Imaged using the Olympus virtual slide system.

3.13.4.3 Chondrogenic differentiation

After 28 days of differentiation, the pellets were fixed using 4% paraformaldehyde, paraffin-embedded, sectioned (4 μ m), and subjected to confirmatory staining.

The following protocols were used for staining the individual samples:

Alcian blue stain: Alcian blue stain was used to assess glycosaminoglycan accumulation. Sections were treated with Alcian blue (pH: 2.5, Cat no: J60122, Alfa Aesar, US) and counterstained with neutral red.

Safranin O fast green: First, the sections were stained with Wiegert's Iron Hematoxylin, followed by incubation with acid alcohol, Fast green solution, acetic acid, and finally with Safranin O solution.

Toluidine blue: 0.1% dye solution for 5 minutes (CI 52040 Qualigens).

PicroSirius red: 0.1% dye concentration counterstained with Hematoxylin

3.13.5 Immunohistochemistry

3.13.5.1 Collagen type II

After chondrogenic differentiation, the sections underwent antigen retrieval by treating the sections with pronase (1mg/mL) and hyaluronidase (2.5mg/mL). The histopathology sections were then incubated with primary antibody mouse monoclonal anti-collagen type II antibody (5 μ g/mL) (DSHB, II-II6B3) at 4°C. Subsequently, they were subjected to a 30-minute incubation with a secondary antibody (HRP labeled goat anti-mouse SCTIMST, Trivandrum

immunoglobulin (31430, Pierce, Wisconsin, USA)). The stained sections treated with a 3, 3-diaminobenzidine solution and then counterstained with Hematoxylin. Histological imaging of the stained sections was performed using an Olympus BX43F microscope.

3.13.5.2 Collagen type X

The osteogenic differentiated paraffin pellet sections were exposed to antigen retrieval using chondroitinase ABC (1mg/mL) and pepsin (2.5mg/mL). The sections were subjected to primary antibody incubation (mouse monoclonal anti-collagen type X antibodies (5µg/mL) (DSHB, II-II6B3)) at 4°C, followed by a 30-minute incubation with HRP labeled goat anti-mouse immunoglobulin antibody (31430, Pierce, Wisconsin, USA) (1:250 dilution) then stained with a 3,3-diaminobenzidine and then counterstained with Hematoxylin. Histological imaging of the stained sections was carried out using an Olympus BX43F microscope.

3.13.6 iCML MSCs functional analysis

For coculture experiments to show the interaction between CML CD34⁺ cells and the MSC stromal layer, the stromal cells were seeded in tissue culture dishes at 20,000 cells/cm² concentration. After 12h, CML CD34⁺ cells were added to the stromal layer at a ratio of 1:10 (stromal cell: leukemic cells). The protective effect was evaluated by measuring apoptosis after 48h with and without imatinib treatment using Annexin V.

Apoptosis analysis was performed by staining the cells with Annexin-V and PI (Annexin-V staining kit, Invitrogen – ThermoFisher Scientific) using the manufacturer's instructions and analyzed using flow cytometry.

3.13.7 Collection of conditioning media from the MSC

MSCs were seeded in tissue culture-treated plates in standard growth media and allowed

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to grow to 60-70% confluency. After the cells reached confluency, fresh, complete media was added to the cells. The conditioned media (CM) containing secreted factors was collected after 24 hrs, centrifuged and filtered through a 0.2µm filter. CM was aliquoted and stored at -20°C until further use (Zhang et al., 2013).

3.14 Cytokine secretion profiling by cytokine antibody array

Human Cytokine Antibody Array C Series 1000 (RayBiotech, Norcross, GA), allows us to detect 274 cytokines, was used per the manufacturer's instructions. Membranes with prespotted capture antibodies were blocked using 1X blocking buffer for two hours and then incubated with a conditioning medium for 3 hours at 25°C. Chemokines were assessed by incubation with biotinylated detection antibodies for 2 hours at 25°C. Following the addition of HRP-conjugated streptavidin and overnight at 4°C, signals were revealed by enhanced chemiluminescence (Cyanogen). Signals were captured by a 3-5sec exposure on a Chemidoc system (Biorad)(Xin et al., 2015).

3.13.1 Semi-Quantification of cytokine antibody array data

Chemiluminescent cytokine array data were semi-quantified with ImageJ software. The intensity of each cytokine spot was measured based on spot signal density. For every spot, the net density gray level was determined by normalizing with the negative controls from the measured density gray levels. The density gray level of six positive controls was used to normalize the results from different membranes. The density for each cytokine was then averaged over a duplicated spot signal.

3.15 Generation of iPSC from CML and healthy donor CD34+

3.15.1 Reprogramming of CML and normal CD34⁺ cells

The CD34⁺ cells were cultured in the expansion medium for 3-days and then nucleofected with episomal reprogramming plasmids (pCXLE-hOCT3/4-shp53 (Addgene: 27077), pCXLE-hSK (Addgene 27078) and pCXLE-hUL (Addgene: 27080) (kind gift from Shinya Yamanaka), as described previously. Electroporation was carried out using a Neon Transfection System (ThermoFisher) at 1600V, 10ms, and 3 pulses. After five days of culture, the nucleofected cells were transferred to Matrigel-coated plates containing Essential-8 (E8) medium (ThermoFisher Scientific). Individual colonies were hand-picked between day 21 and day 32 and passaged and maintained in mTeSR Plus medium (StemCell Technologies) supplemented with 10µM IM (Sigma Aldrich) to generate stable iPSC clones (Miyachi et al., 2018).

3.15.2 Characterisation of iPSCS

The colonies were dissociated using TrypLE (ThermoFisher Scientific) and then stained using a PE-conjugated TRA-1-60 antibody (ThermoFisher Scientific) for 20 mins To analyze the expression of TRA-1-60 in iPSCs. The cells were washed with mTeSR Plus (Stem Cell Technologies) containing Revitacell (ThermoFisher Scientific), and the expression was analyzed using Navios (Beckman Coulter) or FACS Aria (BD Biosciences) flow cytometers. The extent of purity of CD34⁺ cells after their magnetic separation from PBMNCs was measured by flow cytometry using APC-conjugated anti-CD34 antibody (BioLegend)(Manian et al., 2018).

3.15.3 Alkaline phosphatase staining

Alkaline Phosphatase staining was carried out using the Alkaline Phosphatase Detection Kit (Sigma-Aldrich) following the manufacturer's instructions (Bedel et al., 2013).

3.15.4 Immunofluorescence

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Immunofluorescence analysis of the pluripotency marker expression was evaluated as described before. Briefly, iPSC colonies were fixed, permeabilized, and then incubated with primary antibodies in the blocking buffer overnight (16 hours) at 4°C, followed by incubation with suitable labeled secondary antibodies for 2 hours at room temperature (Table 3.8)(Manian et al., 2018).

3.15.5 Trilineage differentiation

Trilineage differentiation of iPSCs to ectoderm, endoderm, and mesoderm lineages was carried out using STEMdiff tri-lineage differentiation Kit (Stem Cell Technologies) per the manufacturer's protocols. Briefly, iPSCs were dissociated using TrypLE™ (Gibco) and cultured in mTeSR + medium (Stem Cell Technologies) supplemented with 10 µM revitacell (ThermoFisher Scientific). On day 1, pre-warmed STEMdiff™ Trilineage medium was added to each well to promote ectoderm, endoderm, and mesoderm differentiation, and the plates were incubated at 37°C for 24 hours. The medium was changed periodically until day 5 (mesoderm and endoderm lineages) or day 7 (ectoderm lineage), after which immunofluorescence was performed according to established protocols (Table 3.8)(Manian et al., 2018).

3.15.6 Hematopoietic Differentiation of iPSCs

For the hematopoietic differentiation of iPSCs, we used the STEMdiff™ Hematopoietic Kit (Stem Cell Technologies). Briefly, iPSCs were either single-cell sorted or seeded as small aggregates in mTeSR Plus (Stem Cell Technologies). Subsequently, the culture medium was changed to STEMdiff™ A medium, with or without IM. After three days, 70,000 cells were reseeded in STEMdiff™ A medium and maintained for another three days. Following this, the medium was switched to STEMdiff™ B. After 12 days of SCTIMST, Trivandrum

culture, the cells present in suspension were harvested and analyzed by flow cytometry for the expression of hematopoietic surface markers(Miyauchi et al., 2018).

3.15.7 Colony forming assay

In each experiment, 7,000 hematopoietic progenitors generated from iPSCs were plated onto the MethoCult GF H4636 medium (Stem Cell Technologies). The number of colonies in each experiment was scored on day 14 following the manufacturer's instructions.

3.16 Quantification and Statistical Analysis

Two-tailed Student's t-test for unpaired samples with unequal variance or 1-way ANOVA with Tukey's correction for multiple comparisons using GraphPad Prism version 8 (GraphPad Software). Differences between various treatment groups were analyzed by 2-way ANOVA, with Tukey's correction for multiple comparisons, using GraphPad Prism version 8 (GraphPad Software). p values < 0.05 were considered statistically significant. Survival curves of mice were compared with the log-rank (Mantel-Cox) test.

Table 3.1 – Reagents used in this study.

Reagents	Company	Cat. No.
HISEP™ LSM 1077	Himedia	LS001
TRIzol	Sigma	T9424-200ML
Cell Lysis	Qiagen	158908
Protein Precipitate		158912
DNA Hydration		158914
1XRBC lysis buffer		52304
EasySep™ Human Whole Blood CD34 Positive Selection Kit II	Stem Cell Technologies	17879
StemSpan™ SFEM II		09655

StemSpan™ CD34+ Expansion Supplement (10X)		02691
UM729		72332
Imatinib mesylate	Sigma	SML 1027
Dasatinib		SML2589
Nilotinib		PHR2833
Ponatinib		TA9H9876F4A8
1X Polybrene		H9268
Doxycycline Hyclate		D9891-1G
Lenti-X-Concentrator		631231
miRNA First-Strand Synthesis KIT	Takara Bio	638313
miRNA TB green Advantage kit		638316
Sybergreen RT PCR Sybergreen RT PCR		4448890
RNA LATER	ThermoFisher Scientific	AM7024
CFSE Kit		C34554
Blasticidin		A1113903
High-Capacity cDNA Reverse Transcription Kit		4368814

Table 3.2 – List of oligos used in this study

Gene/mRNA	Primers/Probe	Primers (5'-3')
<i>GLUT1</i>	Forward Primer	TGCGCCCAACTTCGTCATGTCG
	Reverse Primer	CGTAGCCGTTTTTCGAAGATGCG
<i>VEGF</i>	Forward Primer	GATGAGCTCATTGTAATATGAG
	Reverse Primer	ATATTACCATTAGCTCATCTTT
<i>CTSG</i>	Forward Primer	TCTGCTGGCCTTTCTCCTACC
	Reverse Primer	CACATCTGCTCTGACCTGCTG
<i>SUCNR1</i>	Forward Primer	GGGAGTTGGAAGCAGTATCAG
	Reverse Primer	GCATGTCCCTGAAGTGATCTC
<i>GUS</i>	Forward Primer	CCTGTGACCTTTGTGAGCAA
	Reverse Primer	GTCTGCCGTGAACAGTCCA
<i>BCR::ABL1</i> RQ-PCR primers and primer probes		
BCR - e1a2	Forward Primer	CTG GCC CAA CGA TGG CGA
ABL	Reverse Primer	CAC TCA GAC CCT GAG GCT CAA
BCR b3a2	Forward Primer	TCC GCT GAC CAT CAA YAA GGA
ABL	Forward Primer	GAT ACG AAG GGA GGG TGT ACC A
ABL	Reverse Primer	CTC GGC CAG GGT GTT GAA
ABL	Probe	6-FAM TGC TTC TGA TGG CAA GCT CTA CGT CTC CT -TAMRA
<i>BCR::ABL1</i>	Probe	6-FAM - CCC TTC AGC GGC CAG TAG CAT CTG A - TAMRA

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<i>BCR::ABL1</i> Nested PCR primers	
BCR-e1-A	GACTGCAGCTCCAATGAGAAC
ABL-a3-B	GTTTGGGCTTCACACCATTCC
BCR-e1-C	CAGAACTCGCAACAGTCCTTC
ABL-a3-D	TTCCCCATTGTGATTATAGCCTA
ABL-a3-E3	TGACTGGCGTGATGTAGTTGCTT
BCR-b1-A	GAAGTGTTTCAGAAGCTTCTCC
BCR-b2-C	CAGATGCTGACCAACTCGTGT

Table 3.3 List of shRNAs and gRNAs

Gene	5'-3'
shCTSG	GTGCAGAGGGATAGGCAGTCTCGAGACTGCCTATCCCTC TGCAC
miR 30b dgRNA2 F	ACTGAAACTTGGTTTAAAGC
miR dgRNA2 R	GCTTTAAACCAAGTTTCAGT
mir30b del PCR FP	CAAGTAGATCCCTGCCAGCT
mir30b del PCR RP	TGTGAATGCTGTGCCTGTTC

Table 3.4 List of Restrictions enzymes used for cloning

Restriction enzymes	Company	Cat. No.
<i>Sap I</i>	New England Biolabs	R0569S
<i>Bbs I</i>		R0539S
<i>HindIII</i>		R0104S

Table 3.5 List of flow cytometry antibodies

Flow cytometry reagents	Company	Cat. No.
CD73 APC	Biolegend	344006
CD45 pacific blue		368540
CD34 APC		343608
CD90- PE CY7		328124
CD38 FITC		356610
TRA-1-60	ThermoFisher Scientific	MA1-023

Table 3.6 – List of western blot reagents

Western blot Reagents	Company	Cat. No.
Ladder	Gene Direx	PM0010500
Westar supernova	Cyanagen	xls30100
Stacking Gel Buffer -1ltr	BioRad	161-0799

Resolving Gel Buffer-1ltr	BioRad	161-0798
Blotting grade blocker, 300gm	BioRad	1706404
Acryl/BIS 19:1, 40% SOLN 500ml	BioRad	1610144
Laemmli Sample Buffer 30ml	BioRad	38220090
Immobilon P 26.5x3.75m roll PVDF 45µM	Merck	IPVH00010
PHOSSTOP	Sigma	4906837001
Complete™Ultra Tablets, Mini, Easy protease inhibitor	Sigma	5892970001
TWEEN 20	MP Biomedicals	11TWEEN201
Bovine serum albumin	HiMedia	MB083 100G
Bio-Rad Protein Assay Dye Reagent	BioRad	5000006

Table 3.7 List of Western blot antibodies

Antibodies	Company	Cat. No.
Anti-CTSG	Cell Signalling Technology	63665S
Anti-p-CRKL		3181S
Anti-Cas9		14697s
β-actin	Sigma	A5441

Table 3.8 List of immunofluorescence antibodies

Antibody	Company	Cat. No.
Anti-TRA-1-81	StemLight Pluripotency Antibody Kit (Cell Signaling Technology)	9656S
Anti-SSEA-4		
Anti-NANOG		
Anti-OCT-4		
Anti-HIF1a	Abclonal	A7684
Anti-PAX6	Cell Signaling Technology	60433
Anti-Brachyury		81694
Anti-SOX17		81778

4.RESULTS AND DISCUSSION

4.1 Differential gene expression of CML CD34⁺ vs. healthy donor CD34⁺ hematopoietic progenitor cells.

Imatinib has revolutionized the management of chronic myeloid leukemia (CML) by significantly improving treatment outcomes. However, a significant challenge in CML therapy arises from the development of resistance to TKIs, often attributed to the presence of leukemic stem cells (LSCs). These LSCs can evade standard treatment and are responsible for disease recurrence and progression. They exhibit a remarkable ability to survive through BCR::ABL1 independent mechanisms, suggesting the involvement of alternative signaling pathways in their survival and maintenance. Therefore, it becomes crucial to identify specific gene signatures that are selectively expressed in LSCs. Understanding the molecular mechanisms underlying LSC survival may help develop targeted therapeutic strategies to selectively eliminate LSCs and improve treatment outcomes in CML patients. One way to address this would be to compare the transcriptomic profiles between healthy and CML stem cells and identify specific gene signatures selectively expressed in CML stem cells. ***Although several studies over the years have addressed the differences between CML cells vs. normal HSCs to identify the signatures of these LSCs, very few reports exist using CML CD34⁺ cells.***

In the absence of a clear cell surface marker discriminating CML LSC from normal healthy HSCs, CD34⁺ cells are considered LSPCs and are studied by many groups. CML CD34⁺ cells have been reported to exhibit distinct metabolic differences, showcasing enhanced oxidative phosphorylation (OxPHOS) when compared to healthy HSCs (Kuntz et al., 2017). Tigecycline, an antibiotic, also effectively inhibited OxPHOS

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in treatment-resistant CML LSCs, both in vitro and in vivo, while sparing HSCs. In a Phase I trial involving relapsed/refractory AML patients, tigecycline demonstrated a satisfactory safety profile but failed to yield significant clinical benefits. This outcome was attributed to the shorter half-life of tigecycline (Reed GA et al., 2016).

Previous reports on transcriptomic signatures in CML have highlighted an increased expression of focal adhesion-related genes in CML CD34⁺ cells and have identified ILK as a crucial focal adhesion component required for LSC self-renewal in vivo. The study predominantly utilized in vitro and in vivo models to assess the effects of ILK inhibition. Zhao et al. (2022) reported the role of MS4A3 as a functional tumor suppressor in CML, where its downregulation resulted in the retention of leukemia stem/progenitor cells (LSPCs) in a state of differentiation blockage that rendered them insensitive to TKIs. In this study, exogenous MS4A3 protein was introduced using a targeted delivery strategy, although the feasibility of clinical translation for this approach remains uncertain. Thus, further investigations are imperative to devise more pragmatic methods for inducing MS4A3 reexpression or facilitating the delivery of ectopic MS4A3 (Zhao et al., 2022). Overall, these studies collectively emphasize the complexity of CML and the importance of targeting specific cellular processes and molecules for therapeutic intervention. They also highlight the significance of considering metabolic differences and alternative strategies in addressing treatment-resistant cells while acknowledging the practical challenges in translating laboratory findings into clinical applications.

In the present study, we used the global transcriptomics approach to identify selective gene signatures and pathways differentially expressed in CML CD34⁺ cells to comprehensively understand the mechanisms contributing to CML LSC survival.

4.1.1 Global transcriptome profiling of CML CD34⁺ and normal CD34⁺ cells by RNA-Sequencing

To identify the unique gene signature of CML LSCs, we compared the transcriptomics profiles of CML CD34⁺ and healthy donor CD34⁺ (mobilized peripheral blood CD34⁺ (mPB CD34⁺) cells by NGS in an Illumina NGS platform. Global transcriptome profiling of differentially expressed genes was evaluated by filtering based on the reads per kilobase million (RPKM) > 1 and a minimum of a 2-fold difference (CuffDiff, FDR-adjusted $p < 0.05$) between CML CD34⁺ (n = 10) and mPB CD34⁺ cells (n = 4). Principal Component Analysis (PCA) showed a distinct separation between the two groups, revealing an apparent clustering of samples aligning with their respective groups. (**Figure 4.1.1A**). We observed 1563 genes upregulated and 1400 genes downregulated in CML CD34⁺ cells compared to mPB CD34⁺ (**Figure 4.1.1B**). Top upregulated genes include *GSTM1* (Glutathione S-transferase mu 1), *AZU1* (Azurocidin 1), *LTF* (Lactotransferrin), *CTSG* (Cathepsin G), *HIST14C* (histone proteins) and downregulated gene include *CXCL13* (C-X-C ligand 13), *ITGA3* (Integrin Subunit Alpha 3), *CDH4* (Cadherin 4), *PRMD16* (protein arginine methyltransferase 16) (**Figure 4.1.1C**). Both the up and downregulated genes are involved in immune response, cytokine signaling, and cell adhesion.

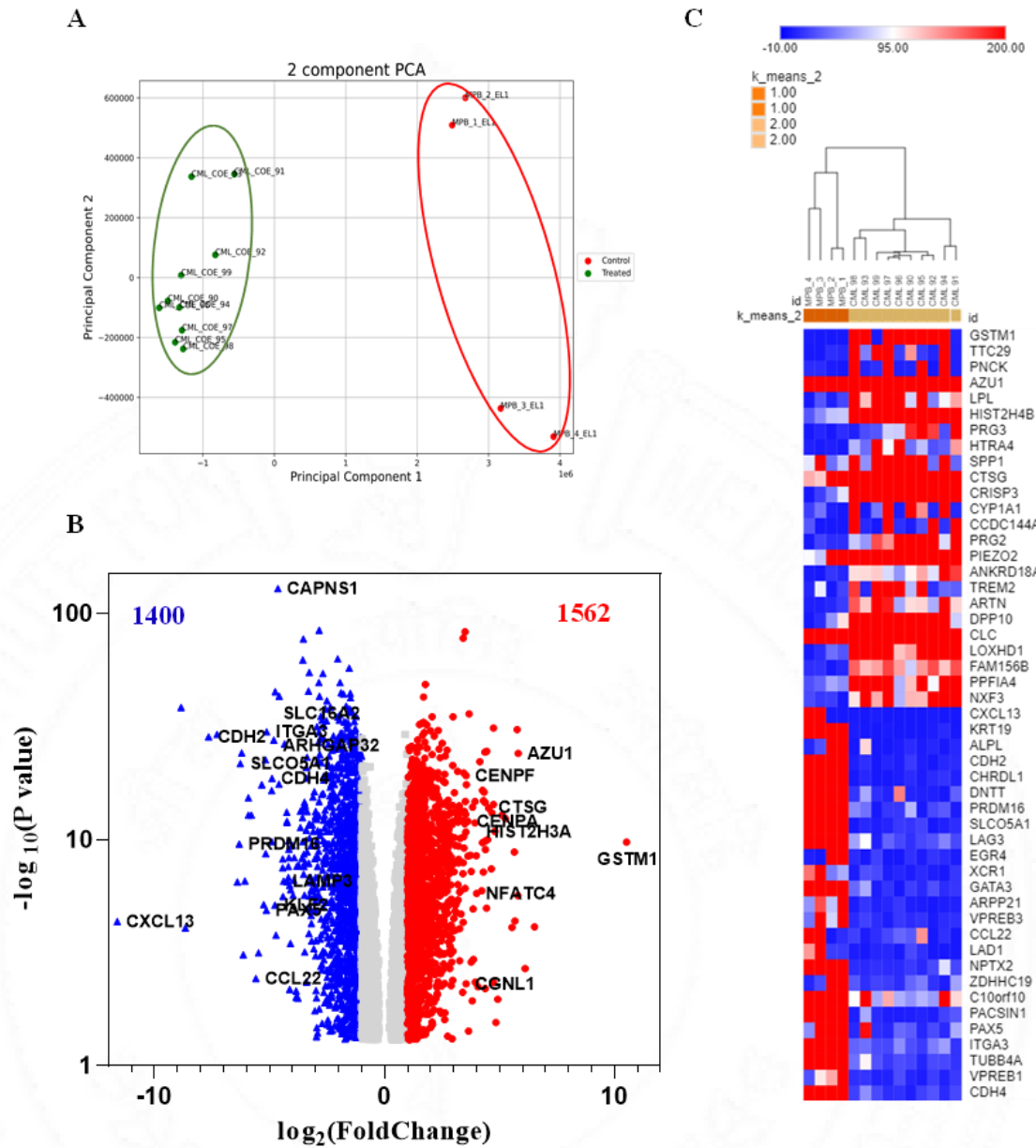


Figure 4.1.1 Global transcriptomics analysis of CML and mPB CD34⁺ cells. (A) Principal component analysis (PCA) plot of CML CD34⁺ and mPB CD34⁺ cells. (B) Volcano plot showing the significant upregulated and downregulated genes in the CML CD34⁺ cells. Red dots indicate upregulated genes, and Blue dots indicate downregulated genes between CML and mPB CD34⁺ cells (C) Heatmap visualizing the expression of the significantly differentially expressed genes showing top-up and downregulated genes with a 2-fold difference (FDR-adjusted $p < 0.05$) between CML and normal CD34⁺.

Pathway enrichment analysis of the top differentially expressed genes ($\log_2 F > 1$, $p < 0.05$) was done using Enrichr (Rothe et al., 2020)(<https://maayanlab.cloud/Enrichr/>). Gene ontology analysis using GO biological process, GO cellular component, and GO molecular function revealed genes associated with mitotic sister chromatid segregation spindle, single strand helicase activity, and mitochondrial matrix to be dysregulated in CML CD34⁺ (**Figure 4.1.2A-C**). Based on Reactome, KEGG, and MSigDB datasets, genes associated with cell cycle, Fanconi anemia pathway, and Metabolism were significantly dysregulated in CML CD34⁺ cells (**Figure 4.1.2D-F**). Similar to the previous studies, we observed enhanced OxPHOS in CML CD34⁺ cells compared to the mPB CD34⁺ (Kuntz et al., 2017). Genes such as *FANCI*, *FANCM*, *FANCB*, *FANCG*, and *FANCD2* associated with the Fanconi anemia pathway were upregulated, which could lead to enhanced DNA repair. In Metabolism, genes related to the ETC-complex I, such as NADH: ubiquinone oxidoreductase subunits and fatty acid β oxidation such as *ACADVL*, *NDUFAB1*, *ACADM*, and *DECR* were significantly upregulated in CML CD34⁺ cells. These results suggest CML CD34⁺ cells have altered Metabolism and enhanced DNA repair mechanisms. As reported earlier, the Fanconi anemia pathway dysregulation was observed in CML CD34⁺ cells (Chakraborty et al., 2012), leading to chromosome instability. For instance, human cord blood CD34⁺ cells transduced with BCR::ABL1 retroviral vectors showed impaired FANCD2 foci formation, whereas FANCD2 monoubiquitination in these cells was unaffected. The mechanism by which c-NHEJ factors disrupt the Fanconi anemia pathway in CML CD34⁺ cells is not yet fully understood, and further research is needed to elucidate this process. Similar to previous studies showing the alteration in the genes related to the mitochondrial respiratory

complex (MRC), we observed 30 genes associated with ETC-I to be significantly upregulated in CML CD34⁺ cells. Metabolic profiling of CML CD34⁺ vs. normal CD34⁺ has revealed enhanced OxPHOS in CML CD34⁺ (Kuntz et al., 2017). Dysregulation of mitochondrial metabolism can increase oxidative stress and DNA damage, leading to genomic instability (Flis et al., 2012). All these studies, including ours, suggest that mitochondrial metabolism can be a potential therapeutic target in CML.

Gene ontology analysis of significantly downregulated genes revealed the genes associated with positive regulation of cytokine production, Focal adhesion, and inhibitory MHCI receptor activity to be downregulated (**Figure 4.1.3A-C**). Pathway enrichment of significantly downregulated genes revealed the genes associated with cytokine signaling, inflammatory response, and TNF α signaling to be downregulated in CML CD34⁺ cells (**Figure 4.1.3D-F**). Together, these results suggest that in CML LSPCs, immune response, inflammation, and cytokine signaling are downregulated.

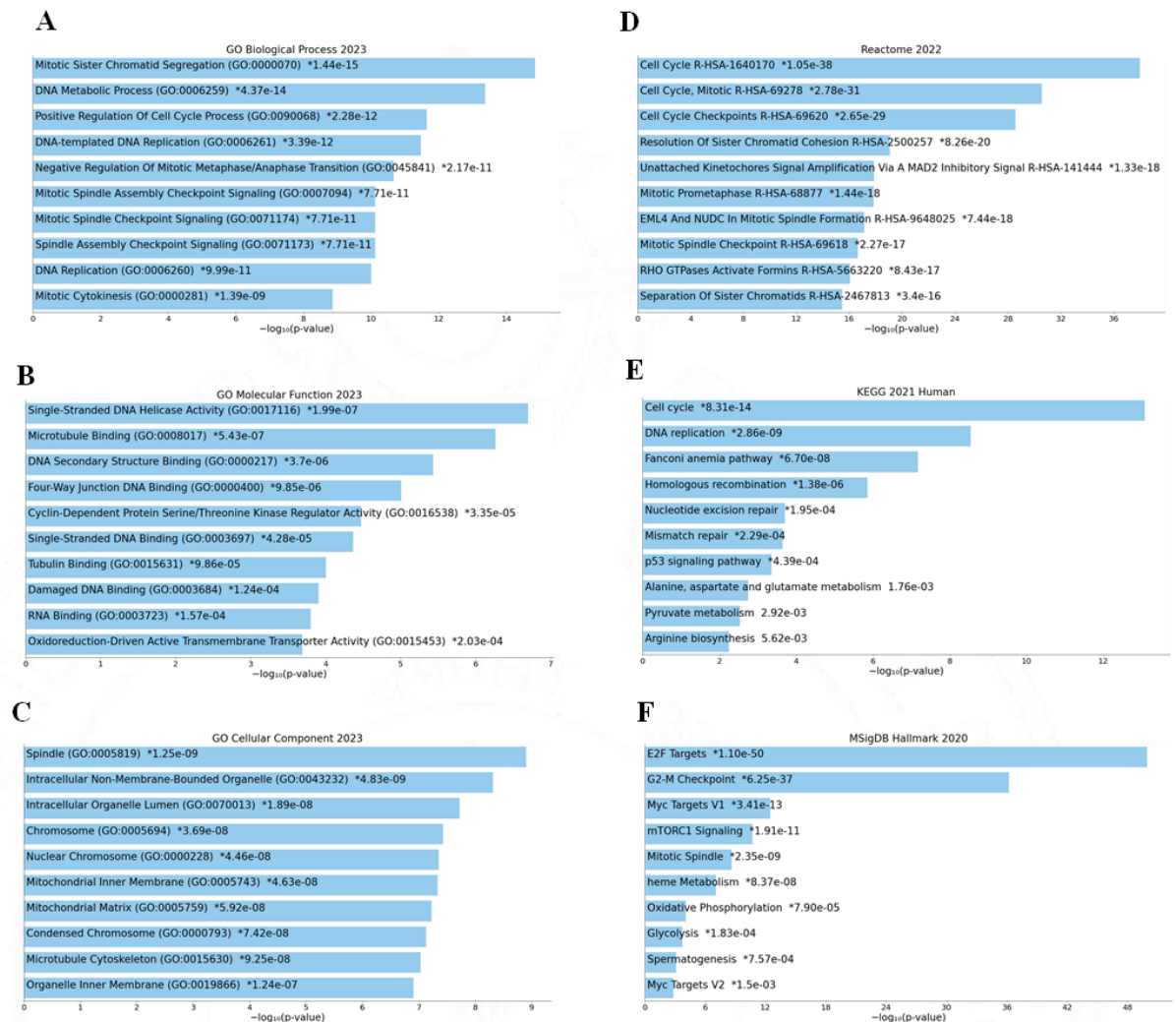


Figure 4.1.2 Pathways dysregulated in significantly upregulated genes: Enrichment analysis was performed using Enrichr A) Reactome 2021 B) MSigDB Hallmark 2020 C) KEGG 2021 D) GO Biological process E) GO cellular components and F) GO molecular functions of significantly enriched pathways was manually curated based on the smallest p-values obtained across all the databases.

4.1.2 Altered cell surface markers

We then evaluated alterations in cell surface markers in CML CD34⁺ cells, as these changes can serve as biomarkers and help distinguish between LSPCs and HSCs. Our analysis revealed that in CML CD34⁺ cells, the expressions of CD200R1, CD276, CD96, and CD36 were increased, while CD79A, CD37, and CD22 were decreased compared to mPB donor CD34⁺ cells (**Figure 4.1.4**). SCTIMST, Trivandrum

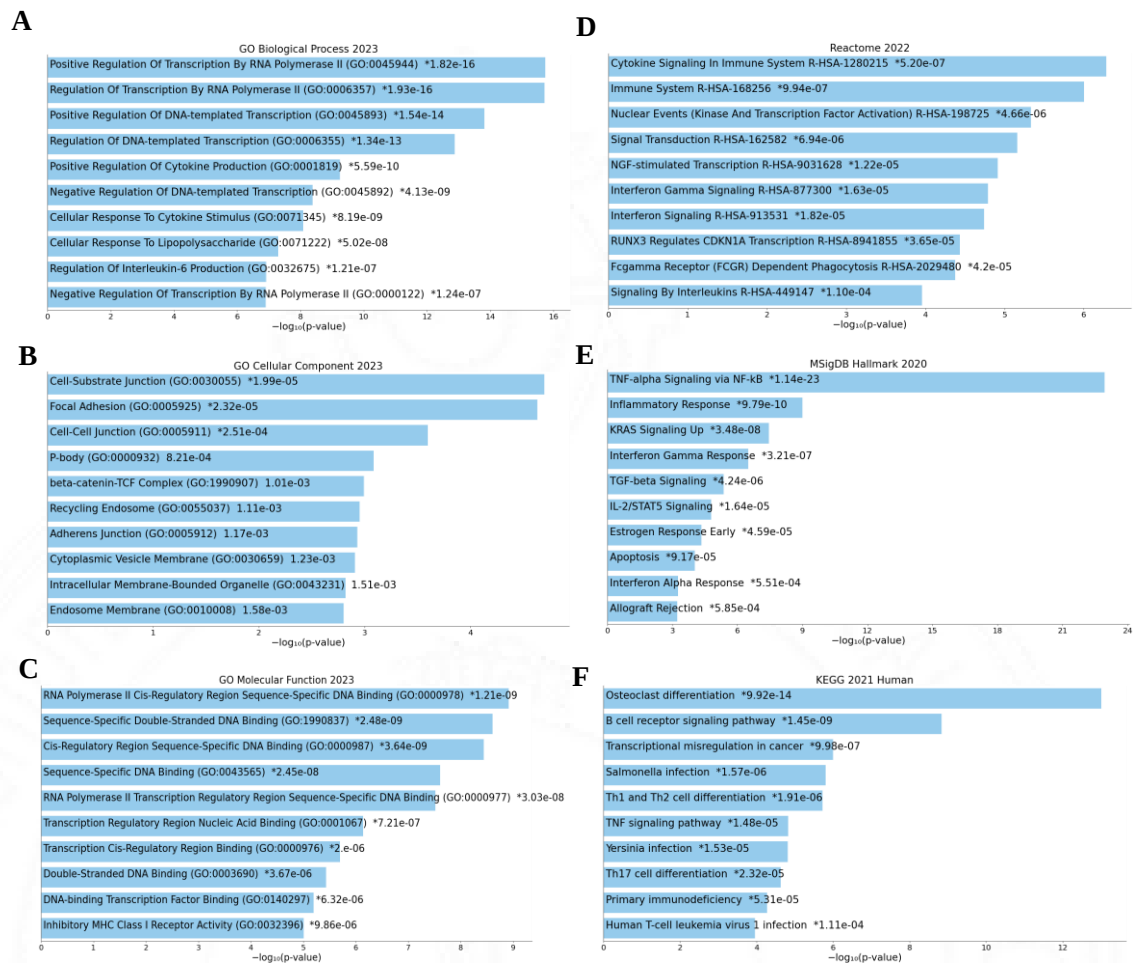


Figure 4.1.3 Pathways dysregulated in significantly downregulated genes: Enrichment analysis was performed using Enrichr A) Reactome 2021 B) MsigDB Hallmark 2020 C) KEGG 2021 D) GO Biological process D) Go cellular components and F) GO molecular functions of significantly enriched pathways was manually curated based on the smallest p-values obtained across all the databases.

CD36 is a receptor for oxidized low-density lipoprotein (ox-LDL) in macrophages and a fatty acid transporter in adipocytes. Interestingly, CD36 is upregulated in CD34⁺CD38^{low} CML cells, and the CD36-expressing subset within this population demonstrates reduced responsiveness to imatinib but heightened vulnerability to antibody-based therapeutic targeting (Landberg et al., 2018). CD276 plays a role in the regulation of proliferation, apoptosis, invasion, cell cycle progression, cell differentiation, cell autophagy, and epithelial-mesenchymal transition across various cancers (Liu et al., 2021). CD96 SCTIMST, Trivandrum

functions as an immune checkpoint that governs the antitumor activity of CD8⁺ T-cells. Overcoming chemoresistance mediated by PD-1 blockade by enhancing CD8⁺ T-cell function in cervical cancer has been achieved through CD96 targeting (Feng et al., 2023). CD96 is also reported as an LSC marker in AML (Hosen et al., 2007). Further research is warranted to elucidate the precise roles of these targets in normal hematopoiesis and determine their potential as therapeutic targets in CML.

CD79A, CD37, and CD22 are expressed on the surface of B-cells and play a crucial role in B-cell signaling and activation (Bobrowicz et al., 2020; Mason et al., 1995; Tedder et al., 2005). The downregulation of these markers in CML CD34⁺ cells suggests altered B-cell signaling and activation in the disease, leading to selective accumulation of myeloid cells and reduced lymphoid lineage. Even though previous studies have shown elevated expression of CD26 and CD25 in CML LSCs, we did not observe any difference (Herrmann et al., 2014; Sadovnik et al., 2017). One possible reason could be that transcriptomics was carried out using CML CD34⁺ (leukemic stem/progenitor cells) instead of CML CD34⁺CD38⁻, which is considered more primitive.

4.1.3 Aberrant expression of serine proteases in CML CD34⁺ cells

Among the significantly upregulated genes in CML CD34⁺ cells, one of the top targets was serine proteases. Notably, genes-associated serine proteases such as Cathepsin G (*CTSG*), Cathepsin L (*CTSL*), neutrophil elastase (*ELANE*), and ribonucleases (*RNASE*) were significantly upregulated in CML CD34⁺ cells (**Figure 4.1.5**). In normal hematopoiesis, serine proteases are expressed from common myeloid progenitors (CMPs) to myelocytes/neutrophils. Serine proteases have been implicated in the development and progression of solid and hematological cancers. In addition, proteinase 3 (*P3*), a serine

protease expressed by myeloid cells, has been shown to inhibit T-cell proliferation. Furthermore, neutrophil elastase (*ELANE*) and proteinase 3 (*PR3*), which are serine proteases, are aberrantly expressed in human myeloid leukemias. Serine proteases may play a role in the CML LSCs survival.

4.1.4 Aberrant expression of Cathepsin G, calcium-binding protein, and cell adhesion

The validation of targets acquired from the gene expression data can pose a substantial challenge, mainly due to the low proportion of CD34⁺ cells in both CML and mPB donors. However, we have successfully addressed this issue by utilizing NanoString technology, which could generate good-quality gene expression results even from 100ng of RNA. Based on Bloodspot (<https://servers.binf.ku.dk/bloodspot/>) and previous publications (Rothe K et al., 2013; Zhang et al., 2013; Bühner, E.D. et al., 2023), a 21-custom gene panel was designed. The expression of these 21 genes was evaluated in an independent cohort of CML-CD34⁺ (N=59) and mPB- CD34⁺ (N=9) samples.

We observed significant upregulation of *CTSG* and *ELANE* (**Figure 4.1.6A**) and considerable downregulation of *CDH2*, *S100A8*, *S100A9*, and *RXRA* in CML CD34⁺ cells compared to the normal (healthy volunteer) CD34⁺ (**Figure 4.1.6B**). *CTSG* and *ELANE* are primary granules, and *S100A8/A9* are secondary granules released into the environment in response to infection. *CTSG* is a multifunctional protease that plays a role in the immune response and can regulate inflammatory responses by stimulating the production of cytokines and chemokines. *CTSG* is also involved in other processes, such as the transmigration of leukocytes, platelet activation, and conversion of angiotensin I to angiotensin II (Rawat et al., 2021). Avilés-Vázquez S et al. have shown high

expression of *CTSG* and *ELANE* comparing CML $CD34^+CD38^-$ vs. normal $CD34^+CD38^-$ cells (Avilés-Vázquez S et al., 2017). Downregulation of *S100A9*, *S100A8*, and *CDH2* was also previously reported to be downregulated in CML $CD34^+$ cells (Buhrer et al., 2023; Zhang et al., 2013).

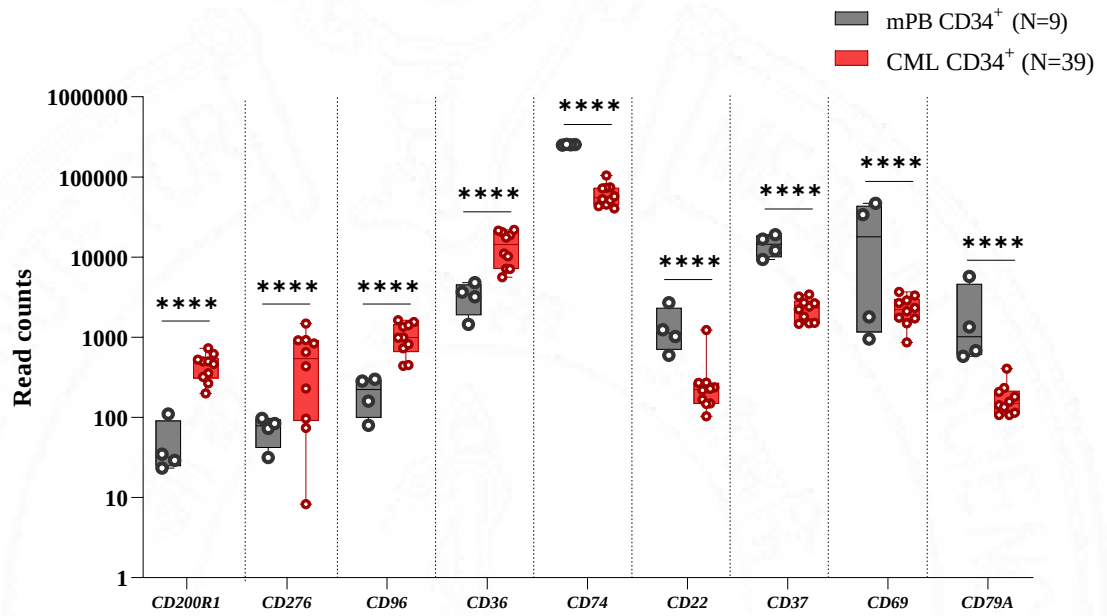


Figure 4.1.4: Altered Cell surface markers in CML $CD34^+$ cells compared with mPB $CD34^+$ cells. The Y-axis is in $\log_{10}$ scale

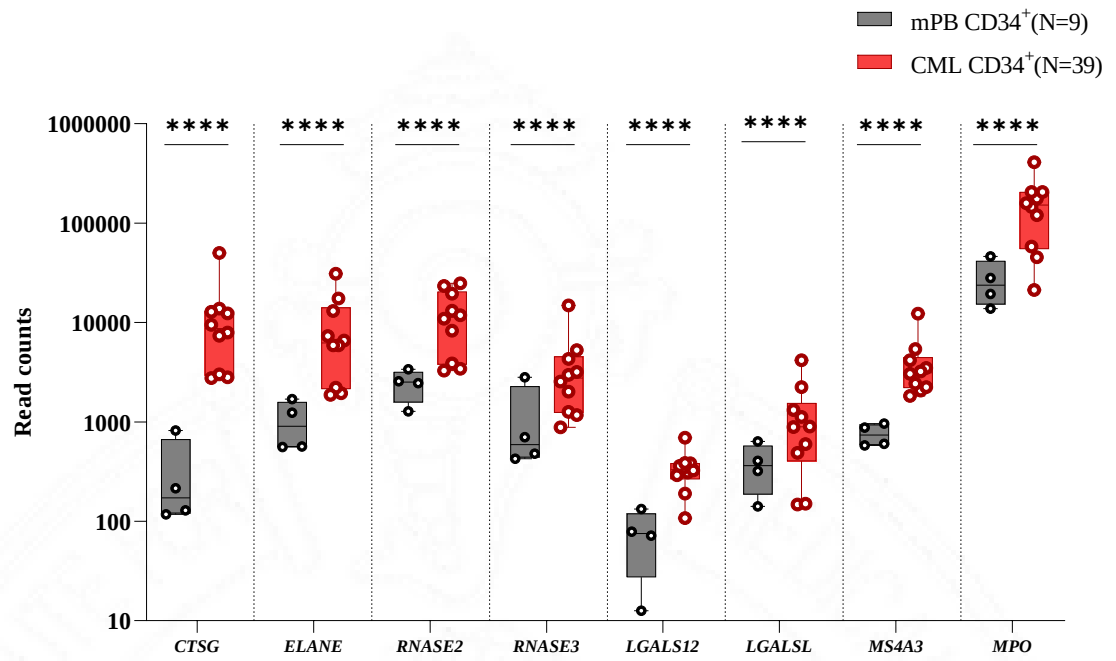
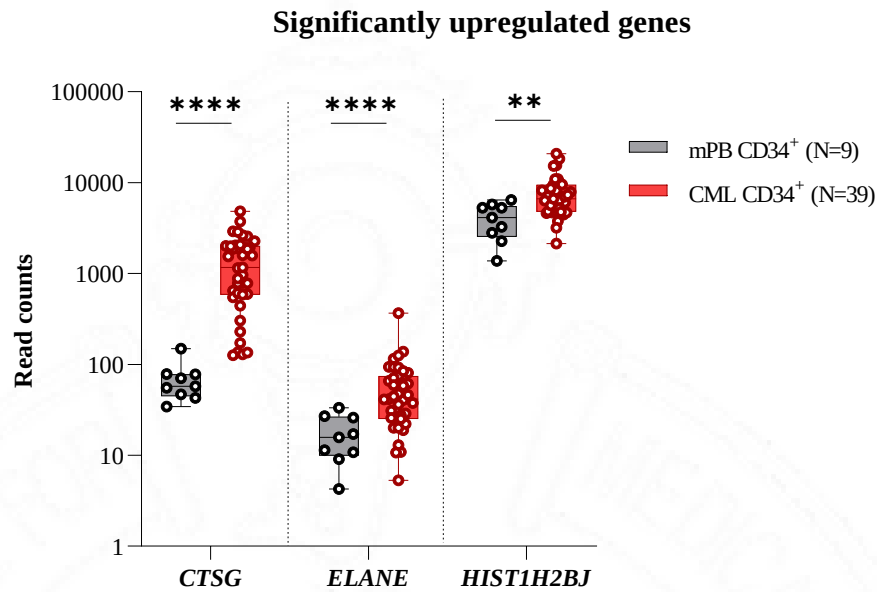


Figure 4.1.5: Aberrant expression serine proteases in CML CD34⁺ cells. The Y-axis is in the log₁₀ scale.

A



B

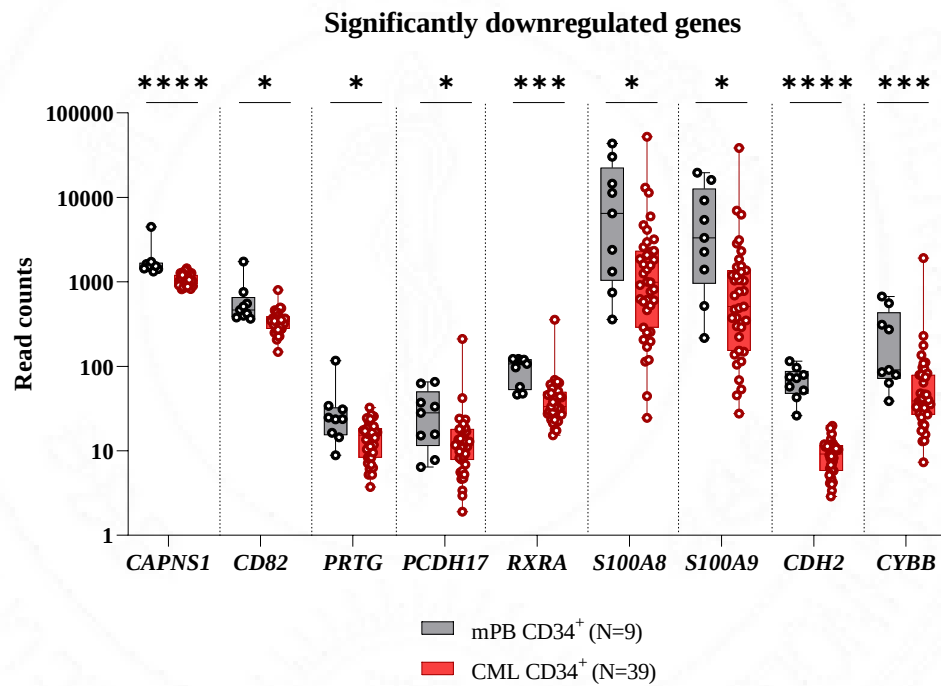


Figure 4.1.6: Validation of differentially expressed gene in CML vs. normal CD34⁺ cells. Bar graph showing log fold change of selected target genes in CML CD34⁺ normalized with the mPB CD34⁺ A) Upregulated B) Downregulated. The target gene expression was normalized with the housekeeping gene (GUSB, GAPDH, and ACTB). Y-axis is in log₁₀ scale.

4.1.5 *CTSG* expression is higher in CML CD34⁺ cells than in mPB CD34⁺ cells.

CTSG, a serine protease, is synthesized and expressed during the promyelocyte stage of hematopoietic development and is stored in the azurophil (primary) granules. It plays a multifaceted role in normal hematopoiesis, encompassing cell differentiation, immune response, antimicrobial activity, and platelet activation (Zhang et al., 2013). However, the precise functions and mechanisms of *CTSG* in hematopoiesis remain unknown. Elevated levels of *CTSG* have been linked to certain inflammatory conditions, autoimmune diseases, and hematological malignancies (Alatrash et al., 2017; Gao et al., 2018).

In the acute myeloid leukemia (AML) context, *CTSG* has been identified as a novel immunotherapeutic target. *CTSG* levels have been assessed in AML patients at diagnosis and relapse and found to be a potential biomarker for disease progression (Alatrash et al., 2017). As CML CD34⁺ cells were found to have high expression of *CTSG*, we aimed to assess it in CML primary samples using q-RT-PCR. There was significant upregulation of *CTSG* in treatment-naïve CML patients compared to healthy donor granulocytes, as depicted in **Figure 4.1.7A**. However, no significant difference in *CTSG* expression levels was observed between CML patients who achieved major molecular response (MMR) and those who did not (**Figure 4.1.7B**), suggesting that *CTSG* expression may not directly correlate with response to TKI therapy or disease prognosis in CML.

Further, the expression of *CTSG* was investigated in CML patients during blast crisis (BC) compared to those in the chronic phase (CP). *CTSG* expression was markedly reduced in CML blast crisis patients (**Figure 4.1.7C**). This result was further

corroborated by an immunoblotting experiment showing increased CTSG protein expression in CML CD34⁺ cells compared to mPB CD34⁺ cells (**Figure 4.1.7D**).

4.1.6 Molecular inhibition of *CTSG* in the CML cell line reduced the colony-forming capacity *in vitro*

To evaluate the functional significance of *CTSG* in CML, we carried out CTSG knockdown (*CTSG* KD) in the CML cell line. The experimental design is outlined in **Figure 4.1.8A**. Our initial screening of CML cell lines revealed that the EM-2 cell line expressed the highest level of CTSG protein expression (**Figure 4.1.8B**). Hence, we transduced EM-2 cells with doxycycline (dox)-inducible *CTSG* shRNAs (*CTSG* KD). After five days of dox induction, *CTSG* KD cells showed a marked reduction in the CTSG protein expression in *CTSG* KD cells compared with the scramble, confirming knockdown (**Figure 4.1.8C**).

We first assessed the difference in the colony-forming capacity after CTSG KD *in-vitro*. EM2 CTSG KD cells exhibited a reduced capacity for colony formation *in-vitro*, supporting the possible role of *CTSG* in the maintenance of CML stemness (**Figure 4.1.8D**).

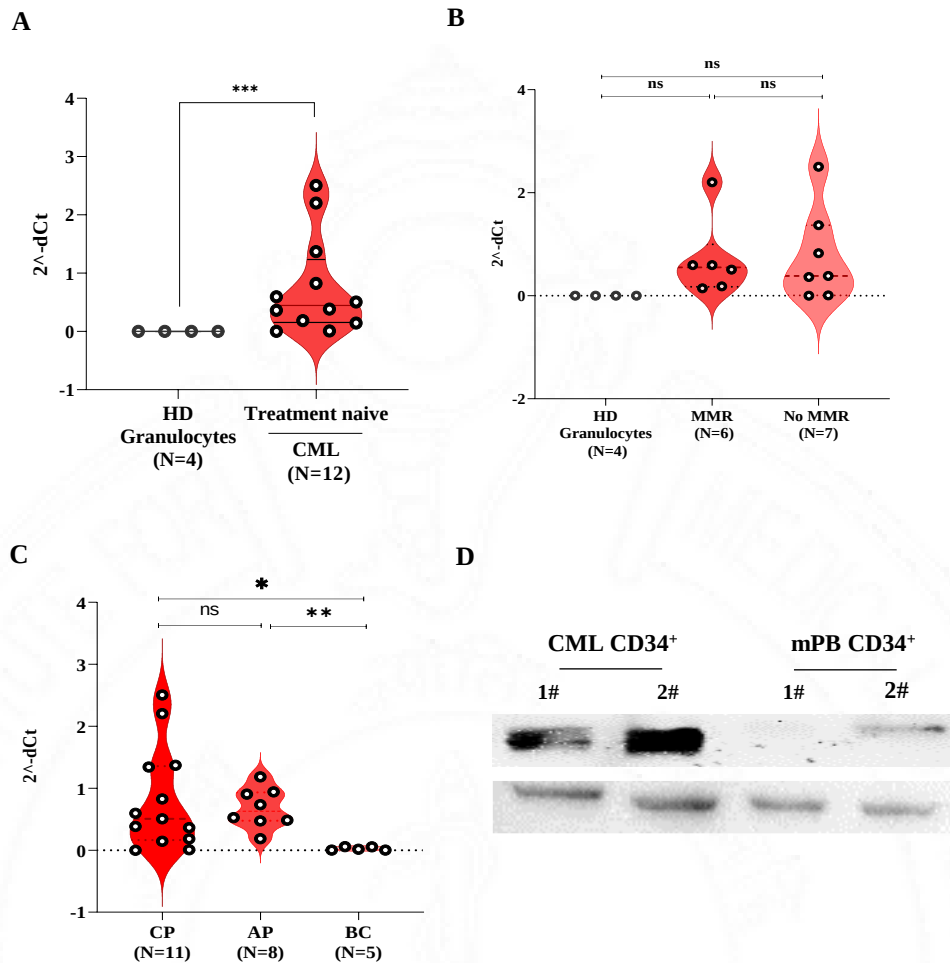


Figure 4.1.7: CTSG expression is high in CML patients. **A)** qRT-PCR analysis of transcript levels of CTSG in **A)** Treatment naïve CP- CML ($n = 12$) vs. Healthy donor (HD) granulocytes ($n = 5$) **B)** CML patients who achieved MMR ($n=6$) vs. no MMR with TKI ($n = 7$) **C)** CML patients at chronic phase (CP) ($n=11$), accelerated phase (AP) ($n=8$) and Blast crisis (BC) ($n=5$) normalized with β -ACTIN (housekeeping gene) **D)** Western blot analysis of CTSG levels in CML CD34⁺ ($n=2$) and normal CD34⁺ ($n=2$). β -ACTIN was used as the loading control.

4.1.7 CTSG KD reduces the leukemogenic potential *in vivo*

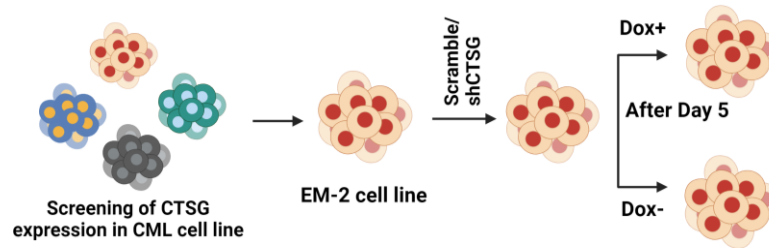
To investigate the impact of CTSG suppression on CML cell persistence and leukemogenic potential *in-vivo*, we transplanted EM2- CTSG KD cells, with and without doxycycline induction, into sublethally irradiated NSG mice (**Figure 4.1.9A**). One week

after transplantation, doxycycline hydrate was added to the drinking water provided to the mice to induce CTSG knockdown *in-vivo*.

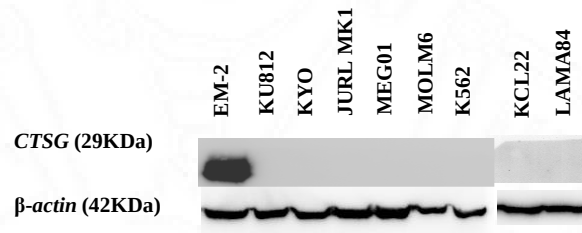
CTSG knockdown significantly increased the overall survival of the mice compared to the control group (**Figure 4.1.9B**), suggesting that the suppression of CTSG could potentially reduce CML cell survival and engraftment *in vivo*. These findings indicate that CTSG is a potential target for therapeutic intervention in CML. Strategies aimed at suppressing CTSG expression or activity could hold promise in reducing CML cell survival and disease burden.

Further investigations are warranted to elucidate the precise mechanisms by which CTSG influences leukemogenesis and to determine the specific downstream pathways or cellular processes affected by its suppression. Understanding the molecular and cellular consequences of CTSG knockdown in the context of CML will provide valuable insights for developing targeted therapies to disrupt the leukemogenic potential of CML cells.

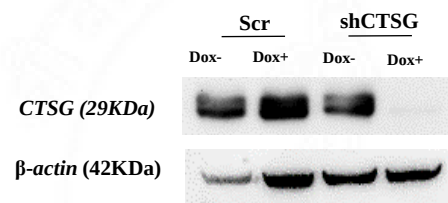
A



B



C



D

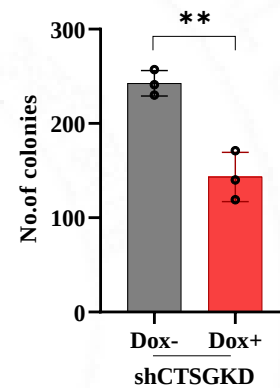


Figure 4.1.8: CTSG knockdown reduces stemness in CML cell line: **A)** Experimental outline of CTSG KD in EM-2 cell line; **B)** Western blot analysis of CTSG protein expression across CML cell lines **C)** CTSG protein expression before and after KD in EM2 cells. β-actin was used as the loading control. **D)** Colony formation assay in EM-2 CTSG KD and scrambled.

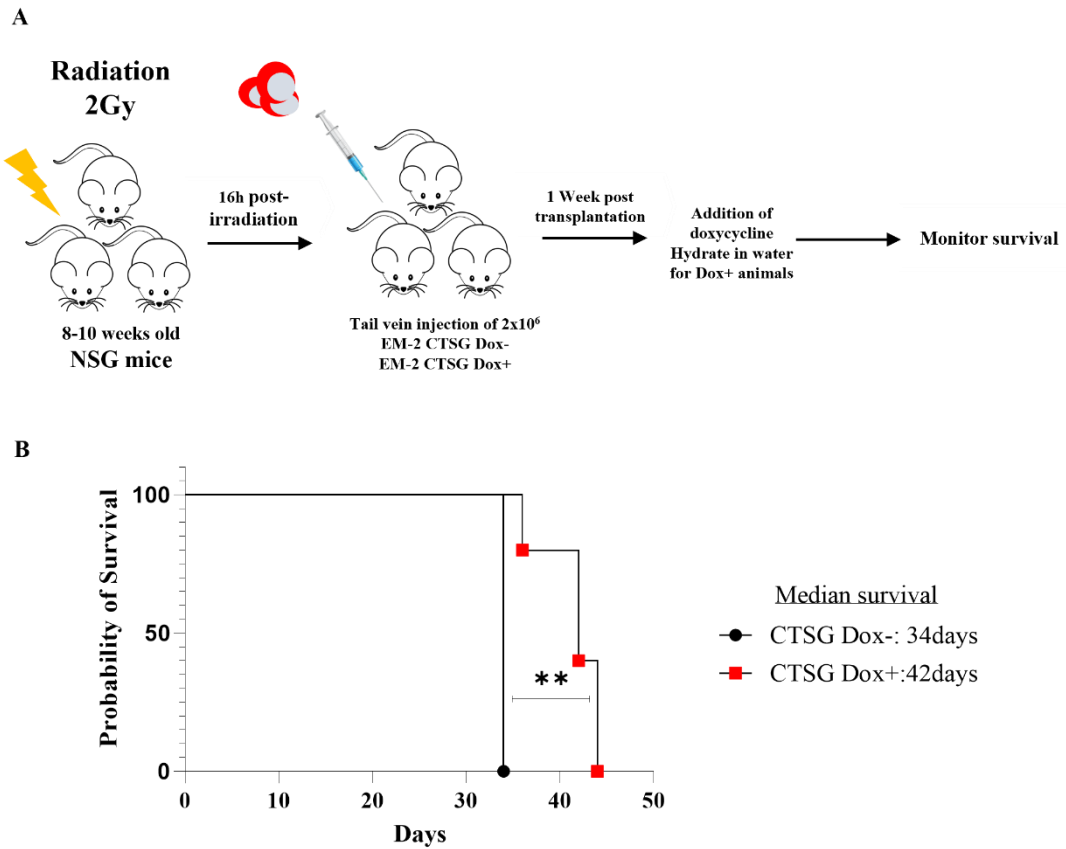


Figure 4.1.9 CTSG knockdown increased survival in NSG mice: A) Work outline of Cell line derived xenotransplant model. Dox-induced shCTSG KD cells were injected into NSG mice kept on dox or normal water and monitored survival. B) NSG mice injected with CTSG KD cells showed significantly improved survival compared to scrambled.

4.2 Role of hypoxia-associated miRNA in CML LSCs

Hypoxia, characterized by reduced oxygen levels, is the defining trait of the bone marrow microenvironment (BMM) where leukemia stem cells (LSCs) reside. Hypoxia-inducible factor 1 α (HIF-1 α) is a crucial transcription factor that gets activated and stabilized under hypoxic conditions (Simsek et al., 2010). It significantly regulates gene expression associated with various cellular processes, including cell proliferation, survival, and stemness (Licausi et al., 2011).

HIFs and hypoxia have diverse tumor-promoting effects in different types of leukemia (CML, CLL, ALL, and AML)(Forristal et al., 2015; Gao et al., 2015; Zou et al., 2013). In the context of CML, leukemic cells cultured under low oxygen conditions demonstrate resistance to IM, heightened clonogenic ability, and greater engraftment potential (Ng et al., 2014; Singh et al., 2022). Hypoxia has been shown to influence the expression of microRNAs (miRNAs) that regulate gene expression post-transcriptionally (Khan et al., 2021; Licausi et al., 2011). Several studies have explored the involvement of miRNAs in the maintenance of CML LSCs. For instance, miRNAs have been shown to mediate the abnormal metabolic characteristics observed in CML stem/progenitor cells(Lin et al., 2020; Salati et al., 2017; Wang et al., 2015). Notably, miR-300, a tumor suppressor miRNA, can induce quiescence in CML LSCs, while miR-126-3p regulates quiescence, self-renewal, and engraftment capacity of CML LSCs (Ruiz et al., 2021). Low oxygen tension induces a distinct shift in a specific group of miRNAs, termed HypoxamiRs. Emerging evidence indicates that hypoxia regulates several facets of HypoxamiR transcription, maturation, and function, influencing miRNA expression and supporting tumor growth(Licausi et al., 2011). ***Although the role of hypoxia-regulated miRNAs is well-documented in solid tumors, its role in CML LSCs survival remains unexplored.*** The current study aimed to determine the role of dysregulated miRNAs under hypoxic conditions, hypothesizing that hypoxia-associated miRNAs contribute to the survival and maintenance of CML LSCs.

4.2.1 Establishment of hypoxic culture condition in CML cell line

We cultured CML cell lines (EM-2 and KU812) and primary CML cells under low-oxygen conditions (1% O₂) to replicate a hypoxic microenvironment. Previous

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studies have shown that cells cultured under hypoxic conditions exhibit reduced proliferation, enter a slow-cycling state, and show induction of HIF-1 α expression and its downstream targets. These cellular responses to hypoxia are part of the adaptive mechanisms that allow cells to survive and function under low oxygen conditions (Hubbi and Semenza, 2015). Both CML cell lines under hypoxia showed reduced cell proliferation after 72 hours compared to cells cultured under normal oxygen conditions (21% O₂) (**Figure 4.2.1A**). Cell cycle analysis revealed an accumulation of cells in the G₀ phase, indicating a slow-cycling or quiescent state similar to stem cells (**Figure 4.2.1B**). We also observed HIF-1 α induction in both the CML cell lines cultured under hypoxic conditions, as evidenced by HIF-1 α translocation from cytoplasm to nucleus (**Figure 4.2.1C**). The expression of HIF-1 α downstream target *GLUT1*, responsible for glucose uptake, was upregulated in response to hypoxia (**Figure 4.2.1D**). Similar to the cell lines, CML primary cells maintained under low oxygen conditions (1% O₂) for 48h also showed induction of HIF-1 α protein expression (**Figure 4.2.1E**) and increased GLUT1 expression (**Figure 4.2.1F**).

4.2.2 miRNA Profiling of CML Primary Samples

To identify differentially expressed miRNA in CML cells under hypoxia, we performed miRNA profiling using the NanoString platform comprising 827 miRNAs previously reported in solid tumors (Armstrong et al., 2015). Briefly, RNA extracted from CML primary cells cultured in normoxic and hypoxic conditions were tested for *GLUT1* expression, and those samples with 2 fold increase in *GLUT1* under hypoxic conditions were taken for miRNA profiling.

NanoString nCounter technology is a platform that can be used for miRNA profiling at cellular levels. The technology utilizes molecular barcodes to quantify individual RNA molecules directly without reverse transcription or amplification. This makes it easier and faster to validate miRNA biomarkers than RNASeq or PCR-based platforms, and the results are highly reproducible. The nCounter miRNA Expression Panels are designed to provide a sensitive, reliable, and robust platform that works well with multiple sample types. We performed miRNA profiling for CML primary samples that showed high *GLUT1* expression under hypoxic conditions, as described in **Figure 4.2.2A**. The data was analyzed using nsolver, and differentially expressed microRNAs were identified using $P < .05$ and absolute $\log_{2}FC > 2$ as the cut-off, and *ACTB*, *B2M*, *GAPDH*, *RPL19*, and *RPLP0* were used as reference genes. Principal component analysis showed an overlapping signature between normoxia and hypoxia (**Figure 4.2.2B**), probably due to the heterogeneity among the primary CML cells. A total of 20 miRNAs were significantly dysregulated, including 5 upregulated and 15 down-regulated in CML cells cultured in hypoxic conditions (**Figure 4.2.2C**). Among the top differentially expressed miRNA, miR-210, known as hypoxia-regulated miRNA, was upregulated considerably under hypoxia ($\log_{2}FC=6.19$).

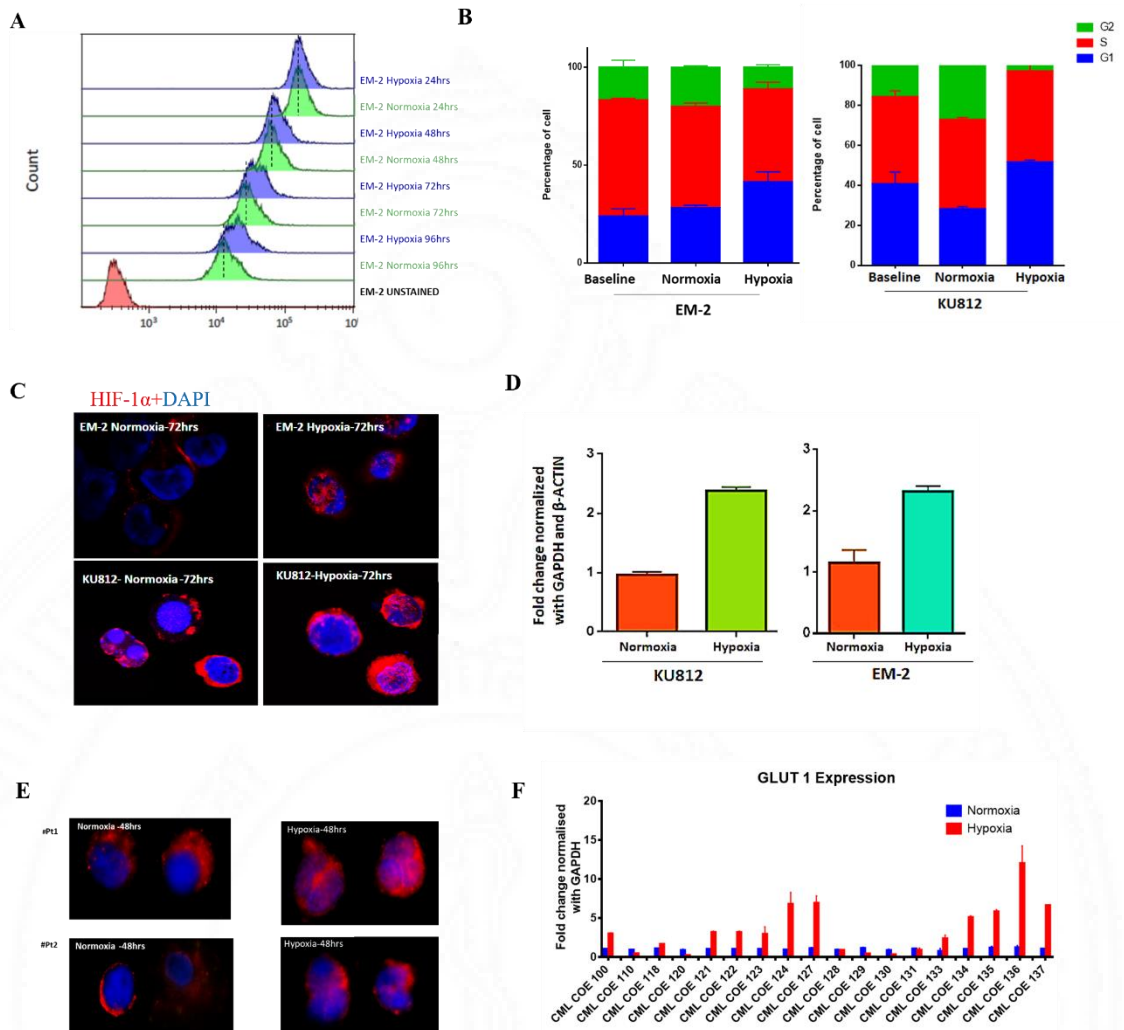


Figure 4.2.1: Establishment of Hypoxic culture conditions in CML cell lines and primary CML cells. CML cell lines cultured in normoxia and hypoxia were assessed for **A)** Cell proliferation of EM-2 cells by staining with CFSE, **B)** Cell cycle analysis, **C)** Immunofluorescence of HIF-1α, and **D)** qPCR for GLUT1 expression. **E)** Immunofluorescence of HIF-1α and **F)** qPCR for GLUT1 after culturing CML primary cells under normoxic and hypoxic conditions for 48h normalized with housekeeping gene GAPDH.

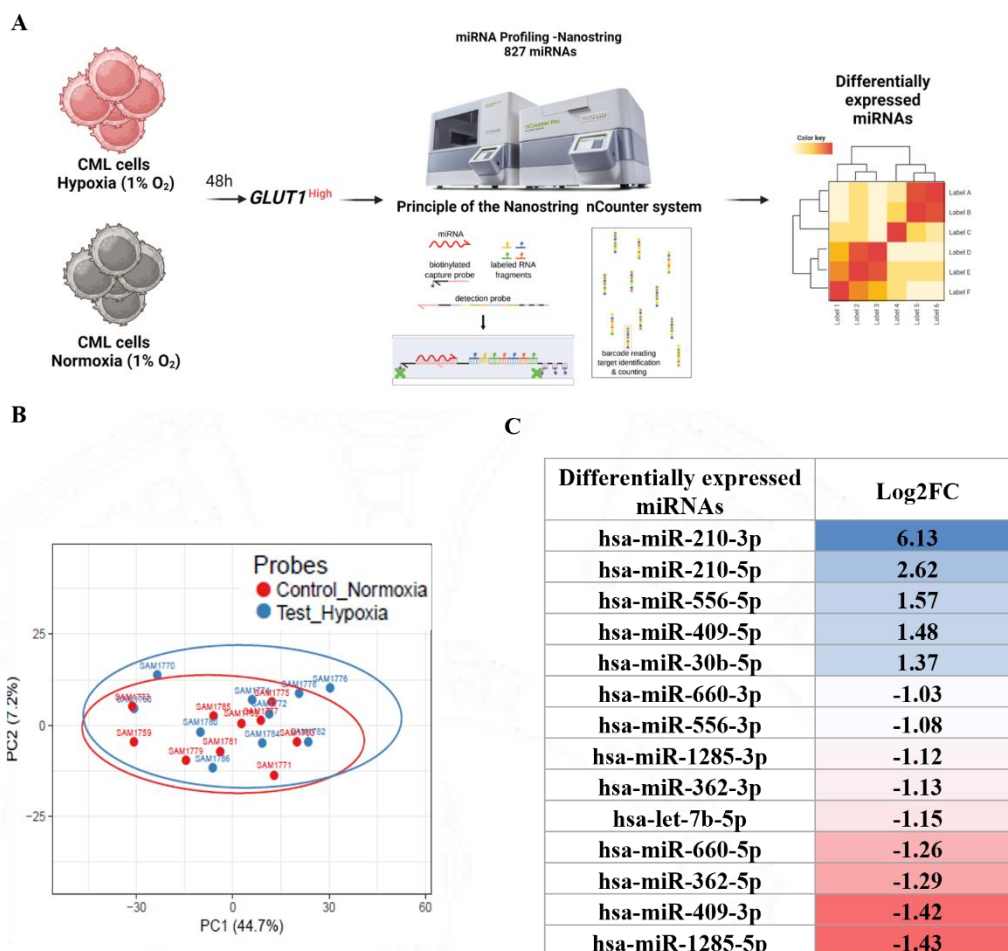


Figure 4.2.2: miRNA Profiling of CML primary cells. **A)** Experimental outline **B)** Principal component analysis **C)** Heatmap showing the differentially expressed miR in CML primary cells cultured under hypoxic conditions.

4.2.3 Validation of differentially regulated miRNAs in CML and healthy donor samples

On further validation by RT-qPCR, miR-30b-5p was found to be significantly upregulated, and miR-362-5p downregulated in CML primary samples cultured under hypoxic conditions (**Figure 4.2.3A-B**). These miRNAs have been extensively evaluated in solid tumors such as breast & hepatocellular cancer; however, the role of

these miRNAs in mediating CML LSCs survival and maintenance is not clear (Cao et al., 2021; Wu et al., 2020; Xiao et al., 2022). To assess whether this upregulated miRNA was CML cell-specific, we evaluated the expression of miR-30b-5p and miR-362-5p in healthy donor PBMNCs maintained under hypoxia vs. normoxia. We observed that miR-30b-5p expression was unaltered or downregulated in healthy donor PBMNCs (Figure 4.2.3D-E), suggesting the possible role of miR-30b-5p in maintaining CML cells under low oxygen tension. Previous studies reported that miR-30b-5p functions as a tumor regulator via target genes. For instance, miR-30b-5p regulates cell proliferation by directly targeting MTDH and HOXA1 in glioma and esophageal cancer (Li et al., 2017; D Zhang et al., 2018). Also, miR-30b-5p influences cell invasion and migration by modulating CTHRC1 in non-small cell lung cancer (Chen et al., 2014).

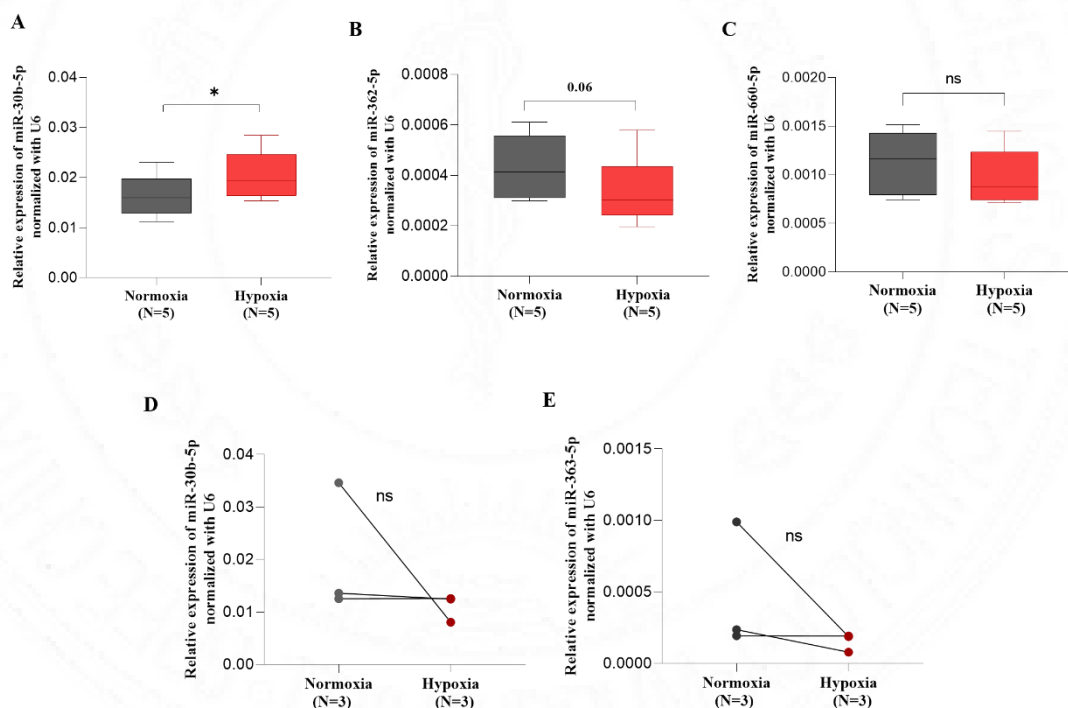


Figure 4.2.3: Validation of differentially expressed miRNAs. qPCR results of A) miR-30b-5p B) miR-362-5p C) miR-660-5p in CML primary cells. D) miR-30b-5p and E) miR-363-5p in healthy donor PBMNCs.

5p in healthy donors cultured in normoxic and hypoxic conditions for 48h. Relative expression was evaluated by normalizing with U6 (housekeeping gene) expression.

4.2.4 CRISPR-Cas9 Knockout of miR-30b-5p reduces proliferation and colony-forming capacity of K562 cell line in vitro.

4.2.4.1. Screening for miR-30b-5p levels in CML cell lines subjected to hypoxic conditions to identify suitable cell lines for knockout experiment

To understand the functional significance of miR-30b-5p in CML cell lines under hypoxic conditions, we initially screened for miR-30b 5p expression in several CML cell lines EM-2, KU812, K562, KYO1, JURL-MK1, LAMA84, KCL22, MOLM6, and MEG01 cultured under normoxic and hypoxic conditions. After 72h, RNA samples were extracted, cDNA was synthesized and tested for *GLUT1* expression. Increased *GLUT1* expression was observed in MEG01, KYO1, MOLM6, KCL22, K562, and EM-2 cell lines, confirming hypoxia induction. Next, miR-30b-5p expression was evaluated in these cell lines. Among the CML cell lines tested, K562 showed enhanced miR-30b-5p expression in hypoxic conditions compared to the other CML cell lines (**Figure 4.2.4A**). Hence, we chose the K562 cell line as a model to evaluate the effect of miR-30b-5p KO (**Figure 4.2.4B**).

4.2.4.2. Generation of K562 cell line expressing Cas9 protein

We employed CRISPR Cas9 dual guide RNA mediated knockout of miRNA as it results in efficient generation of short deletions within miRNA genomic regions and complete loss of gene function, allowing for precise targeting of specific genes or gene regions and resulting in more specific and controlled gene manipulation.

The cells were first transduced with lenti-Cas9 to generate Cas9 expressing K562 cell line. After five days of transduction, cells were subjected to blasticidin selection for two weeks. The cas9-expressing K562 cell line was transduced with lentivirus containing PLKV2.2-BFP cloned with dual guide RNA targeting miR-30b-5p. After 48h, transduction efficiency was found to be 90%, as assessed by the percentage of BFP-expressing cells. BFP^{Bright} cells were flow sorted, and then the knockout of miR-30b-5p was confirmed at the genomic level by amplifying the target genomic locus using specific primers flanking the guide RNA binding regions. PCR results showed a ~60 bp reduction in the product size compared to the wild type (320bp vs. 378bp), confirming the deletion (**Figure 4.2.4C**). To further validate the effect of miR-30b-5p knockout at the mRNA level, the expression levels of miR-30b-5p were quantified using qPCR in Scramble vs. miR-30b-5p KO. The results demonstrated a significant reduction of 70% in miR-30b-5p levels in K562 KO cells compared to K562 scramble cells, as depicted in Figure 4.2.4D.

To assess the functional impact of miR-30b KO, K562 scramble and miR-30b KO cells were cultured in normoxia and hypoxia for 96 hours, and cell proliferation and colony-forming capacity were compared (**Figure 4.2.4E-F**). K562 miR-30b KO cells cultured under hypoxic conditions showed reduced proliferation compared to those cultured under normoxia (2×10^5 cells vs. 9×10^5 cells, $P < 0.0078$) at 96h. miR-30b KO altered the proliferation of K562 cells in hypoxia, confirming its specific role in hypoxic conditions altering cell proliferation. Further, the colony-forming assay revealed a significant reduction in the colony-forming capacity of K562 miR-30b KO cells

maintained in hypoxia vs. normoxia (123 vs. 92 colonies, $P < 0.0022$), suggesting the role of miR-30b-5p in maintaining the stemness of CML cells in low oxygen tension.

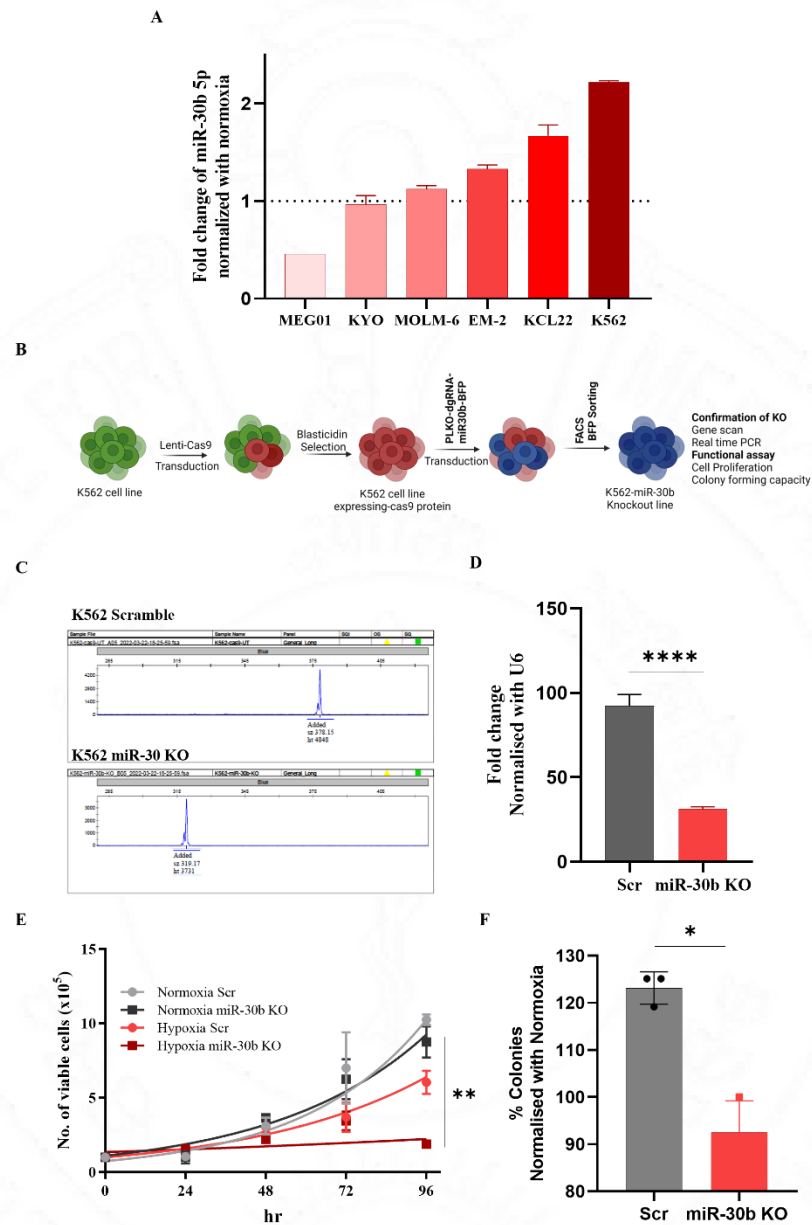


Figure 4.2.4: CRISPR-Cas9 dual guide RNA knockout of miR-30b in K562 cell line. A) Real-time PCR showing increased miR-30b expression in K562 cells cultured under hypoxic condition **B)** Experimental outline CRISPR-Cas9 dual-guide RNA mediated knockout of miR-30b-5p **C)** GeneScan analysis showing indel confirming KO **D)** qPCR for miR-30b expression in K562 scramble and miR30b KO cells. **E)** Cell proliferation by

Trypan blue dye exclusion F) Colony-forming capacity of K562 scramble and miR-30b-5p KO cells.

4.2.4.3 Target gene prediction of miR-30b-5p

miRNA functions by negatively regulating the mRNA expression at the post-transcriptional level through base-pairing with complementary sequences of the 3' untranslated region (UTR). To identify the potential target genes of miR-30b, we used target prediction tools such as miRWalk, TargetScan, miRDB, and miRTarBASE. Seventy genes were predicted to be the target for miR30b, including *PPARGC1B*, *MTDH*, *ELOVL15*, and *EED*. These genes were associated with mitochondrial biogenesis, promoting tumor progression, biosynthesis of very long-chain fatty acids, and epigenetic modification. While these genes have been studied in various contexts, there is currently no clear evidence to suggest that they play a significant role in the pathogenesis or progression of CML. Further research is needed to determine whether these genes are involved in the development or treatment of CML. Gene Ontology analysis revealed genes associated with “positive regulation of T-regulatory cell differentiation” and “regulation of transcription by RNA polymerase II” were dysregulated (**Figure 4.2.5A-B**). Pathway analysis showed that genes associated with post-transcriptional silencing by small RNAs and TGF-beta Receptor Complex might be a target of miR-30b (**Figure 4.2.5C-D**). TGF- β (transforming growth factor-beta) signaling plays a complex role in tumor progression, including in hematologic malignancies such as CML. Specifically, TGF- β is known to maintain CML-initiating cells through the Akt-FoxO pathway (Naka et al., 2010b). Further validation of these targets is needed to understand the role of TGF-beta signaling and mir-30b in maintaining CML cells under hypoxic conditions. One of the significant limitations of this study is

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the use of primary CML PBMNCs for studying miRNA, as there is inherent heterogeneity in the data due to patient-specific variability. Previous studies in CML on miRNA regulating CML stem cells using cell lines have provided repeatable and comparable results. While Cell lines are cost-effective and provide repeatable and similar results, primary samples are more representative of the clinical features of the disease but are more variable and less reproducible.



Figure 4.2.5: Gene ontology and pathway enrichment analysis of predicted target genes of miR-30b-5p. A) GO biological processes B) Interactive network view of significantly enriched target genes. C) Reactome 2021 D) Interactive network view.

We investigated the role of hypoxia-related miRNAs in the survival and maintenance of CML cells. Notably, miR-30b-5p exhibited significant upregulation under hypoxic conditions in CML cells. Previous studies have identified miR-30b as a prognostic

marker and have been reported to influence the proliferation and migration of solid tumors. Additionally, the knockout of miR-30b led to reduced proliferation and colony-forming capacity of CML cells cultured in hypoxic conditions. Further, Target prediction showed that dysregulated miRNA was associated with TGF- β signaling, which is already reported to be associated with CML progression. One of the significant limitations is the use of primary CML patient samples due to their inherent variability. Despite these limitations, this research illuminates the role of hypoxia-related miRNAs, particularly miR-30b-5p, in the survival of CML cells, thereby offering insights into potential avenues for therapeutic intervention.

4.3 Generation and characterization of immortalized CML MSC line and assessing its role in the maintenance of CML LSCs

Despite the impressive efficacy of TKIs in inducing remission and controlling the disease, a subset of leukemic cells known as Leukemia Stem Cells (LSCs) has emerged as a major obstacle to achieving treatment-free remission (TFR) (Corbin et al., 2011b; Hamilton et al., 2006; Holyoake et al., 1999; Perl and Carroll, 2011). The bone marrow mesenchymal stromal cells (BMMSCs) play a crucial role in leukemogenesis and the protection of leukemic stem cells (LSC) from TKIs (Guarnerio et al., 2018; Zhang et al., 2012). Recent evidence suggests that the persistence of LSCs might be attributed to the sheltered environment provided by the BM niche. The BM hematopoietic microenvironment is replete with growth factors and cytokines that potentially offer survival signals to the residual CML cells (Hou et al., 2020; Nair et al., 2010; Shahrabi et al., 2014). Williams et al. have reported that cytokines present in the bone marrow microenvironment (BMM) can stimulate leukemic cell growth and contribute to imatinib SCTIMST, Trivandrum

resistance in the mouse model of BCR/ABL, Arf-null lymphoblastic leukemia (Williams et al., 2007). Zhang et al. demonstrated that MSCs derived from imatinib-resistant CML patients exhibited protective effects against cell death induced by IM or NI in K562 and BV173 cells compared to MSCs from IM responders. This protection was attributed to elevated IL-7 secretion from the MSCs (Zhang et al., 2019).

Furthermore, MSCs derived from CML patients displayed an enhanced capacity to support normal and CML BM CD34⁺CD38⁻ cells (Dolinska et al., 2023). Despite these findings, there is limited knowledge of how the MSCs are altered due to their interaction with CML LSCs; further research is needed. It is challenging to conduct extensive research to study the alterations in CML patient-derived BMMSCs. ***Primary MSCs possess several drawbacks, including cell senescence and the loss of multipotent differentiation capacity. Immortalizing primary MSCs offers several potential benefits and applications to overcome these limitations.*** Immortalized MSCs enable the generation of a consistent and abundant cell supply, provide a source for long-acting therapeutic cells, avoid cell senescence, and maintain differentiation capacity. In the present study, we characterize MSCs derived from CML patients at diagnosis, post-treatment, and from normal healthy volunteer bone marrow to assess alterations in their trilineage differentiation potential. Since primary CML MSCs have finite growth, and HS-5 (healthy donor-derived MSCs) cannot replicate the CML microenvironment, we generated and characterized immortalized MSCs from a CML patient (iCML MSCs), comparing them with primary CML MSCs. The iCML MSCs can be valuable for investigating the mechanisms underlying MSC-mediated protection in CML.

4.3.1 Comparison of BM MSCs from CML patients vs. Normal BM

To understand the changes incurred by the stromal cells during CML, mesenchymal stem cells were isolated from the bone marrow of CML-CP patients at diagnosis, post-treatment, and normal bone marrow cells. The CML and NBM-derived MSCs were spindle-shaped and reached confluency approximately in 2-3 weeks (**Figure 4.3.1A**). When the immunophenotype profile between NBM and CML patients was compared, all were positive for stromal markers CD73 and CD90 and negative for hemopoietic lineage CD45 and CD34 (**Figure 4.3.1B**).

MSCs are multipotent progenitors that can differentiate into osteocytes, adipocytes, and chondrocytes (Somoza et al., 2014). The MSCs' ability to differentiate into various lineages is crucial in maintaining the structure and composition of the BM niche (Woods and Guezguez, 2021). To assess any alteration in the differentiation capacity of MSCs derived from CML patients at diagnosis, post-treatment, and NBM, we differentiated the MSCs into adipocytes, osteocytes, and chondrocytes by supplementing specific differentiation media. Both CML and NBM-derived MSCs could be differentiated into all three lineages.

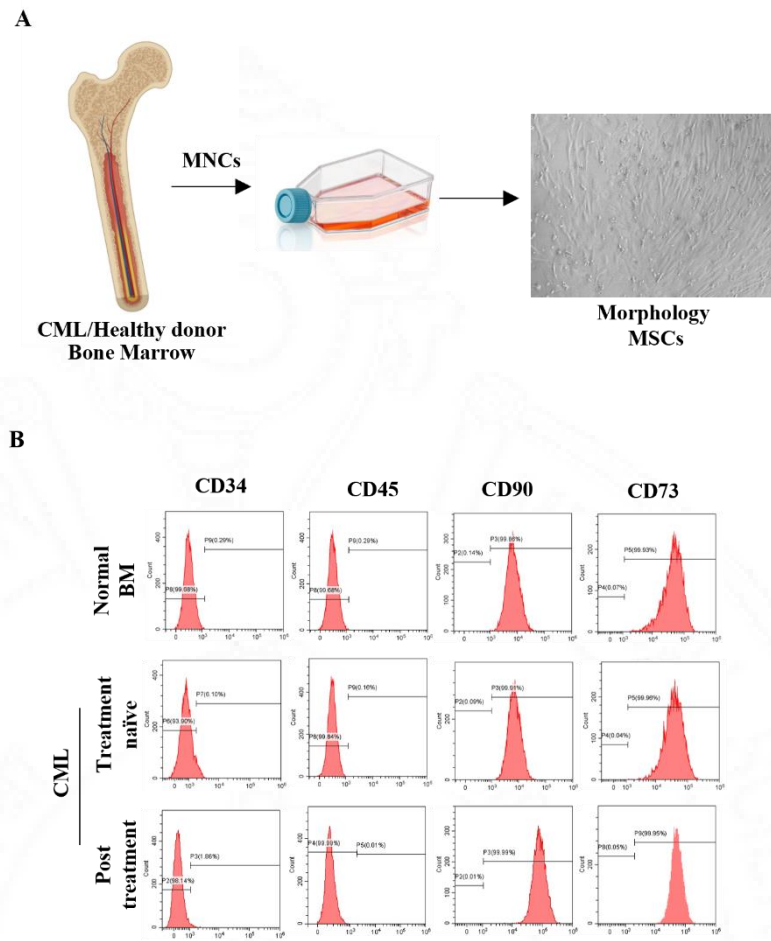


Figure 4.3.1: Propagation and characterization of CML and healthy donor-derived MSCs. A) Schematic representation of the experimental outline of CML MSC propagation B) Immunophenotypic characteristics of CML vs. Healthy MSCs. MSCs were positive for CD73 and CD90 and negative for hematopoietic lineage markers CD45 and CD34.

Although there was no significant difference in the adipogenic (**Figure 4.3.2A-B**) or osteogenic differentiation potential (**Figure 4.3.2C**) between CML MSCs vs. NBM MSCs, chondrogenic differentiation potential was enhanced in CML MSCs compared to NBM MSCs (**Figure 4.3.2D**). There was no difference in the trilineage differentiation potential between the MSCs derived from treatment naïve and post-treatment BM. Previous studies have reported that leukemic-stromal interaction alters the trilineage differentiation potential of MSC in leukemic patients (Dolinska et al., 2023; Zhang et al., SCTIMST, Trivandrum

2019). The altered chondrogenic differentiation potential of MSC derived from CML patients might have been caused by leukemic cell-stromal cell interaction. These changes may collectively contribute to the functional alterations of CML MSCs, including their hematopoiesis-supportive function and differentiation (Zhang et al., 2012). Further studies are needed to investigate the role of altered chondrogenic differentiation in CML MSCs and its role in the LSCs maintenance.

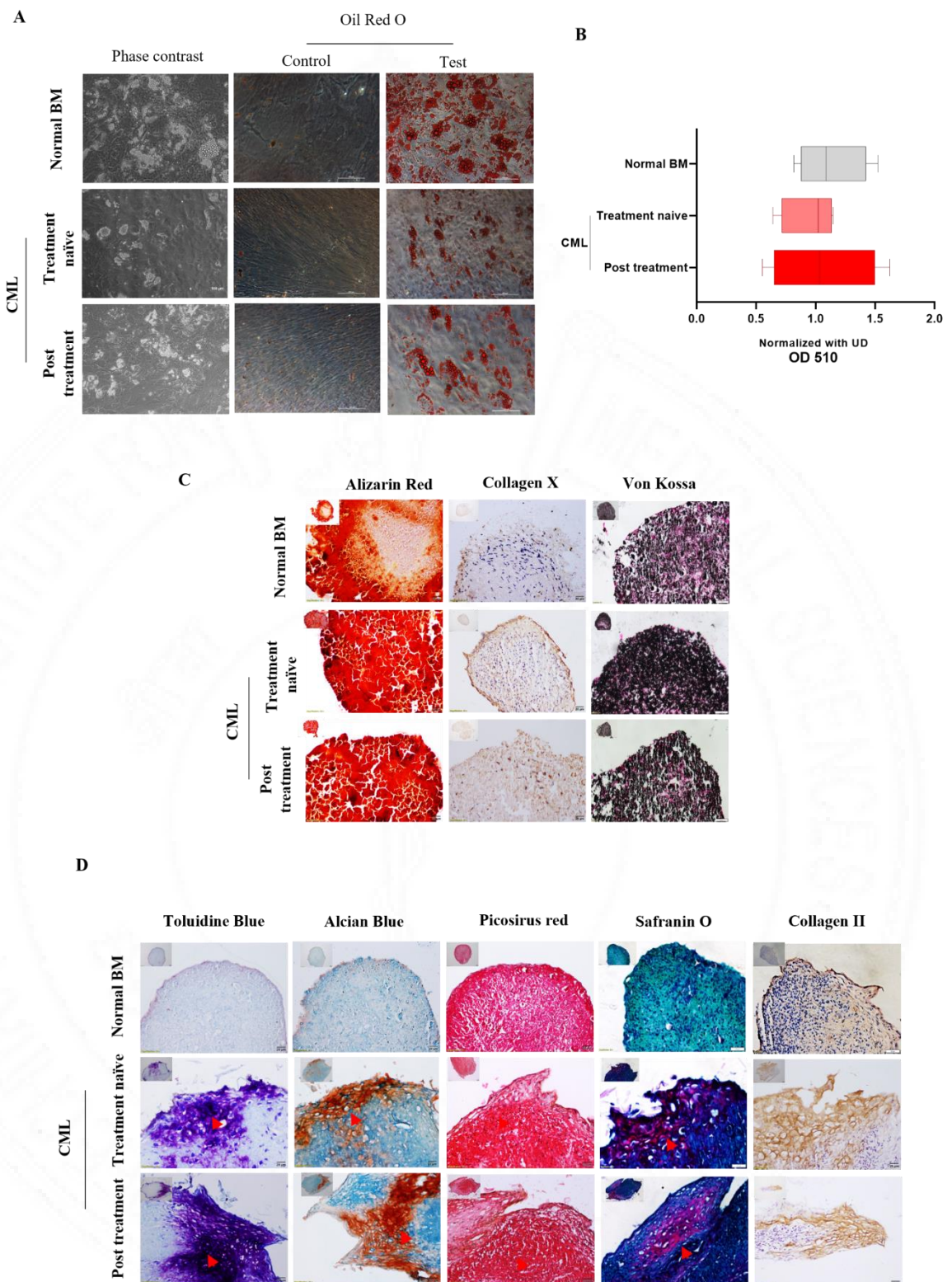


Figure 4.3.2: Trilineage differentiation potential of CML and Normal BM MSC. Representative image of A) adipogenic differentiation (after oil Red O stain) B)

Quantification of in vitro adipogenic differentiation potential of NBM and CML MSC C) Osteogenic differentiation (by Alizarin Red stain and immunohistochemistry of collagen X) D) Chondrogenic differentiation potential (by Toluidine blue, Alcian Blue, Picosirus red, Safranin O and Collagen II protein expression).

4.3.2. Generation of immortalized CML MSCs (iCML MSC)

The healthy donor-derived MSC line (HS-5) has been extensively used as a model to investigate the interplay between the leukemic and stromal cells. However, it has been reported that HS-5 cells lack the ability to differentiate into chondrocytes (Roecklein and Torok-Storb, 1995). CML MSCs showed enhanced chondrogenic differentiation potential, and primary CML MSCs have a limited lifespan and tend to undergo senescence, losing their functional abilities after a few passages (typically 2-3). To address these limitations, we developed an immortalized CML MSC line and showed that the iCML MSC line can be cultured up to 25 passages, allowing for long-term investigations and screening of molecules that can target CML LSCs and their interactions with the BMM.

For immortalizing MSCs, primary CML MSCs were transduced with HEE lentivirus carrying Human Kusabira Orange (huKO⁺) fluorescent protein, and the medium was supplemented with doxycycline (dox) to induce the expression of HEE virus (**Figure 4.3.3A**). After four days of dox induction, huKO⁺^{bright} cells were single-cell-sorted and then characterized further. After isolating 62 single cells expressing the huKO⁺ fluorescent protein, we carefully examined their characteristics (**Figure 4.3.3B**). Among these clones, we chose one that displayed spindle-shaped morphology and positive expression of CD73 and CD90, recognized as MSC markers (**Figure 4.3.3D**). Karyotyping analysis to assess further the genetic stability of the immortalized CML

MSC clone showed no additional chromosomal abnormalities after the immortalization process (**Figure 4.3.3C**).

Immortalized CML MSCs (iCML MSCs) retained the capacity for trilineage differentiation, closely resembling the differentiation potential observed in primary CML MSCs (**Figure 4.3.4A-C**); however, the HS-5 could not be differentiated into chondrocytes (**Figure 4.3.4C**). Further, the expression of *PPARG*, *RUNX2*, and *ACAN* genes was evaluated as markers after adipogenic, osteogenic, and chondrogenic differentiation, respectively. Compared to undifferentiated cells, we observed increased *PPARG* and *RUNX2* expression in adipo and osteo-differentiated cells (**Figure 4.3.5A-B**). As expected, *ACAN* expression was higher in CML MSCs than HS-5, confirming the enhanced chondrogenic differentiation potential observed in CML MSC (**Figure 4.3.5C**). The iCML MSCs retained the chondrogenic differentiation potential as the primary CML MSCs, suggesting that the iCML MSC clone can be an appropriate model for studying the interaction between BMM and CML cells.

4.3.3 iCML MSCs reduce imatinib-induced apoptosis of primary chronic myeloid leukemia CD34⁺ cells

We then studied the functional role of iCML MSCs in regulating the growth of CML CD34⁺ cells and their protective effect against TKI. MSCs interact with the leukemia cells through their secreted factor/direct cell contact to regulate the growth of leukemic cells (Nair et al., 2010) and chemoprotect the leukemic cells from therapeutic insult (Zhang et al., 2019). To confirm iCML 'MSCs' role in mediating the protection of CML LSCs, we cocultured CML CD34⁺ cells with iCML MSC and then treated them with IM as described in **Figure 4.3.6A**. CML CD34⁺ cells cocultured with iCML MSCs

showed significantly enhanced cell viability compared to the CML-CD34⁺ cultured alone (132% vs 100%, $p=0.0199$). CML-CD34⁺ cells cocultured with iCML MSC and maintained in hypoxia showed a significant increase in the percentage of viable cells compared to the control (137% vs. 100%, $p=0.0252$). We also observed that CML-CD34⁺ cells cultured with MSCs and then treated with imatinib (IM 20nM) showed reduced cell death in comparison to the control (85% vs 53%, $p<0.0001$) (**Figure 4.3.6B**). These results suggest that iCML MSC retained its functional property and protected the CML CD34⁺ cells from IM treatment, similar to primary CML MSCs.

We then tested if the secretory factors released from the iCML MSC line support the growth of leukemic cells. Conditioning media was collected, and the leukemic cells were cultured with and without the conditioning medium. The proliferation and percentage of CD34⁺ cells were measured sequentially. The growth of leukemic cells increased gradually by 15 times when cultured in the presence of conditioning media (**Figure 4.3.6C-D**). Also, the percentage of CD34⁺CD38⁻ cells gradually increased from 16% to 56%. In contrast, the leukemic cells cultured in basal media showed a subtle increase in proliferation but did not show an increase in CD34⁺ percentage (**Figure 4.3.6C-D**). These results suggest that the secretory factor released from the iCML MSC may selectively support the growth and proliferation of CD34⁺ leukemic cells.

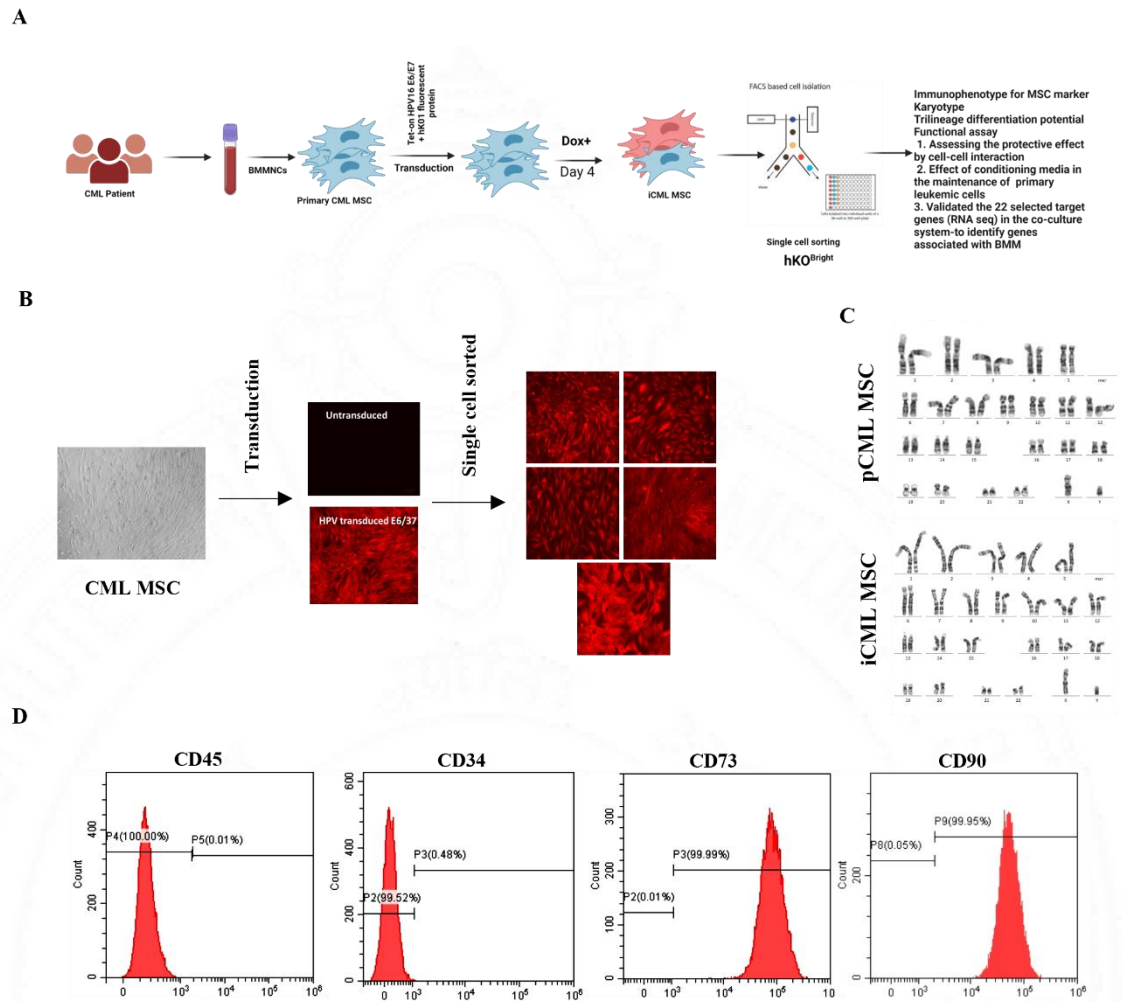
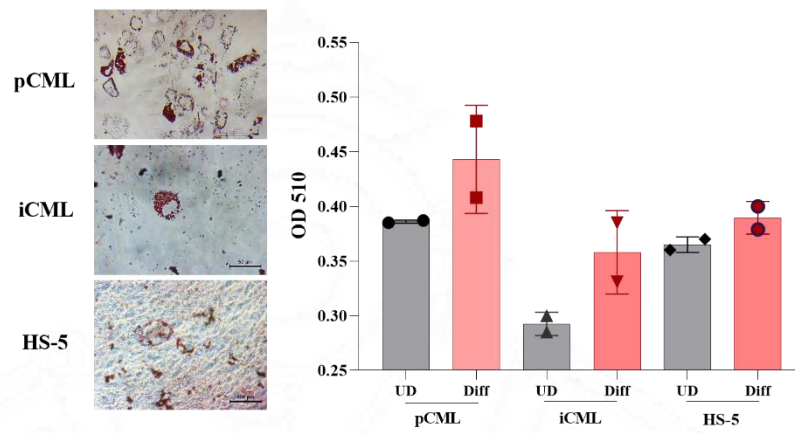
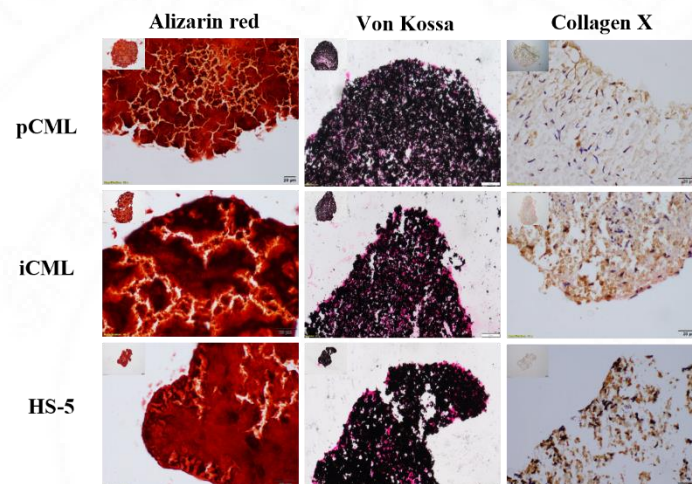


Figure 4.3.3: Generation and characterization of immortalized CML MSCs. A) Experimental outline B) Representative image of MSC transduced with HEE lentivirus expressing huKO+ after dox induction C) Karyotypic analysis of primary CML MSC before and after immortalizing D) Immunophenotypic characterization of immortalized CML MSCs expressing MSCs marker and negative for hematopoietic lineage markers.

A



B



C

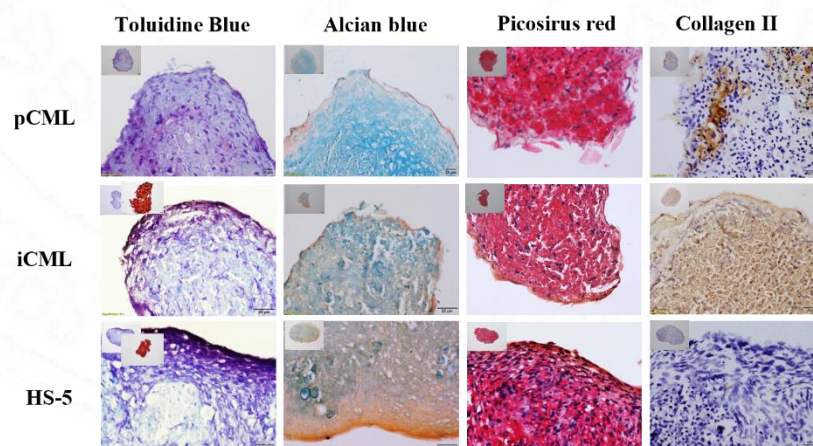


Figure 4.3.4: *i*CML MSC retained adipogenic differentiation potential. Representative image of A) Oil Red O stain and absorbance at OD540 showing adipogenic differentiation B) osteogenic differentiation by Alizarin Red stain, Von kossa stain and immunohistochemistry of collagen-X C) Chondrogenic differentiation by toluidine blue, Alcian blue, Picosirus red, and Collagen II in p-CML, *i*CML MSCs, and HS-5 after adipogenic differentiation. pCML – Primary CML MSCs, *i*CML MSC – immortalized CML MSCs, and HS- 5 – Healthy donor-derived immortalized MSCs.

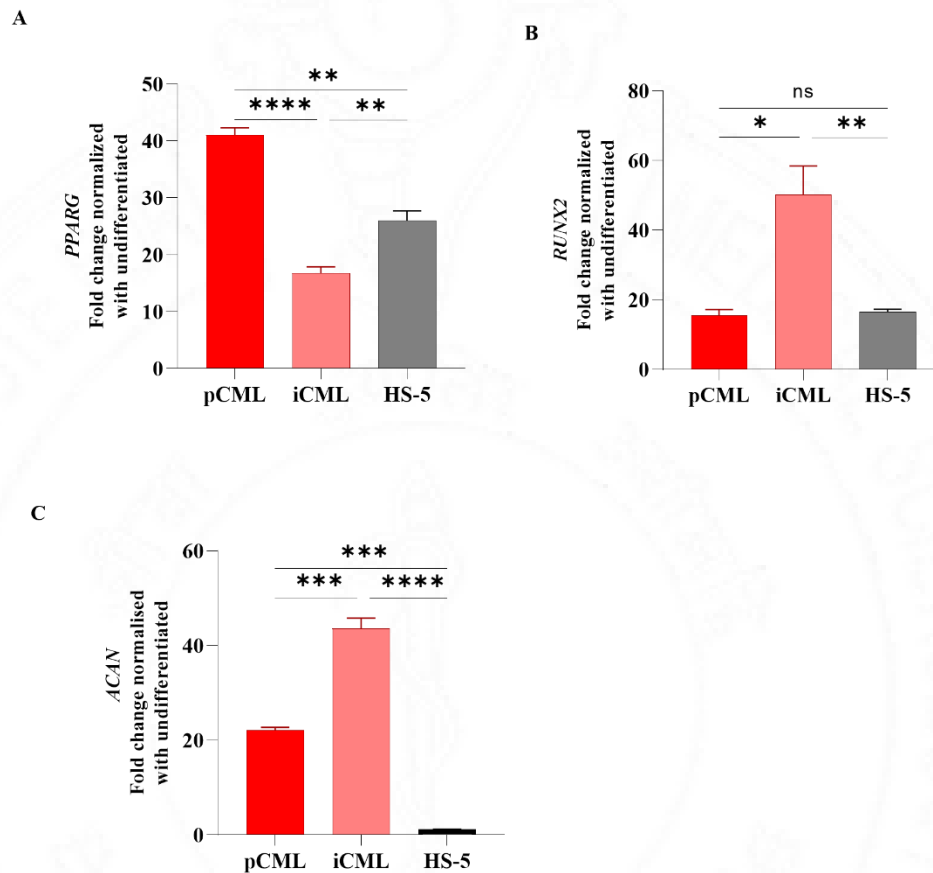


Figure 4.3.5: *i*CML MSC retained the osteogenic differentiation potential. Quantitative RT-PCR results showing A) PPARG, B) RUNX2, and C) ACAN expression in p-CML, *i*CML MSCs, and HS-5 after adipogenic, osteogenic and chondrogenic differentiation respectively. pCML – Primary CML MSCs, *i*CML MSC – immortalized CML MSCs, and HS- 5 – Healthy donor-derived immortalized MSCs.

4.3.4. Coculture of CML CD34⁺ with iCML MSCs showed dysregulation of genes associated with Calcium metabolism (S100A8, S100A9)

Our RNA seq data revealed that genes associated with chemotaxis, cell migration, and adhesion are differentially expressed in CML CD34⁺ compared with healthy donor CD34⁺. To identify the genes related to bone marrow microenvironment-mediated protection in CML LSC, we cocultured CML CD34⁺ cells with iCML MSC and tested the expression of differentially expressed genes using NanoString technology.

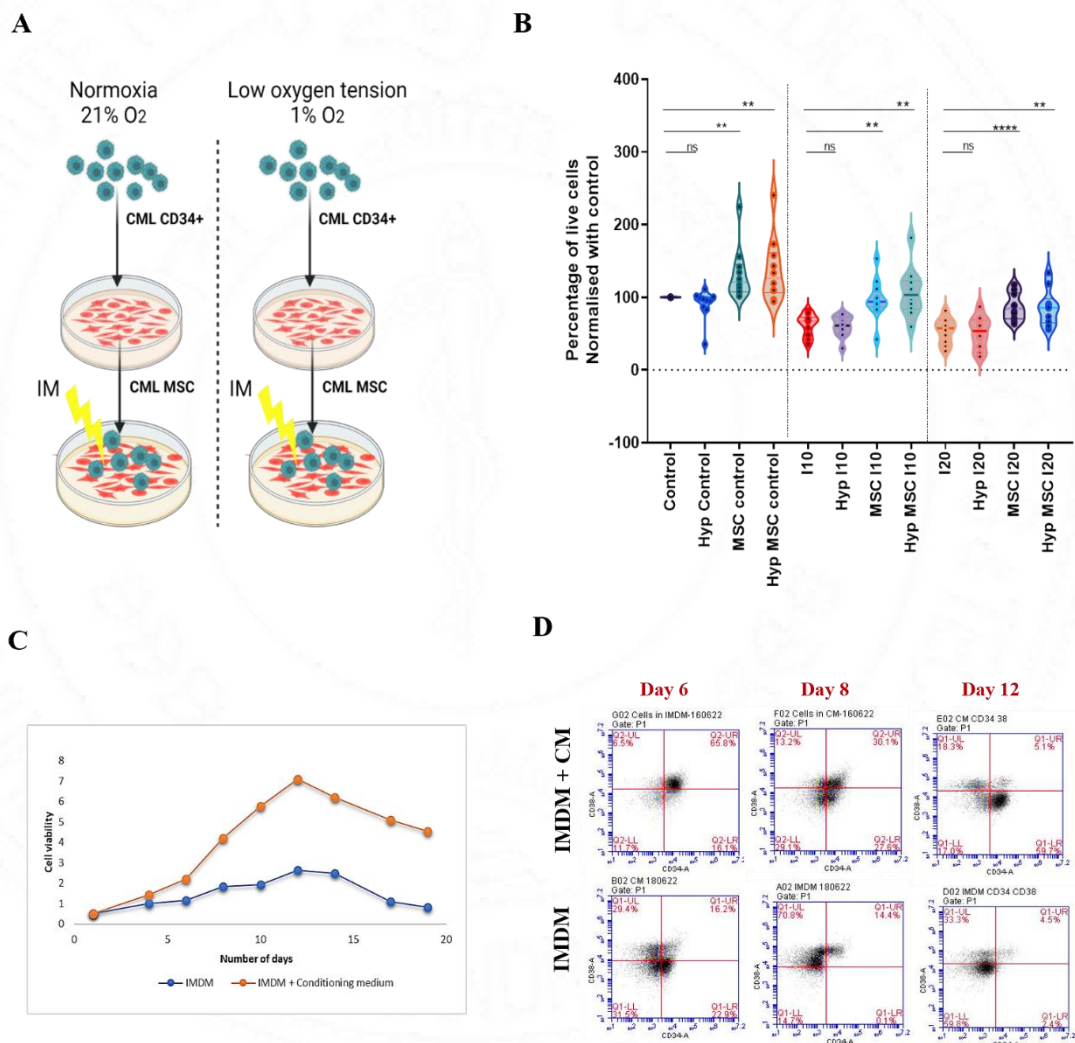


Figure 4.3.6: iCML MSC protected the CML CD34⁺ cells from TKI treatment and selectively increased the growth of CD34⁺ cells in ex vivo. A) Experimental outline of iCML MSC coculture with CML CD34⁺ B) Violin plot showing the percentage of live cells with and without IM treatment C) Growth rate of leukemic cells cultured with and without conditioning medium D) Representative flow plots showing the increase in the percentage of CD34⁺ cells cultured with conditioning medium. CM: Conditioning medium

As reported earlier, we observed upregulation of *CDH2* in CML CD34⁺ cocultured with CML MSCs (Zhang et al., 2012). Furthermore, genes associated with calcium metabolism were also increased in CML CD34⁺ cells cocultured with iCML MSCs (**Figure 4.3.7A-C**). Investigating the functional role of these genes may provide valuable insights into identifying novel therapeutic targets for eradicating CML CD34⁺ cells.

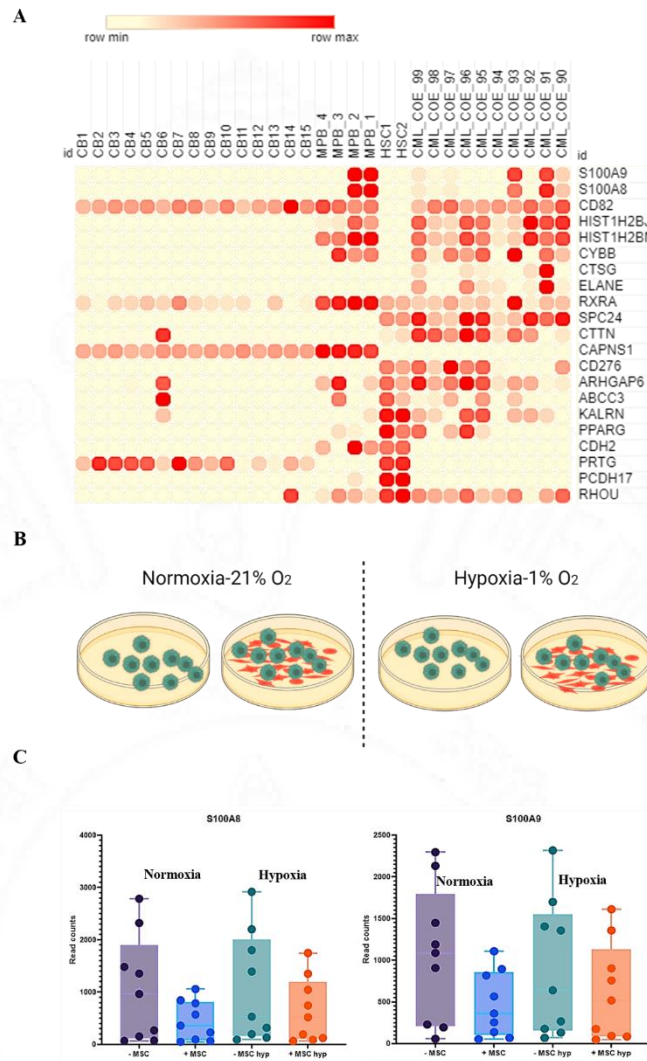


Figure 4.3.7: Genes associated with calcium metabolism upregulated in CML CD34⁺ cocultured with iCML MSCs. *A)* Heatmap showing the 21 gene panel differentially expressed in CML-CD34⁺ cells *B)* Experimental outline of coculture system *C)* Significant downregulation of S100A8, S100A9 in CML-CD34⁺ cocultured with MSCs.

4.3.5 Cytokine profiling of iCML MSCs

MSCs secrete various cytokines, growth factors, and chemokines, contributing to immune modulation, tissue remodeling, and cellular homeostasis during regeneration. Since the conditioned media isolated from CML MSC lines have been shown to protect and selectively improve the growth of leukemic cells, we conducted cytokine profiling

to assess the cytokines released by these MSCs that support the growth of leukemic cells. Cytokine profiling revealed that the CML MSC lines abundantly secreted 13 cytokines. As previously reported, IL-6 was abundant in the CML MSCs lines. Additionally, IGFBP6, known to support the growth of CML cells, as reported earlier, was also found to be secreted by CML MSCs (Cambria et al., 2023). In addition, cytokines associated with cell adhesion, such as TIMP1 and TIMP-2, were also seen (Ries et al., 1999). The cytokines abundantly secreted by CML MSCs were significantly involved in immune regulation, supporting the hypothesis that CML MSCs create an immune modulatory microenvironment that promotes growth and protects against TKIs.

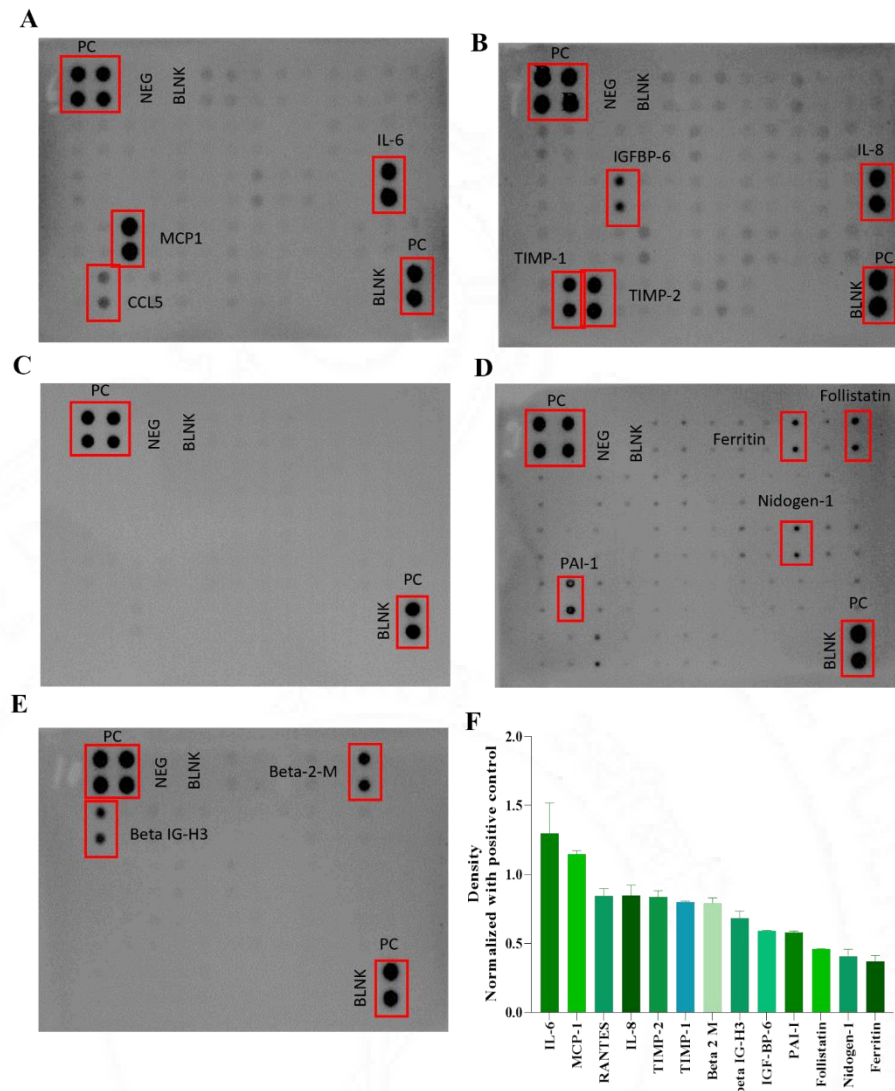


Figure 4.3.8 Cytokine profiling in iCML MSC. A-E) Representative pictures of the cytokine antibody array F) Relative expression of secreted factors detected using the cytokine array.

Like normal hematopoietic progenitors, leukemia cells physically interact with the BM niche and deliver signals capable of re-programming MSCs and endothelial cells (ECs), consequently altering the hematopoietic niche (Zehentmeier and Pereira, 2019). The present study shows that CML MSCs have morphology and immunophenotypes similar to NBM MSCs. As reported earlier, CML MSCs at diagnosis and post-treatment showed

no trilineage differentiation potential (Estrada-González et al., 2014) but exhibited enhanced chondrogenic differentiation compared to NBM MSCs. This altered differentiation potential might be caused by leukemic-stromal interaction. In normal hematopoiesis, chondrocytes play an essential role in the secretion of inflammatory cytokines, and the increased chondrogenic differentiation potential in CML MSCs might replicate an inflammatory microenvironment in CML patients (Nievergall et al., 2016).

The healthy donor-derived MSC line (HS-5) is extensively utilized as a model for exploring the interaction between leukemia and stromal cells. It has a limitation of the reported lack of chondrogenic differentiation ability. Due to the increased chondrogenic differentiation potential observed in CML MSCs, HS-5 might not be optimal for investigating the BMM-mediated protection in CML leukemic stem cells (LSCs)(Roecklein and Torok-Storb, 1995). Meanwhile, Primary CML MSCs, despite their relevance, suffer from a limited lifespan and a tendency to undergo senescence after a few passages (typically 2-3), leading to a loss of functional abilities. We developed an immortalized CML MSC line to overcome this challenge. Immortalization enables indefinite culturing, facilitating long-term investigations and hence the ability to be used for screening molecules capable of targeting CML LSCs and their interactions with the bone marrow microenvironment. iCML MSCs showed no change in chromosomal aberration compared to the primary CML MSCs.

Further, trilineage differentiation revealed that it had retained the trilineage differentiation potential as the primary CML MSCs. Functional analysis revealed that the iCML MSCs retained the capacity for trilineage differentiation, closely resembling the differentiation potential observed in primary CML MSCs. This characteristic makes the

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iCML MSC clone an appropriate model for studying the interaction between bone marrow microenvironment (BMM) and CML cells. iCML MSCs protected CML CD34⁺ cells from IM treatment, confirming that iCML MSCs support the CML CD34⁺ cells from chemotherapy. The secretory factor released from the iCML MSC may selectively support the growth and proliferation of CD34⁺ cells. Genes associated with calcium metabolism were increased in CML CD34⁺ cells cocultured with iCML MSCs. Investigating the functional role of these genes may provide valuable insights into identifying novel therapeutic targets for eradicating CML CD34⁺ cells. In solid tumors such as Gliomas, immortalized MSCs and their plasma membranes can be used as natural drug carriers for targeting gliomas and providing a safe, adequate, quality-controlled, and continuous source for the treatment of gliomas (Zhang et al., 2022). MSCs from elderly patients are immortalized by transducing them with SV40LT and hTERT. These iMSCs have a high osteogenic potential and are used in *in vitro* models to study bone tissue engineering (Piñeiro-Ramil et al., 2021). Human Adipose Tissue Mesenchymal Stem/Stromal Cells 1 (HATMSC1) cell line phenotype was found to be consistent, and the cytokine production appeared to be higher compared to the primary MSCs (Paprocka et al., 2021). iCML MSC showed enhanced chondrogenic potential because of clonal selection, and retaining the trilineage differentiation potential may be beneficial in studying leukemia-stromal cell interaction.

Further cytokine profiling of CML MSCs revealed that cytokines associated with immune response and inflammation are enhanced compared with HS-5, supporting the finding that CML MSCs might provide an inflammatory microenvironment to support the growth of CML CD34⁺. Further studies are warranted to confirm the results.

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In summary, the immortalized CML line cell maintains the main characteristics observed in primary CML MSCs, thus making this a valuable model for studying leukemic-stromal interaction and functional studies of candidate genes involved in mediating TKI resistance in CML LSCs.

4.4 Generation and characterization of iPSCs from primary CML cells for modeling CML LSCs.

The persistence of leukemic stem cells (LSCs) remains a primary factor contributing to disease recurrence in CML (Corbin et al., 2011b). Owing to the rarity of LSCs, obtaining a sufficient number of cells for high throughput screening to understand TKI resistance mechanisms and screening drugs to eliminate them poses a challenge (Corbin et al., 2011b; Holyoake et al., 1999). Induced pluripotent stem cells (iPSCs) derived from patients with genetic diseases or cells with genetic alterations can be banked and differentiated into large quantities of disease-relevant cells, making them perfect for studying disease development and drug screening (Takahashi et al., 2007b). In the context of CML, iPSCs generated from patients help researchers understand how oncogenes and patient-specific chromosomal abnormalities influence leukemia-like phenotypes (Papapetrou, 2019b). CML-iPSCs derived from the same patient at different stages of the disease and from patients with distinct responses to TKI therapy offer valuable resources for investigating the molecular and epigenetic mechanisms underlying CML progression and drug resistance. This approach aids in identifying novel therapeutic targets and potential drug candidates for CML treatment (Corbin et al., 2011b; Miyauchi et al., 2018). Additionally, iPSCs can be effectively genetically

manipulated using gene editing methods to study specific genes involved in disease pathogenesis (Imeri et al., 2023).

Earlier studies have successfully generated CML-iPSCs from peripheral blood mononuclear cells and CD34⁺ hematopoietic stem and progenitor cells isolated from CML patients (Hosoi et al., 2014; Hu et al., 2011; Kumano et al., 2012; Miyauchi et al., 2018; Telliam et al., 2016). These CML-iPSCs exhibit resistance to IM, similar to CML-LSCs, making them cellular models for investigating the mechanisms responsible for IM resistance (Bedel et al., 2013; Charaf et al., 2017; Kumano et al., 2012). Moreover, hematopoietic progenitors derived from CML-iPSCs demonstrated some of the pathophysiologic characteristics of the disease (Bedel et al., 2013; Hosoi et al., 2014; Hu et al., 2011; Kumano et al., 2012; Miyauchi et al., 2018). However, it remains unclear how BCR::ABL1 fusion protein expression and TKI treatment affect the efficiency and quality of iPSC generation from CML cells.

This study presents the successful generation of iPSCs from CD34⁺ cells of CML patients with varying responses to TKI therapy. The purpose was to model the disease and explore the impact of BCR::ABL1 oncoprotein expression on the maintenance of CML-iPSCs. Unlike previous studies, we discovered that the expression and activity of BCR::ABL1 influences the survival and maintenance of CML-CD34-iPSCs. It was crucial to suppress the TKI activity of the BCR::ABL1 fusion protein through TKI supplementation to ensure the viability of CML-iPSCs. The CML-iPSCs produced using our protocol could be further differentiated into BCR::ABL1-positive hematopoietic progenitors. These patient-specific CML-iPSCs and their differentiated hematopoietic cells hold great potential for many applications, including high throughput CRISPR-Cas9

screening, which can help identify novel pathways involved in CML pathogenesis and drug resistance.

4.4.1 Generation of iPSCs from normal and CML CD34⁺ cells

CD34⁺ cells from two CML patients are known to constitute the leukemia stem/progenitor cells associated with disease recurrence. Our goal was to reprogram these cells from the patients who achieved complete cytogenetic response and one who achieved suboptimal response after TKI therapy. The patients' demographics are shown in Table 4.4.1. In addition, we reprogrammed CD34⁺ cells from a healthy volunteer as a control.

Table 4.4.1: Patient demographics.

Sample ID	Age/Gender	TKI/Dose	Phase	Response status
G13	58/M	DA/50mg	CP	CCYR*
G15	53/M	DA/50mg	CP	SOR**
G17	28/M	DA/50mg	CP	CCYR*
G18	51/F	IM/400mg	CP	REFRACTORY***

*Complete cytogenetic response - <1% BCR::ABL1 cells

**Sub-optimal response ->1% BCR::ABL1 cells

***No hematological response at 3 month evaluation

CD34⁺ cells were purified from the patients and the healthy donor and cultured in a CD34⁺ expansion medium. The percentage of CD34⁺ cells remained consistent while the number of cells increased by 15 to 20-fold (Mean: 20.2±3.34) (**Figure 4.4.1A**). Actively proliferating cells from day 3 of the culture were nucleofected with episomal plasmids(Miyauchi et al., 2018). After 2 days of nucleofection, the cells were transferred to a Matrigel-coated plate. Subsequently, the half-culture media was replaced with reprogramming media on alternating days. Between day 14 and day 18, iPSC colonies with typical morphology were generated from normal CD34⁺ cells (as shown in **Figure 4.4.1B**), and two clones were picked between day 20 and day 24 (**Figure 4.4.1B**). Normal

iPSC clones retained typical iPSC morphology and expressed high levels of pluripotency markers without differentiation for 12 passages (**Figures 4.4.1C and 1D**). In contrast, CML-CD34⁺ cells from TKI-responsive patients formed several "dome-shaped" colonies surrounded by adherent cells formed by the spontaneous differentiation of iPSCs (**Figures 4.4.1B and C**). Eleven "dome-shaped" colonies were isolated from 3 patients, and all these clones expressed TRA-1-60 in the initial passages (**Figure 4.4.1D**). Such dome-shaped colonies were described by a few authors (Bedel et al., 2013; Kumano et al., 2012) and have not been described by other studies. After 3-4 passages, the "dome-shaped" colonies peeled off from the bottom of the plates and floated in the medium, leaving the differentiated cells attached to the plate.

The "dome-shaped" CML-CD34-iPSC colonies expressed the same types of *BCR::ABL1* fusion transcripts (e13a2 and e14a2) present in the peripheral blood mononuclear cells used for reprogramming (**Figure 4.4.2A and B**). Karyotyping confirmed all iPSC clones had t(9;22) presence (**Figure 4.4.2C**). Furthermore, no mutations were detected in the *BCR::ABL1* kinase domain among any clones.

4.4.2 IM supplementation aids in the maintenance, stability, and pluripotency of CML iPSCs

Previous studies have shown that TKI treatment enhances pluripotency marker expression in *BCR::ABL1*⁺ iPSC clones (Charaf et al., 2017). However, it was unclear in these studies whether TKI supplementation improves the stability of CML-CD34-iPSCs. We hypothesized that the atypical morphology and high rate of spontaneous differentiation of CML-CD34-iPSC colonies were due to the constitutive expression of the *BCR::ABL1* fusion protein and its tyrosine kinase (TK) activity. To address this, we

supplemented the medium with 10 μ M IM to inhibit the TK activity of the BCR::ABL1 fusion protein. Within two days of IM supplementation, we observed that several attached colonies with typical iPSC morphology derived from the dome-shaped colonies (**Figure 4.4.3A**), and there was an increase in the TRA-1-60+ cell population (**Figures 4.4.3B and C**), with all clones showing positive pluripotency marker expression (**Figure 4.4.3D**). These clones could also differentiate into three germ layers: ectoderm, endoderm, and mesoderm (**Figure 4.4.4**). However, when IM was withdrawn, the IM-supplemented CML-CD34-iPSC colonies reverted to their original "dome-shaped" morphology within 2-4 days (**Figure 4.4.5A**), with a decrease in cell viability (**Figure 4.4.5B**) and TRA-1-60 expression (**Figure 4.4.5C**). Conversely, when control iPSCs were treated with IM, there was a reduction in the colony number, indicating that IM supplementation explicitly enhances the maintenance of CML-CD34-iPSCs (**Figure 4.4.6**).

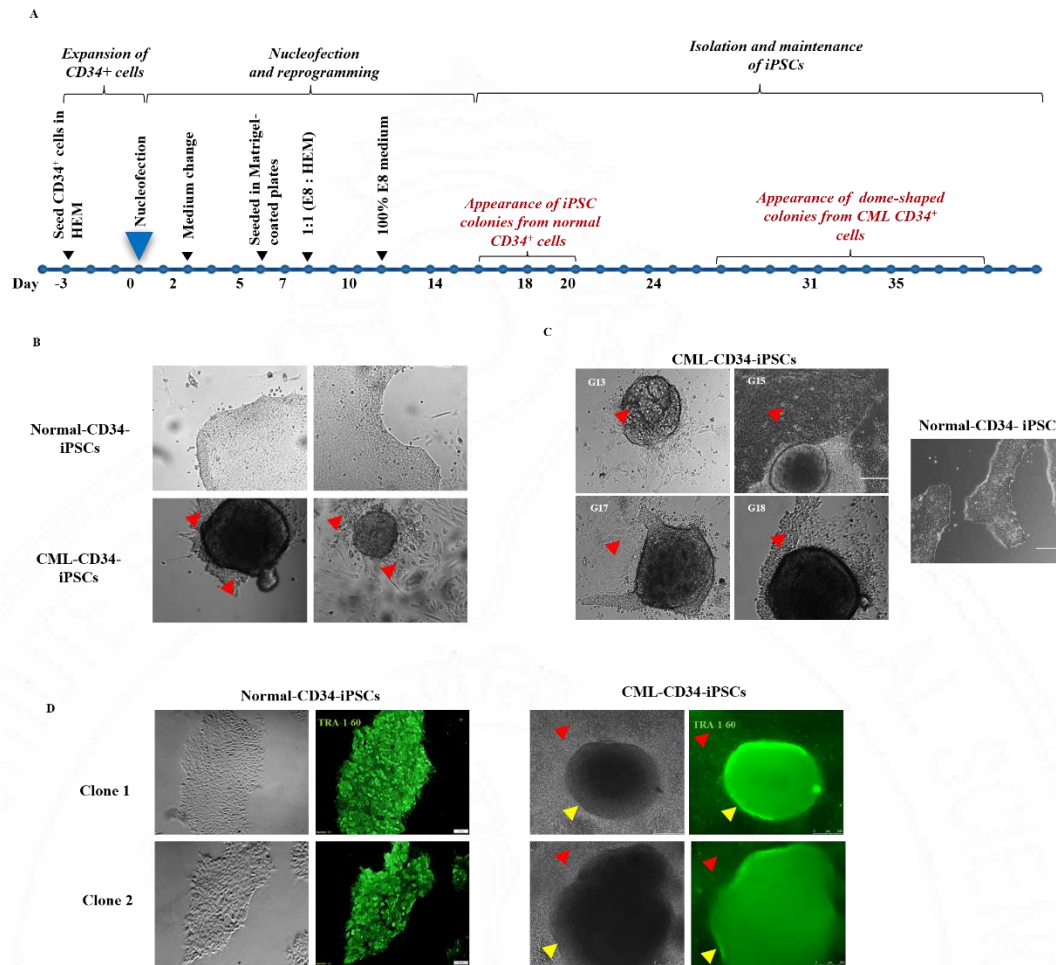


Figure 4.4.1: Reprogramming of Normal and CML CD34⁺ cells. (A) Experimental outline for reprogramming of CML-CD34⁺ and normal-CD34⁺ cells. The purified CD34⁺ cells were expanded in culture, nucleofected with episomal plasmids, transferred to Matrigel-coated plates containing the reprogramming medium, and isolated colonies and passaged. HEM: Hematopoietic expansion media comprising SFEM II containing CD34⁺ expansion supplement and UM729. (B) Representative images of the emerging iPSC colonies generated from CML-CD34⁺ and normal-CD34⁺ cells. CML-CD34-iPSCs had "dome-shaped" colonies, and normal-CD34-iPSCs had typical flat morphology. (C) CML-CD34-iPSC and normal-CD34-iPSC colonies. Red arrows indicate the non-iPSC cells spontaneously differentiated from CML-CD34-iPSCs. Scale bar: 50 μ M. (D) TRA-1-60 immunofluorescence of normal-CD34-iPSC and "dome-shaped" CML-CD34-iPSC colonies. Scale bar: 250 μ M. The green color indicates TRA-1-60. Yellow arrows indicate TRA-1-60⁺ "dome-shaped" CML-CD34-iPSC colonies and red arrows indicate differentiated cells.

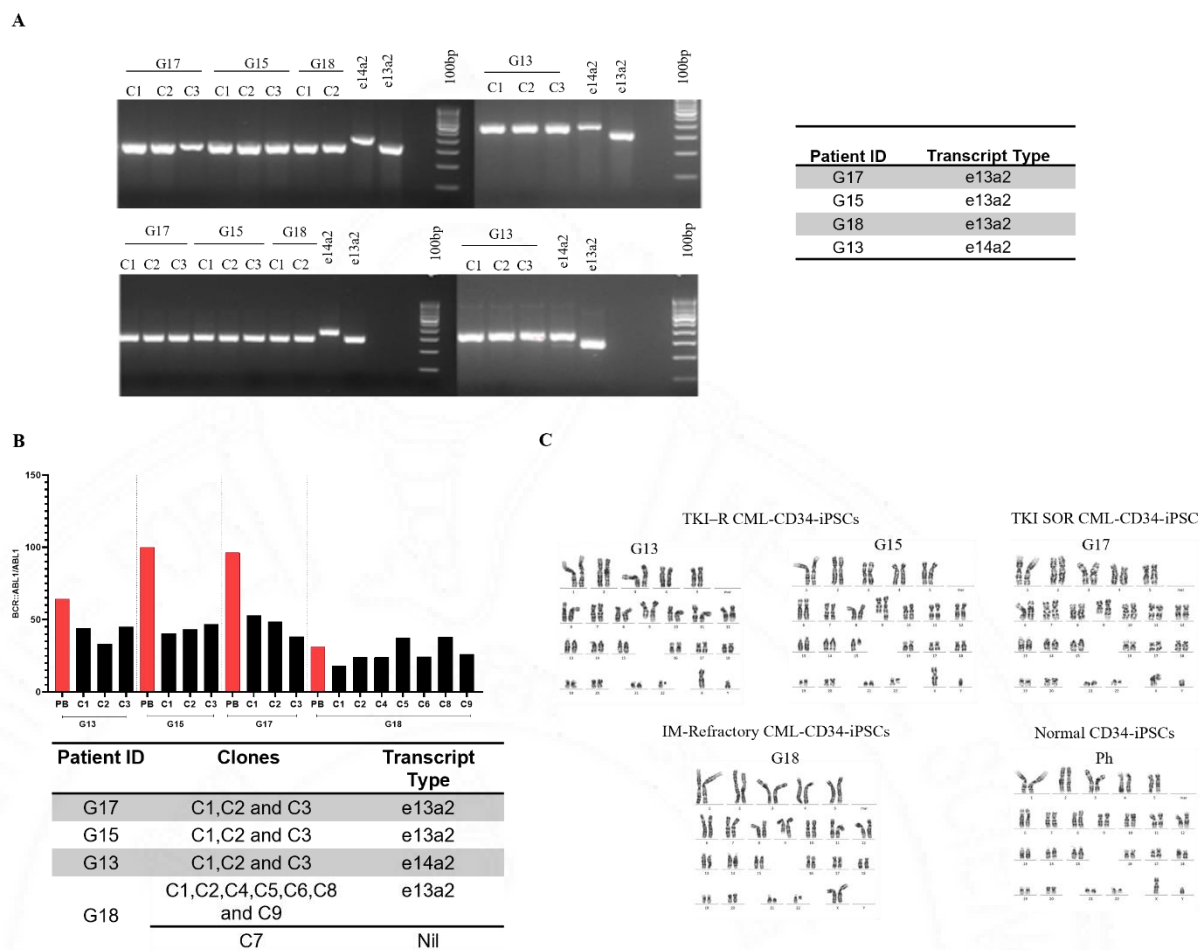


Figure 4.4.3: Characterization of CML-CD34-iPSC clones. (A) Agarose gel image of *BCR::ABL1* transcripts in CML-CD34-iPSC clones. **(B)** Quantification of *BCR::ABL1* transcripts present in the CML-CD34-iPSC clones from 4 patients (normalized to *ABL1* expression) by ddPCR. Red bars indicate the *BCR::ABL1* transcripts % in the peripheral blood samples collected at presentation, and the black bars indicate those in the CML-CD34-iPSC clones (cultured with 10 μ M IM supplementation). **(C)** Karyotyping analysis of iPSC lines showing *t*(9;22) (*q*34;*q*11) translocation in the CML-CD34-iPSC lines except one clone showing normal karyotype. An iPSC line generated from normal-CD34⁺ cells showing normal karyotype is also shown. #Ph+G13, G17 represents CML-CD34-iPSC clones derived from CML patients, TKI- Tyrosine kinase inhibitor; SOP- Suboptimal response, R-Responder, IM-Imatinib Mesylate, Ph- represent clone showing normal karyotype.

The CRK-like proto-oncogene (CRKL) is a downstream target of *BCR::ABL1* and can be phosphorylated by the fusion protein (Hamilton et al., 2006; Nichols et al., 1994). The expression of p-CRKL was analyzed in CML-CD34-iPSCs before and after IM treatment

to determine the status of BCR::ABL1 kinase activity (**Figure 4.4.5D**). The results showed that the expression of p-CRKL decreased in CML-CD34-iPSCs after IM treatment, suggesting that the suppression of BCR::ABL1-mediated tyrosine phosphorylation is needed for the stable maintenance of CML-CD34-iPSCs.

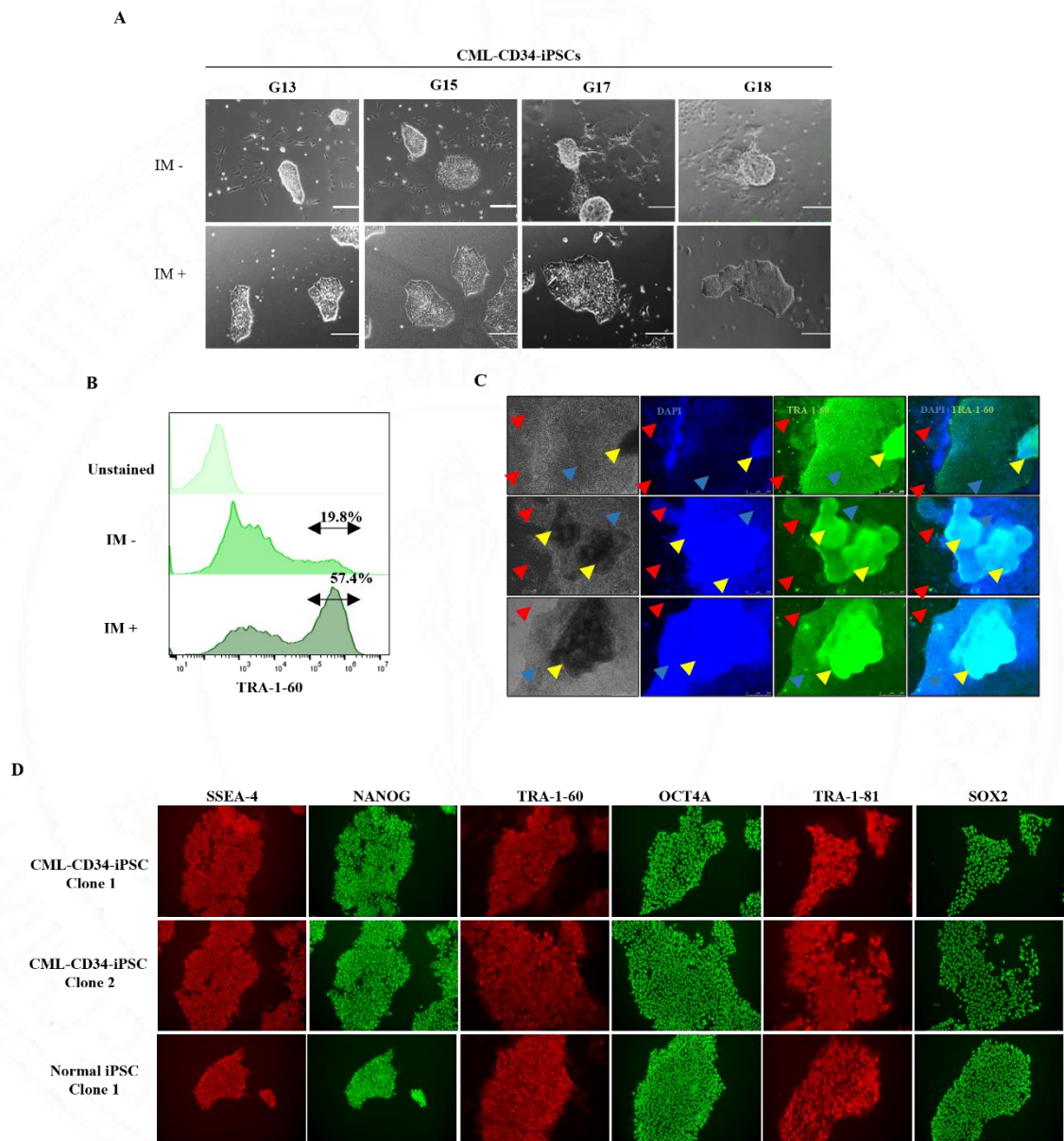


Figure 4.4.3: IM aids in the maintenance of CML-CD34-iPSCs. (A) Representative images of the "dome-shaped" CML-CD34-iPSC colonies exhibit significant differentiation generated without IM and with typical iPSC morphology after IM supplementation for 24 hours. Scale bar: 20 μ M. (B) Flow cytometry analysis of

TRA-1-60 expression in CML-CD34-iPSCs cultured in the presence and absence of IM. CML-CD34-iPSCs cultured in the presence of IM showed a significantly reduced level of differentiation compared to the colonies maintained in the absence of IM. (C) Transformation of "dome-shaped" colonies to typical iPSC colonies after IM treatment. "Dome-shaped" colonies transform to TRA-1-60+ iPSC colonies with flat morphology. Red arrows: differentiated cells, yellow arrows: "dome-shaped" TRA-1-60+ colonies, and blue arrows: typical iPSC colonies formed beneath the "dome-shaped" colonies. Scale bar: 250µM. (D) Representative immunofluorescence of pluripotency markers in CML-CD34-iPSC and normal-CD34-iPSC clones stained with anti-SSEA-4, anti-NANOG, anti-TRA-1-60, anti-OCT4A, anti-TRA-1-81 and anti-SOX2 antibodies. Scale bar:100µM

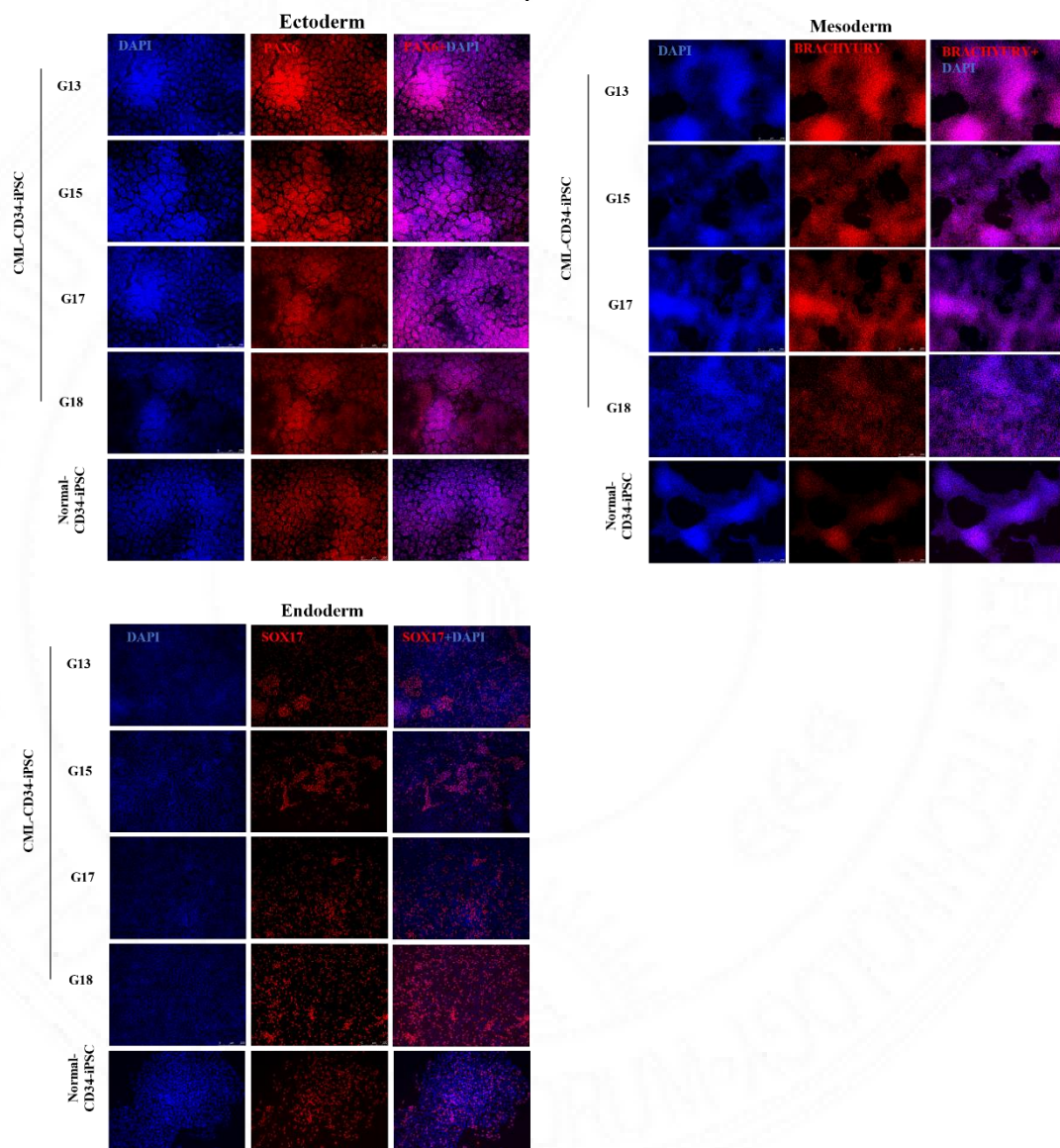


Figure 4.4.4: Trilineage differentiation of CML-iPSC lines (G13, G15, G17, and G18) and a normal iPSC line. The cells differentiated to ectoderm, endoderm, and mesoderm

exhibited the expression of Brachyury, SOX17, and PAX6, as measured by immunofluorescence analysis. DAPI was used for staining the nuclei; Scale bar: 250µM.

4.4.3 The effect of IM in reprogramming CML-CD34+ cells

We assessed the impact of IM supplementation on the reprogramming of CML-CD34+ cells, as we observed that the reduction of BCR::ABL1 kinase activity improves the stability and pluripotency of CML-iPSCs. CML CD34+ cells from two IM-responsive patients were nucleofected and cultured in the reprogramming medium supplemented with 10µM IM (**Figure 4.4.7A**). Reprogramming of CML-CD34+ cells without IM supplementation resulted in "dome-shaped" colonies. After IM supplementation, these colonies transformed into typical iPSC morphology, expressing the TRA-1-60 pluripotent marker (**Figure 4.4.7B**). However, IM supplementation throughout reprogramming failed to yield iPSC colonies with either the typical morphology or those with "dome-shaped" morphology. Our findings indicate that while IM facilitates the stability of CML-iPSCs by inhibiting BCR::ABL1 kinase activity, it hinders the reprogramming of CML-CD34+ cells. Overall, our results demonstrate that IM supplementation transforms the "dome-shaped" colonies formed during the reprogramming of CML-CD34+ cells into iPSCs with flat morphology. Additionally, IM improves the pluripotency of CML-CD34-iPSCs by inhibiting BCR::ABL1-mediated TK activity.

4.4.4. Reprogramming of CD34+ cells from an IM refractory CML patient

The HPCs (Hemopoietic progenitor cells) derived from the CML iPSCs generated from therapy-resistant patients offer a valuable tool to explore the underlying resistance mechanisms. Therefore, CD34+ cells obtained from IM-refractory patients were subjected to reprogramming with and without IM supplementation (**Figure 4.4.7A**).

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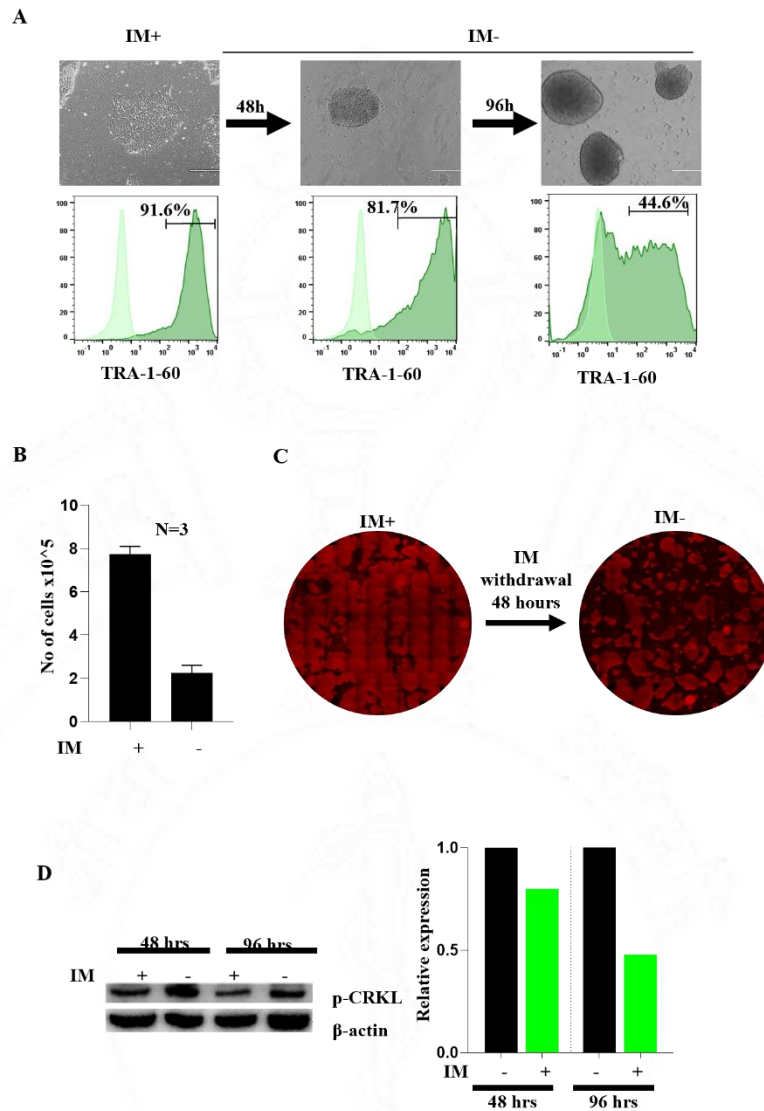


Figure 4.4.5: Withdrawal of IM affects cell proliferation, morphology, and pluripotency of CML-CD34-iPSCs. (A) The morphology and flow cytometry analysis of CML-CD34-iPSCs after IM withdrawal for 48 and 96 hours. The IM-supplemented CML-CD34-iPSCs formed "dome-shaped" colonies after IM withdrawal, resulting in reduced TRA-1-60 expression. **(B)** Number of CML-CD34-iPSCs after 48h of IM withdrawal. CML-CD34-iPSCs showed reduced cell viability after IM withdrawal **(C)** Immunofluorescence analysis showing TRA-1-60 expression in the CML-CD34-iPSC clones cultured with (IM+) and without IM (IM-). **(D)** Immunoblot analysis of phospho-CRKL expression in CML-iPSC clones cultured with and without IM. Relative Phospho-CRKL protein expression was estimated using Image J software.

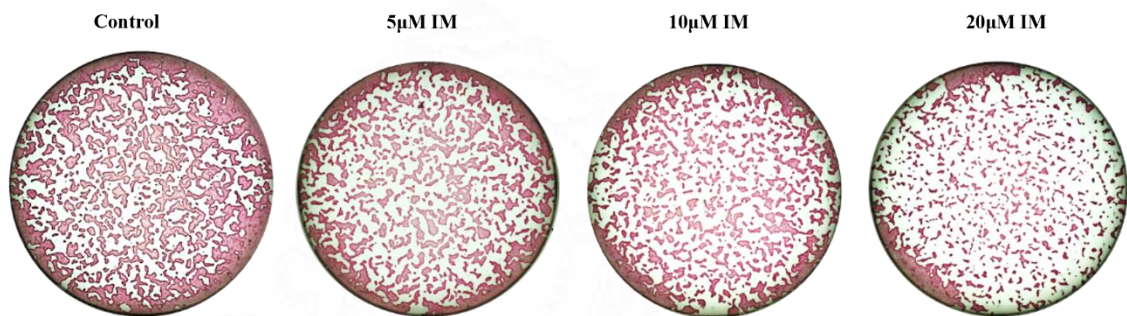


Figure 4.4.6: Alkaline phosphatase staining of a healthy donor CD34-iPSC line treated with different concentrations of IM showing a reduction in the colony number after IM treatment.

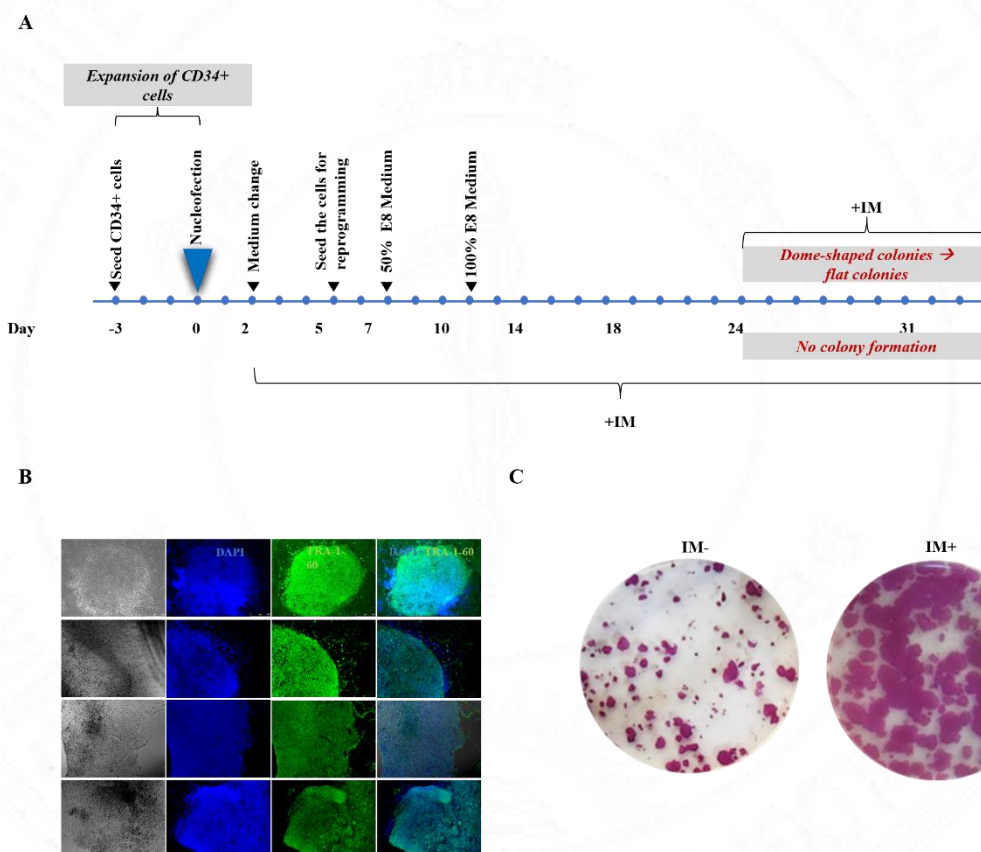


Figure 4.4.7: Supplementation of IM did not improve the reprogramming of CML-CD34-iPSCs. (A) Experimental outline to assess the effect of IM in reprogramming CML-CD34-iPSCs. (B) Immunofluorescence showing TRA-1-60 expression in CML-CD34-iPSCs after IM supplementation during reprogramming. (C) Alkaline Phosphatase

staining of CML iPSC generated from an IM-refractory CML patient's CD34⁺ in the absence (a) and presence of IM (b). Scale bar: 50μM

In contrast, we observed iPSC colonies emerge earlier in the IM-refractory patients' sample than the responder (~3 weeks vs. ~4 weeks). However, IM refractory patients' sample generated more colonies with continuous IM supplementation than without IM (**Figure 4.4.7C**). We isolated a total of 9 colonies, with 8 of them displaying a distinct "dome-shaped" appearance (as shown in **Figure 4.4.1C**), while the remaining colonies exhibited the typical iPSC morphology (**Figure 4.4.8A**). Upon conducting karyotyping analysis on the "dome-shaped" colonies derived from the IM refractory patient, we observed the presence of t(9;22). Conversely, the clone with the typical iPSC morphology showed a normal karyotype (**Figure 4.4.8A and 4.4.8B**). These findings strongly indicate that the "dome-shaped" morphology observed in CML-CD34-iPSCs results from BCR::ABL1 expression in these cells.

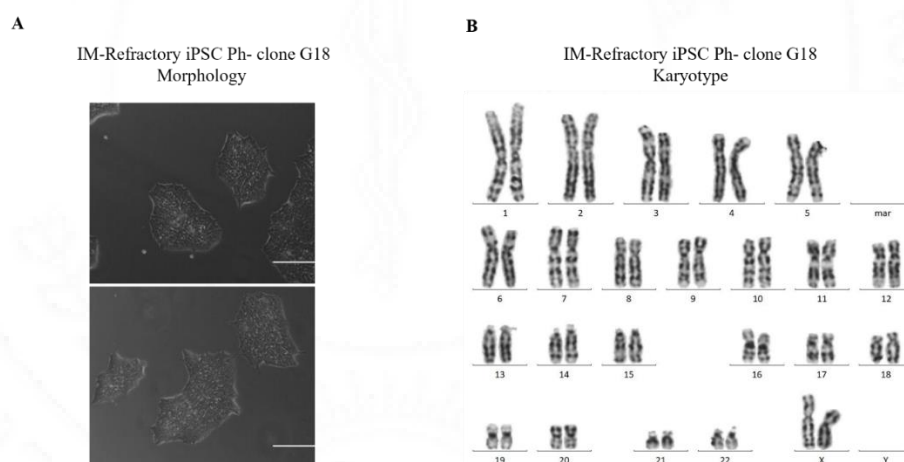


Figure 4.4.8: Reprogramming of IM refractory CML-CD34⁺ cells (A) Representative images of one Ph-CML-CD34-iPSC colony with typical morphology generated from an IM refractory patient. (B) Karyotyping analysis of IM refractory CML-CD34-iPSC clone with typical iPSC morphology showing normal karyotype. IM- Imatinib mesylate, G18-CML patient sample id. Scale bar:50μM.

4.4.5 Second and third-generation TKIs also aid in the maintenance of CML iPSCs

We investigated the effects of second-generation TKIs, DA and NIL, and third-generation TKI ponatinib on the maintenance of CML-CD34-induced pluripotent stem cells (CML-CD34-iPSCs). We replaced IM with different DA, NIL, and ponatinib concentrations and observed their effects on CML-CD34-iPSCs. However, when used at lower concentrations, these TKIs formed "dome-shaped" colonies (**Figure 4.4.9**). Our findings collectively indicate that suppressing BCR::ABL1 TK activity enhances the pluripotency of CML-iPSCs.

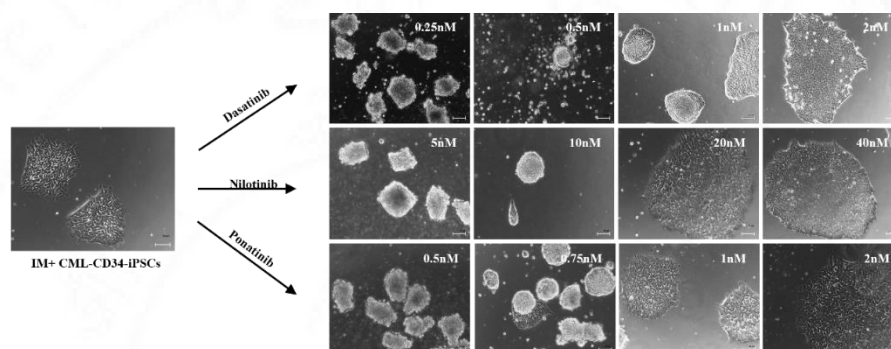


Figure 4.4.9: Effect of Second and third-generation TKI in maintaining CML-CD34-iPSCs. Images of iPSCs after treating the IM-supplemented CML-CD34-iPSCs with different concentrations of Dasatinib (0.25,0.5,1 and 2nM), Nilotinib (5,10, 20 and 40nM) and Ponatinib (0.5,0.75, 1 and 2 nM). Scale bar: 50µM

4.4.6 Hematopoietic differentiation of CML iPSCs

Previous research has shown that CML-iPSCs can be successfully transformed into hematopoietic progenitor cells (HPCs) despite the presence of BCR::ABL1 oncoprotein and its TK activity (Bedel et al., 2013; Kumano et al., 2012; Miyauchi et al., 2018). However, the efficiency of hematopoietic differentiation of CML-iPSCs has been reported to be lower than that of normal iPSCs (Hu et al., 2011; Miyauchi et al., 2018). Additionally, the HPCs obtained from CML-iPSCs have only partially regained TKI

sensitivity (Hosoi et al., 2014) (Hosoi et al., 2014). In light of our findings that IM supplementation is necessary to maintain CML-CD34-iPSCs, we studied whether IM supplementation is also essential for differentiating CML-iPSCs into HPCs.

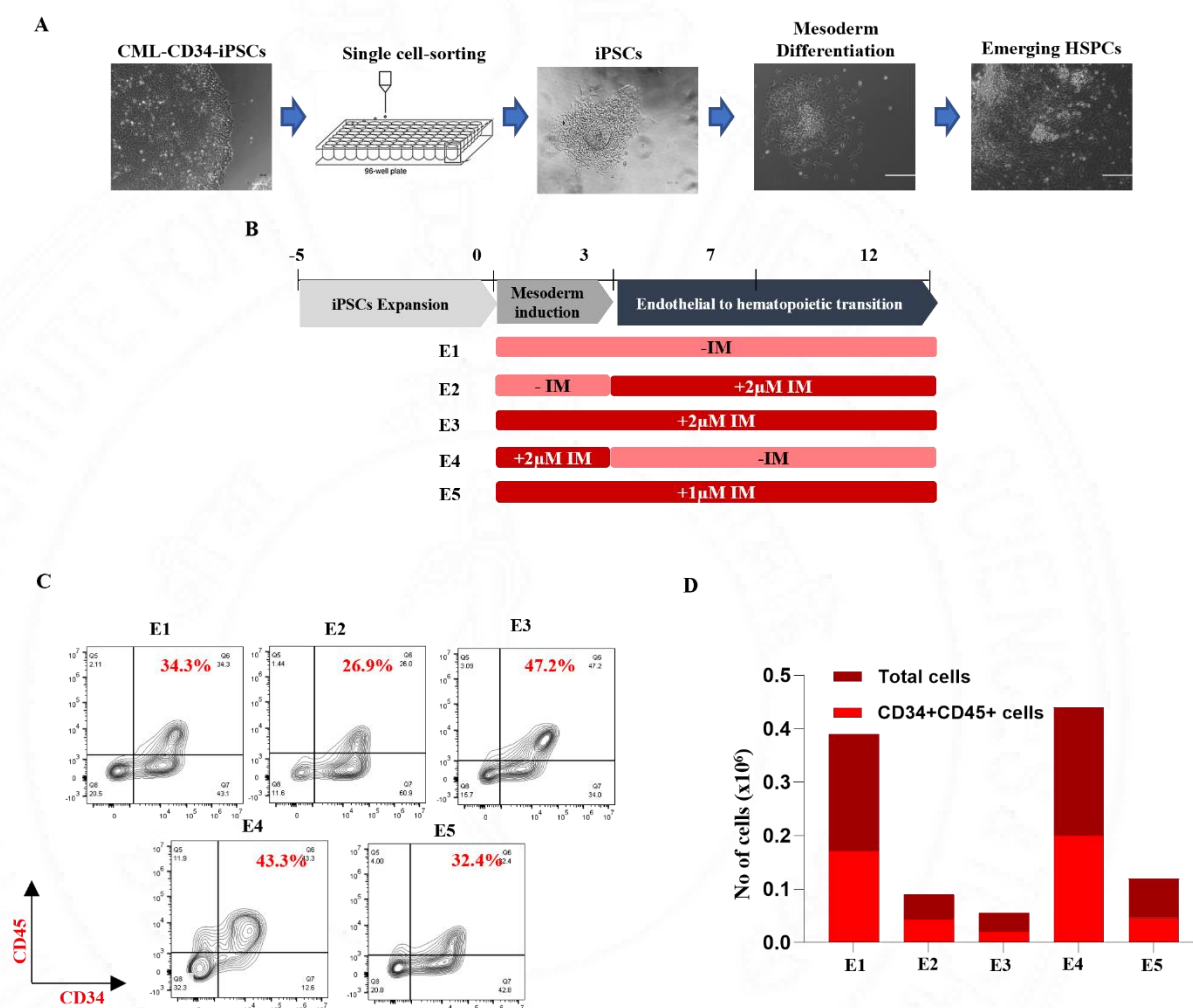


Figure 4.4.10: Effect of IM on hematopoietic differentiation of single cell expanded CML-CD34-iPSCs. (A) Experimental outline of hematopoietic differentiation from single-cell sorted CML-CD34-iPSCs. (B) Representative work outline of different experiment groups (E1 to E5) with IM supplementation during various stages of hematopoietic differentiation. (C) Representative flow cytometry results showing the percentage of CD34⁺ and CD45⁺ generated under different conditions of hematopoietic differentiation. (D) Total cells and CD34⁺CD45⁺ cells generated during hematopoietic differentiation.

To answer our question, we sorted single cells to obtain iPSC colonies with an equal number of cells (**Figure 4.4.10A**). These colonies were subjected to hematopoietic differentiation, and then IM was supplemented during different stages (**Figure 4.4.10B**). We differentiated CML-CD34-iPSCs using two different concentrations of IM (1 μ M and 2 μ M) and without any IM supplementation. We obtained suspension cells consisting of HPCs both with and without IM. Interestingly, CML-CD34-iPSCs differentiated without IM before and after mesoderm induction (E1) showed a 5-fold increase in the yield of CD34⁺CD45⁺ HPCs and the total number of suspension cells compared to those differentiated in the presence of IM throughout the entire differentiation (E3 and E5) (**Figure 4.4.10D**). Flow cytometry analysis of hematopoietic markers revealed that approximately 41% of the suspension cells were CD34⁺CD45⁺ HPCs (**Figure 4.4.10C**). By utilizing single-cell sorting of iPSCs, we generated a sufficient number of HPCs for a drug screening experiment.

Based on the previous results, iPSCs were seeded as small aggregates and then subjected to differentiation with and without IM supplementation after mesoderm induction (**Figure 4.4.11A**). Flow cytometry analysis showed that around 50% of the suspension cells were CD34⁺CD45⁺ HSPCs (**Figure 4.4.11B**). We found that CML-CD34-iPSCs that were differentiated without IM resulted in approximately 30 times more HPCs than those with IM supplementation. The decline in HPC differentiation with IM can be attributed to the sensitivity of CML-iPSC-HPCs to IM (**Figure 4.4.11C**). Hematopoietic colony formation revealed that the HPCs generated without IM had more myeloid progenitors than those from a normal iPSC clone (**Figure 4.4.11D**). We validated these findings by differentiating CML-CD34-iPSCs from two more patients

(Figure 4.4.11E), resulting in more CD34⁺CD45⁺ cells without IM (Figure 4.4.11F, G). Consistently, differentiation without IM resulted in more HPCs than the other experimental groups.

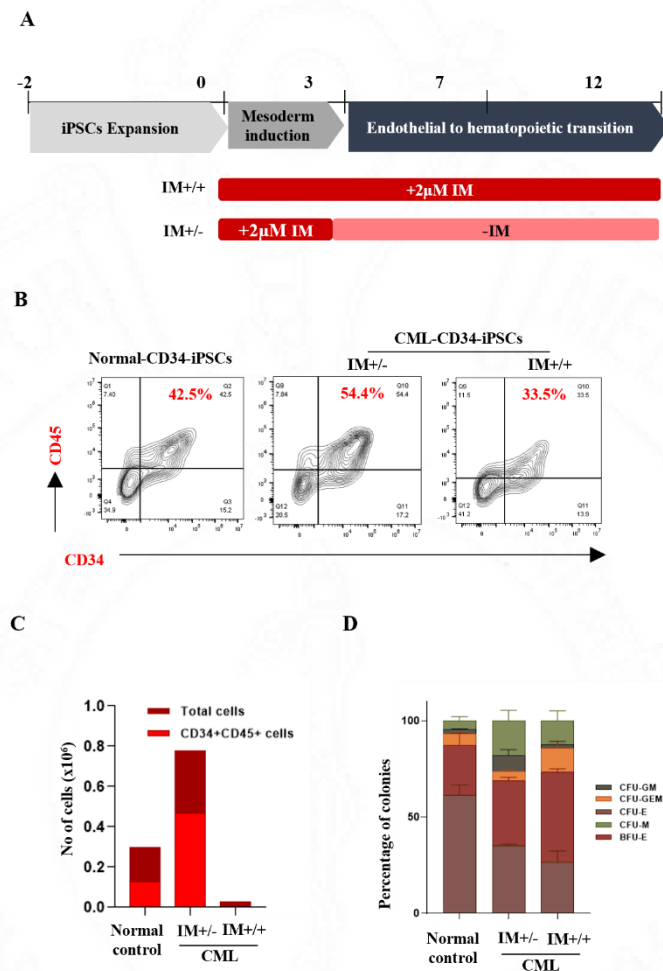
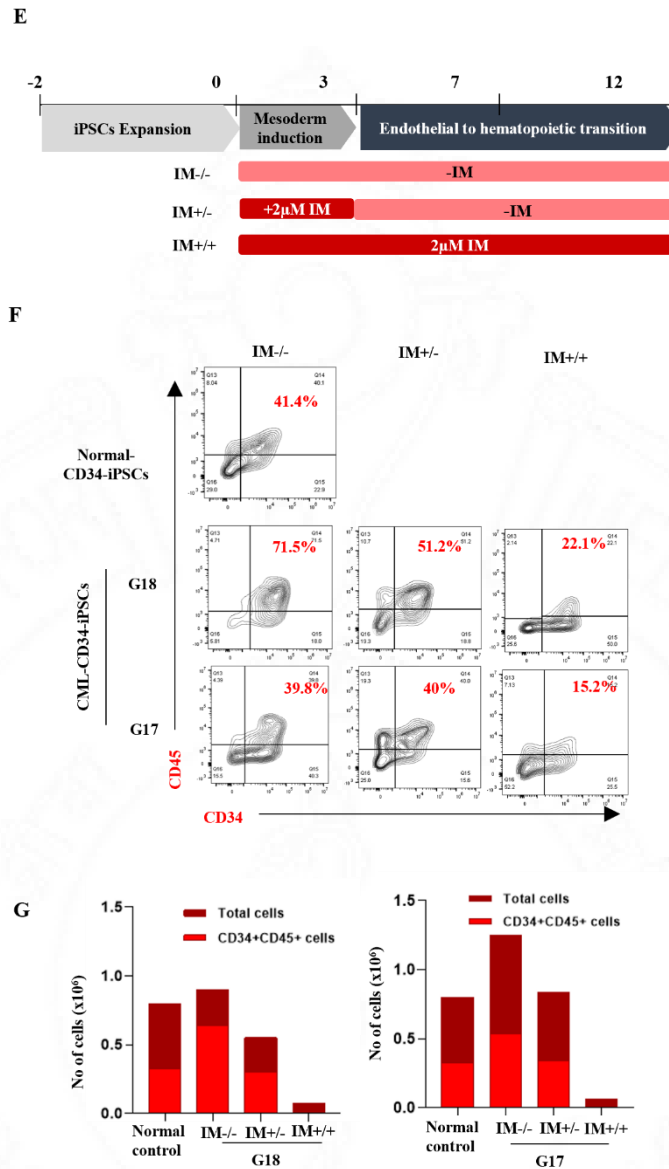


Figure 4.4.11: IM affects the HSPCs differentiation of CML-CD34-iPSCs. (A) Schematic representation of hematopoietic differentiation of CML-iPSCs to analyze the effect of IM after mesoderm differentiation (B) Representative flow cytometry results of the percentages of CD34⁺CD45⁺ cells generated from healthy donor and CML-CD34-iPSCs. (D) Hematopoietic colony forming capacity of HSPCs derived from CML-CD34-iPSCs and normal CD34-iPSCs.



(E) Experimental outline for HSPC differentiation with and without IM at different stages of differentiation. (F) Representative flow cytometry results of the percentages of CD34⁺CD45⁺ cells generated from healthy donor and CML-CD34-iPSCs generated from two patients. (G) Bar graphs showing the relative cell numbers and CD34⁺CD45⁺ cells generated by CML-iPSC colonies (G17 and G18) generated from two patients during differentiation under different conditions. IM-/- represents CML-CD34-iPSCs was subjected to HSPC differentiation without IM; IM+/- represents IM was withdrawn after mesoderm induction. IM+/+ represents IM was supplemented throughout HSPCs differentiation.

A crucial goal in treating CML is to eliminate CML-LSCs, as doing so can help prevent the disease recurrence, prolong survival, enhance the quality of life, and enable curative treatments (Corbin et al., 2011b; Etienne et al., 2017; Mahon and Etienne, 2014). However, due to the scarcity of these cells, studying the molecular mechanisms contributing to their survival has been challenging. A potential solution to this obstacle is generating iPSCs from CML CD34⁺ cells. These iPSCs can be expanded and differentiated into hematopoietic cells, providing a continuous and abundant supply for in-depth proteome, epigenome, transcriptome, and drug screening studies.

Previous studies have successfully generated CML iPSCs from PBMNCs (Hu et al., 2011) and CD34⁺ cells (Bedel et al., 2013; Kumano et al., 2012; Miyauchi et al., 2018; Telliam et al., 2016) using various vectors such as lentiviral (Bedel et al., 2013), retroviral (Hosoi et al., 2014; Kumano et al., 2012), Sendai (Telliam et al., 2016) or episomal vectors (Hu et al., 2011; Miyauchi et al., 2018). However, these studies did not address the atypical morphology and low reprogramming efficiency observed in CML iPSCs. Additionally, the impact of BCR::ABL1 expression on the morphology and stabilization of CML-iPSC colonies was not investigated (Imeri et al., 2023).

In our observations, the reprogramming efficiency of CML CD34⁺ cells was approximately 50 times lower than that of normal CD34⁺ cells, as previously reported (Hu et al., 2011; Miyauchi et al., 2018). This lower efficiency could be attributed to the presence of the BCR::ABL1 oncogene, which might interfere with the reprogramming process of CML CD34⁺ cells (Ito et al., 2022). Furthermore, CML CD34⁺ cells formed colonies later than normal CD34⁺ cells and exhibited atypical colony morphology (Bedel et al., 2013). Other researchers, such as Bedel et al., reported that CML iPSCs displayed

a distinct morphology similar to cell aggregates found in mouse embryonic stem cells (Bedel et al., 2013; Kumano et al., 2012).

The instability of CML-CD34-iPSCs may be due to the presence of the *BCR::ABL1* oncogene, which might disrupt the action of reprogramming factors required to generate stable iPSC colonies. Previous research by Ito et al. indicated that the use of molecularly targeted drugs like IM, capable of suppressing the activity of the BCR-ABL oncogene, could enhance the transcriptional regulation of reprogramming factors and boost pluripotency marker expression (Ito et al., 2022). This study also demonstrated that IM supplementation increased pluripotency marker expression in BCR::ABL+ K562 cells, further confirming that oncogene suppression might enhance cancer cells' reprogramming. Our study corroborated these findings, revealing that IM supplementation was essential for generating stable CML iPSCs without spontaneous differentiation. The presence of IM improved the stability and maintenance of all isolated CML-CD34-iPSC colonies, enabling them to be cultured for over 20 passages without significant differentiation. CML-CD34-iPSCs cultured with IM showed increased proliferation, while IM withdrawal led to reduced proliferation and TRA-1-60 expression. These results align with previous findings indicating faster growth of CML-iPSCs in the presence of TKI (Bedel et al., 2013; Charaf et al., 2017).

Interestingly, the phosphorylation of CRKL was decreased in IM-treated primary CD34+ HPCs from CML patients (Suknuntha et al., 2015) and CML iPSCs (Figure 4.4.3D), suggesting that BCR-ABL TK activity in hematopoietic cells and CML iPSCs may involve similar pathways. The discovery that IM can stabilize the pluripotency of CML-iPSCs unveils a novel mechanism of pluripotency maintenance. Both CML-iPSCs and

LSCs express the BCR-ABL fusion protein but are resistant to IM. Therefore, CML-iPSCs generated using our approach, along with the HPCs derived from these iPSCs, offer a valuable platform for CRISPR-Cas9 screening to investigate the mechanisms of TKI resistance in these cells and identify potential drugs to eliminate CML-LSCs effectively.

5. SUMMARY AND CONCLUSIONS

The introduction of targeted therapy with tyrosine kinase inhibitor (TKI) imatinib in chronic myeloid leukemia (CML) has revolutionized the management of this disease, resulting in patients on TKI therapy having life expectancy similar to healthy populations. However, one major challenge to TKI therapy's success in CML is the persistence of leukemic stem cells (LSCs) that TKIs do not eliminate. In this doctoral work, we sought to understand the mechanisms by which CML LSCs escape TKI therapy. We explored ways to modulate identified targets in eliminating these LSCs, as outlined in Figure 6.

We took three approaches: First, we performed transcriptomic profiling in primary CML CD34⁺ cells and healthy donor CD34⁺ cells to look for differentially expressed gene signatures. Subsequently, we performed molecular inhibition of one of the topmost upregulated targets in CML CD34⁺ cells. We assessed whether this would reduce the stemness in-vitro and leukemogenic potential in-vivo in a cell line-derived xenograft mouse model. Further, as LSCs are a rare population of cells, we used iPSC-based disease modeling of CML and found an exciting observation that to form stable iPSCs, BCR::ABL has to be inhibited.

Second, we attempted to unravel the contribution of dysregulated miRNAs in primary CML cells cultured under hypoxia, postulating that hypoxia-associated miRNAs play a role in the survival and maintenance of CML LSCs.

Third, to explore the role of the bone marrow MSC niche in protecting CML LSCs, we successfully generated and characterized an immortalized CML MSC cell line and assessed its role in maintaining CML LSCs.

The salient findings from each objective are listed below:

Objective 1: Transcriptomic profiling of CML CD34⁺ cells to study the differentially expressed genes between CML LSCs and normal hematopoietic stem cells.

- ❖ Transcriptomics profiling revealed 1563 genes upregulated and 1400 genes downregulated in the CML CD34⁺ cells.
- ❖ Pathway enrichment analysis showed genes associated with the cell cycle, Fanconi anemia pathway, and metabolism were significantly altered in CML CD34⁺ cells. These changes pointed towards enhanced oxidative phosphorylation, indicating potential metabolic vulnerabilities that could be exploited for therapeutic intervention. in CML CD34⁺ cells.
- ❖ Genes associated with serine proteases, including cathepsin G (*CTSG*), cathepsin L (*CTSL*), neutrophil elastase (*ELANE*), and ribonucleases (*RNASE*) were upregulated in CML CD34⁺ cells. Subsequent validation in an entirely new cohort of (n=59) primary CML CD34⁺ cells corroborated the upregulated genes.
- ❖ Molecular modulation of CTSG markedly reduced colony-forming potential *in vitro*, underscoring its probable role in promoting LSC stemness and clonogenicity. These observations were further corroborated in an *in vivo* xenotransplantation model, wherein CTSG knockdown showed a significant increase in overall survival, suggesting its role in leukemogenesis.

Objective 2: The role of hypoxia-associated miRNAs in CML LSC survival

- ❖ Apart from CML cell lines, we established hypoxic culture conditions in primary CML cells, which is quite challenging. We further confirmed that the cells were

maintained in hypoxic conditions by showing reduced proliferation, entry into a slow-cycling state, and elevated expression of HIF-1 α and its downstream targets.

- ❖ MiRNA profiling of CML primary cells cultured under hypoxia showed dysregulation of 20 miRNAs. Among the differentially expressed miRNAs, miR-210, a well-known hypoxia-regulated miRNA, was significantly upregulated.
- ❖ miR-30b-5p were explicitly upregulated in CML cells under hypoxic conditions, whereas unaltered in healthy donor PBMNCs. The role of miR-30b is exclusively studied in solid tumors, but its role in CML LSC is unknown. MiR-30b-5p knockout reduced proliferation and colony-forming capacity in K562 cells under hypoxic conditions, suggesting that mir30b5p might play a role in the maintenance of CML cells.
- ❖ Target prediction revealed genes associated with TGF- β as a potential target of miR-30b-5b. TGF- β signaling is reported to be associated with CML LSCs; further studies are needed to confirm the findings.

Objective 3: Generation and characterization of immortalized CML MSC lines and assessing their roles in maintaining LSCs.

- ❖ CML-MSCs and normal MSCs were confirmed immunophenotypically for MSC markers. Although both normal and CML MSCs retained the trilineage differentiation potential (Adipogenic, Osteogenic, and Chondrogenic), CML MSCs exhibited enhanced chondrogenic differentiation compared to NBM MSCs.
- ❖ The iCML MSCs expressed all the MSC markers, retained the trilineage differentiation potential as the primary CML-MSCs, and did not acquire any additional chromosomal aberrations due to immortalization.

- ❖ iCML MSCs protected the CML cells from Imatinib-induced apoptosis and selectively increased the growth of CD34⁺ leukemic cells in *ex vivo* conditions. iCML MSC could be ideal for studying cell-cell interaction.
- ❖ CML MSCs secreted cytokines associated with immune response and inflammation, suggesting the possibility of an immune microenvironment in CML BMM. These results need to be validated further.

The iCML MSC is a valuable model for studying leukemic-stromal interaction and functional studies of candidate genes mediating TKI resistance in CML LSCs.

Objective 4: Generation and characterization of iPSCs from primary CML cells for modeling CML LSCs.

As LSCs are a rare population of cells, we generated and characterized induced Pluripotent Stem Cells (iPSCs) from CML CD34⁺ cells. These iPSCs can be expanded and transformed into hematopoietic cells, providing a steady supply for in-depth proteome, epigenome, transcriptome, and drug screening studies.

- ❖ The reprogramming efficiency of CML CD34⁺ cells was significantly lower than that of normal CD34⁺ cells, likely due to the presence of the BCR::ABL1 oncogene, which may interfere with the reprogramming process. CML CD34⁺ cells also exhibited delayed colony formation and atypical morphology.
- ❖ CML-iPSCs are highly unstable, and supplementation with imatinib mesylate (IM) aided in the maintenance of CML-iPSCs. The suppression of BCR::ABL1 fusion protein is essential for maintaining CML-iPSCs in the pluripotent state.

- ❖ Hematopoietic differentiation of CML-iPSCs in the absence of imatinib resulted in an increased generation of hematopoietic cells. Colony forming assay showed bias towards myeloid lineage compared with the CML-iPSCs differentiated with imatinib.
- ❖ The CML-iPSCs generated could be differentiated into BCR::ABL1 positive hematopoietic progenitors.

The CML-iPSCs and the resulting HSPC are excellent tools for conducting high-throughput screening assays such as CRISPR-Cas9 or small molecule compound library.

Conclusions

Global transcriptomic analysis of CML vs. normal CD34⁺ cells provided valuable insights into the molecular intricacies of CML, highlighting altered pathways, cell surface markers, and the potential role of *CTSG* in LSC survival. These findings offer a foundation for future research to develop targeted therapeutic strategies to disrupt these survival mechanisms and improve treatment outcomes for CML patients.

MiRNA profiling in primary CM cells in hypoxic conditions showed upregulation of miR-30b-5p. miR-30b-5p expression in hypoxic conditions was restricted to CML cells without alteration in healthy donor PBMNCs. Further, miR-30b-5p knockout in the K562 cell line markedly reduced colony-forming capacity and proliferation in hypoxic conditions. In-silico analysis revealed that miR-30b-5p might alter the TGF- β signaling in hypoxic conditions, suggesting that miR-30b-5p could serve as a potential biomarker.

Limitations of the present study

The transcriptomic study was performed in CD34⁺ fraction, which could be a mixture of progenitors and stem cells in both healthy donor and CML cells. The gene signatures obtained need validation in CML LSCs (CD34⁺CD38⁻) fraction to identify bonafide CML LSCs signatures.

One of the major limitations in the miRNA study is the use of primary CML patient bulk cells for miRNA profiling; the inherent variability among the primary samples would have masked the prominent dysregulated miRNA. An ideal scenario would have been to use a cell line model for discovery and validation in primary CML cells.

Factors influencing CML LSC survival

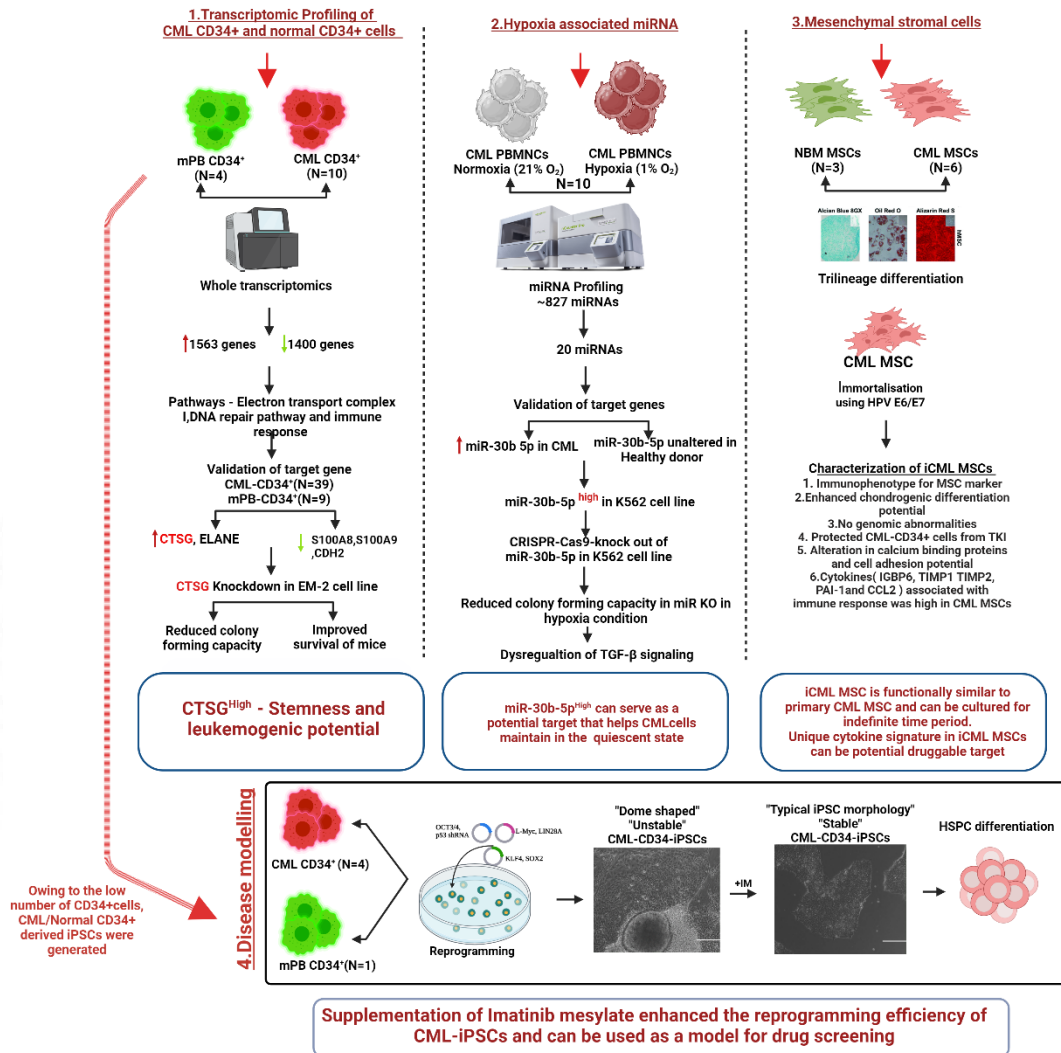


Figure 6: Summary of the significant findings

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7.LIST OF PUBLICATION

1. **Esther Sathya Bama Benjamin**, Elizabeth Vinod, Stallon Illangeswaran, Bharathi M. Rajamani, Rakhi Thalayattu Vidhyadharan, Abhirup Bagchi, Arnab Maity, Ajith Mohan, Ganesh Parasuraman, Soosai Manickam Amirtham, Aby Abraham, R.V. Shaji, Poonkuzhali Balasubramanian "Immortalised chronic myeloid leukemia (CML) derived mesenchymal stromal cells (MSC) line retains the immunomodulatory and chemoprotective properties of CML patient-derived MSCs", Cellular Signaling, in Press, 2024. **IF-4.8**
2. **Esther Sathya Bama Benjamin**, Dinesh Babu, Gaurav Joshi, Bharathi M Rajamani, Kritika Nandy, Sonam Rani, Senthamizhselvi Anandan, Chitra Premkumar, Madhavi Maddali, Aby Abraham, Shaji R Velayudhan, Poonkuzhali Balasubramanian "Inhibition of BCR-ABL1 tyrosine kinase activity Aids in the Generation of Stable Chronic Myeloid Leukemia Induced Pluripotent Stem Cells", Biorvix, 2023.
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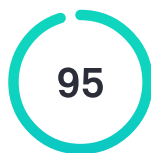
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1. INTRODUCTION

In 1960, Nowell and Hungerford made a groundbreaking revelation in blood samples obtained from individuals affected with chronic myeloid leukemia¹ (CML), uncovering a tiny chromosome known as a minute² chromosome (Peter C. Nowell and David A. Hungerford, 1960). This pivotal discovery marked a fresh era in hematology³ and triggered the current emphasis on molecular medicine.

291 | This genetic anomaly was subsequently identified as a balanced translocation of chromosomes 9 and 22 t(9;22)(q34;q11), resulting in the fusion of Abelson tyrosine-protein kinase 1 (ABL1) and breakpoint cluster region protein (BCR). This fusion triggered constitutive tyrosine kinase activity, leading to the abnormal accumulation of granulocytes(Deininger et al., 2000; Nowicki et al., 2003).⁴

Efforts to improve the quality of life for CML patients began with arsenicals⁵ in the late 1800s and treatments like radiation therapy, anti-leukocyte sera, and benzene in the early 1900s(Hehlmann et al., 1993).⁴ In 1953, Busulfan⁶ was introduced and showed promising results, which was then replaced⁷ by hydroxycarbamide due to safety concerns in the 1960s(Galton, 1953).⁴

Interferon alpha (IFN- α) gained popularity in the mid-1980s, with studies in the 1990s demonstrating its survival benefits(Allan et al., 1995).⁴ Imatinib replaced IFN- α as the preferred treatment in 2001, followed by the development of next-⁸ generation tyrosine kinase inhibitors (TKIs)(Druker et al., 1996, 2001). TKIs have radically changed the treatment paradigm of CML for most patients, turning it from a fatal to a chronic disorder(Druker et al., 2001).⁴ Currently, CML patients who receive proper TKI treatment, undergo adequate monitoring and display

⁹favorable treatment responses can anticipate an existence that ¹⁰closely resembles ¹¹normal life expectancy. Despite their promising results, TKI-based therapies do not eradicate leukemic stem cells (LSC) and have significant drug toxicities(Soverini et al., 2019).⁴ ~~Irrespective of these, the treatment recommendations, until recently, were to continue therapy indefinitely for all patients.~~

Risks of prolonged TKI treatment include noncompliance, associated with the risk of relapse and progression, toxicities including serious vascular complications and teratogenic effects, ¹²precluding pregnancy while on ¹³treatment(Kumar and Krause, 2021a).⁴ A significant area of research in CML ¹⁴is focused on targeting the quiescent and self-renewing LSCs implicated in refractoriness and disease progression. These LSCs can persist even when no measurable residual disease is present, ¹⁵and upon changes in the bone marrow (BM) microenvironment, they can exit quiescence and drive relapse, representing a bottleneck to cure. Some survival mechanisms of LSCs involve altering the BM microenvironment, transitioning from the quiescent state to the proliferative state through intracellular ¹⁶signaling changes and the secretion of cytokines(Camacho et al., 2017; Jin et al., 2008; Manshouri et al., 2011; Zhang et al., 2013, 2019; Zhao et al., 2009, 2022).⁴ These changes can disrupt metabolic processes, creating an anti-apoptotic microenvironment and dysregulated immune system(Brück et al., 2018; Kuntz et al., 2017; Zhao et al., 2022).⁴

Various strategies for combinatorial therapies to target and eradicate CML stem cells are ¹⁷being tested. These strategies include (a) targeting specific LSC surface markers (CD26, CD25, IL1-RAP),(Herrmann et al., 2014; Landberg et al., 2016; Sadovnik et al., 2017)⁴ (b) modulation of ¹⁸signaling pathways (i.e., Wnt or Hh)(Zhang et al., 2013; Zhao et al., 2009)⁴ to reverse LSC quiescence by

interfering with the activity of transcription factors (i.e., FoxO, BCL6, or PML) (Ito et al., 2008; Lagadinou et al., 2013),⁴ (c) interaction with the bone marrow microenvironment, (d) inhibition of drug-efflux pumps (e.g., ABC transporters), (e) targeting epigenetic factors distinguishing normal and LSCs(Abraham et al., 2019; Li et al., 2012).⁴ Despite an improved understanding of LSC biology, they remain elusive¹⁹ and difficult to target. Not all LSCs can be characterized²⁰ by surface markers, and the LSCs are heterogeneous in comprehending the mechanisms and pathways that lead to the survival and resistance of CML LSCs, necessitating further research for new drugs and treatment option.²¹ The interplay between CML LSCs and the BMM plays a significant role in malignant transformation(Colmone et al., 2008; Zhang et al., 2012).⁴ The specific mechanisms and molecules involved in this communication are not yet² fully understood. Although potentially druggable targets have been identified in CML to eradicate LSCs, drugs against these targets have not yet been implemented²³ in the clinic as a standard of care. These limitations highlight the need for further research to deepen our understanding of CML LSCs and develop more effective treatments for CML aiming toward treatment-free remission.

1.1 Rationale and Hypothesis

1. Genome-wide transcriptome analysis of CML and normal stem cells will enable us to identify novel targets for eradicating CML LSCs.
2. Hypoxic microenvironment-associated miRNA induced in CML stem cells could provide a survival advantage for CML LSCs
3. Mesenchymal stromal cells derived from CML patients will differ from those of healthy donors. The generation²⁴ of a CML MSC line will be of great advantage in studying the leukemia-stromal²⁵ cell interaction.

4. CML LSCs are rare, making it difficult to study the survival mechanisms. CML patient-derived iPSCs will be an ideal ²⁷ model for drug and CRISPR library screening, as it can retain the ²⁶ patient's pathophysiology without altering the genetic makeup.

1.2 Objectives

1. To elucidate the survival mechanisms of CML-LSCs by

§ Transcriptomic profiling of CML CD34+ cells to study the differentially expressed genes between CML LSCs and normal hematopoietic stem cells.

§ To study the role of hypoxia-associated miRNAs in CML LSCs.

2. Models to study LSCs by

§ ²⁴ Generation and characterization of immortalized CML MSC lines and assessing their roles in maintaining LSCs

§ ²⁴ Generation and characterization of iPSCs from primary CML cells for ²⁸ modeling CML LSCs

1.3 Brief overview of the thesis chapters

1.3.1. Literature review

²⁹² Chapter 2 is the literature review, which elaborates on the CML, its pathology and molecular mechanisms, and the treatment options. The literature review highlights the evolving understanding of LSC biology, including genetic and epigenetic alterations, ²⁹ signaling pathways, and microenvironment interactions. It emphasizes the significance of LSC-targeted therapies, especially tyrosine kinase inhibitors, in managing CML. Despite progress, challenges in LSC isolation and persistence remain. Ultimately, the review underscores the need for continued research to advance personalized treatments targeting CML LSCs to enhance patient outcomes and eradicate the disease.

1.3.2. Materials and Methods

This section describes the various techniques and methods used in the experiments and analyzing data. The study design and methods utilized for each experiment, methods used to assess molecular modulation by knockdown and knockout assays, and protein and mRNA expression techniques are described here. Detailed protocols describing the establishment of an immortalized MSC line and the generation²⁴ of CML iPSCs are also described³⁰ in this section.

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1.3.3. Results and Discussion

The results section is divided into four parts based on each objective, as listed above. The first part focuses on the differential gene expression between CML CD34+ cells and normal CD34+ cells. It also highlights the significance of CTSG in maintaining CML LSCs and its impact on LSC survival. In the second part, the emphasis is on the role of hypoxia-associated miRNAs and their effects on stemness. The third part highlights the importance of CML MSCs and the creation of an immortalized MSC line for studying BMM interaction. Lastly, the fourth part describes the generation²⁴ of CML iPSCs for drug screening.

1.3.4. Summary, Conclusion, and Bibliography

This chapter comprehensively summarises the observations from this doctoral research and outlines the ongoing research and future directions to expand understanding of this disease.

The references are listed³¹ in the Bibliography.

2. LITERATURE REVIEW

2.1. Hematopoiesis

The hematopoiesis process involves the precise regulation³² of hematopoietic stem cells (HSCs) to generate fully developed blood cells, categorized into

either myeloid or lymphoid lineages. This intricate differentiation of HSCs into specific lineages is a continuous journey encompassing a sequence of³³ progressively committed progenitor stages (**Figure 2.1**). These distinct progenitors traverse separate pathways, culminating in the production of diverse mature blood cells. The instructive model postulates that HSCs react to external cues, particularly cytokines, which actively prompt them to embark on a differentiation pathway of a particular lineage. This model implies a directed and responsive process where specific signals dictate the fate of HSCs, guiding them toward becoming specialized blood cells. In contrast,³⁴ the stochastic model presents an alternative perspective. According to this theory, the commitment of HSCs arises from spontaneous stochastic variations in cell phenotype. These intrinsic fluctuations are then followed by a selective step driven by cytokines.³⁵ In this scenario, the selection process refines the initial stochastic variations, determining the eventual lineage of the differentiated blood cell(Dussiau et al., 2022; Olson et al., 2020).⁴

Figure 2.1: Models of hematopoietic stem cell (HSC) lineage commitment. (A) Classical tree-like model (B) The continuous differentiation model. Boxes represent progenitor states based on phenotypic markers and illustrate the heterogeneity within each population. MPP-multipotent progenitor; CMP-common myeloid progenitor; CLP-common lymphoid progenitor; MEP-megakaryocyte-erythroid progenitor; GMP - granulocyte-macrophage progenitor (Olson et al., 2020) .^{4 36}

HSCs are regulated³⁷ by both intrinsic and extrinsic mechanisms. Disruptions to this regulatory mechanism, either through single or multiple driver mutations, can lead to malignant transformation.

2.2. Origins of Leukemia

A hierarchical organization exists in normal, self-renewing tissues, featuring a specific group of stem cells responsible for maintaining tissue integrity throughout an individual's²⁶ lifetime. Studies have identified cells with stem cell properties in various types of cancer, known as cancer stem cells (CSCs)

The initial evidence supporting cancer stem cells originated from investigations in leukemia³⁸ research. Earlier studies showed a subset of leukemic cells displayed clonogenic activity *in vitro* and demonstrated the ability to initiate the disease upon transplantation into mice Subsequently³⁹, these cells were termed leukemic stem cells (LSCs). Interestingly, the cell surface marker phenotype of these LSCs was identical to that of HSCs.

LSCs are leukemia-initiating⁴⁰ cells and, through serial transplantation, can propagate leukemia⁴¹, recapitulating the primary tumor⁴² and its heterogeneity in the recipient mouse. Additionally, LSCs can arise from transformed

294 | HSCs/progenitor cells that regain the capacity for self-renewal (Eppert et al., 2011; Goardon et al., 2011; Jamieson et al., 2004; Wang et al., 2010).⁴³ Leukemic cells can remodel the bone marrow microenvironment to support their maintenance and activity (Hou et al., 2020; Roeklein and Torok-Storb, 1995; Zhang et al., 2013).⁴

LSCs have displayed significant resistance to the majority of existing treatment approaches. Traditional therapies such as irradiation and cytotoxic drugs primarily target rapidly dividing cells, rendering them ineffective against the mostly quiescent LSC population. Furthermore, LSCs have exhibited resistance not only to irradiation and chemotherapy but also to targeted treatments like immunotherapy and tyrosine kinase inhibitors (Corbin et al., 2011a; Goff et al., 2013; Lagadinou et al., 2013; Sykes et al., 2011).⁴ Consequently, LSCs are⁴⁴ believed to be the main contributors to a significant proportion of treatment

failures and instances of relapse. As a result, focusing on targeting LSCs holds paramount importance from a clinical perspective.

Figure 2.2: Simplified representation of the hematopoietic hierarchy. The driver lesion of CML, the t(9;22) chromosomal translocation giving rise to the BCR-ABL1 fusion gene, is gained at the HSC level. Abbreviations: LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; MPP, multi-potent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor (Soverini.S et al., 2021).

2.3.Chronic myeloid leukemia⁴⁵

Chronic myeloid leukemia⁴⁶ (CML) is a hematopoietic stem cell disorder characterized by t(9;22) resulting in uncontrolled proliferation of myeloid cells (Kantarjian et al., 2019)⁴(Figure 2.2). It is a relatively rare disease, with 1-2 individuals per 100,000 people being diagnosed with CML annually, accounting for 15% of all newly diagnosed cases of leukemia⁴⁷ in adults (Elias Jabbour, Hagop Kantarjian 2020). Despite its rarity, CML is one of the most extensively studied malignancies and was among the first to be linked⁴⁸ to LSCs. Consequently, it now serves as a model disease for stem cell-based malignancies. The development of CML is associated with a reciprocal translocation between chromosomes 9 and 229, resulting in the formation of "Philadelphia chromosome"⁴⁹ (Ph) and the BCR::ABL1 fusion protein²⁶(Clarke and Holyoake, 2017; Deininger et al., 2020; Hochhaus et al., 2020)⁴ (Figure 2.3).

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Figure 2.3: The origin of the Philadelphia chromosome. The Philadelphia chromosome results from the translocation between the Abelson murine leukemia⁵⁰ (ABL1) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22. (Adapted from https://www.brainkart.com/article/Causes-of-Cancer_16408/)

2.2.1 Epidemiology

CML typically manifests at a median age of 60 in Caucasians while appearing at a younger age (35–45 years) in Asians. It is uncommon in children, accounting for only 2–3% of all pediatric leukemia⁵¹ cases (Jabbour and Kantarjian, 2014a)⁴. The global incidence rate of CML is 15 cases per 1,000,000 people annually, with a male-to-female ratio of 1.34 (Suttorp et al., 2021)⁴. The age-adjusted incidence rate for individuals under 18 is 1.0 cases per 1,000,00 (Suttorp et al., 2021)^{4 52}. The introduction of imatinib⁵³ in the year 2000 significantly contributed to a decrease in⁵⁴ annual mortality rates, reducing them from 10%-20% to 1%-2% (Goldman, 2010)⁴.

2.2.2 Disease phase

CML is mostly diagnosed⁵⁵ in the chronic phase (CP),⁵⁶ but if left untreated, it can progress to an accelerated phase⁵⁸ (AP) and eventually advance to a terminal phase⁵⁹ known as blast crisis (BC), which resembles acute leukemia⁶⁰ (Clarke and Holyoake, 2017; Sawyers, 1999)⁴ (Figure 2.4). The initial comprehensive data on the development of the disease originated from the study of atomic bomb survivors (Preston et al., 1994)⁴. It was observed⁶¹ that following an extended latent period, most patients (85%) initially presented with the CP, yet within a

5-year timeframe, they transitioned from accelerated⁶² phase to a blast crisis. However, the duration it takes for patients to progress to the blast crisis stage varies significantly among individuals, with some remaining in the CP for up to 20 years, while others advance to blast crisis within a few months (Cortes and O'Dwyer, 2004).²⁶ The reason for disease progression in some patients is worth exploring (Branford et al., 2018).⁴ Recent NGS-based studies show that additional gene mutation may drive this progression (Branford et al., 2019).⁴ *Figure 2.4. Progression of chronic myeloid leukemia.*⁶³ CML is characterized⁶⁴ *by an excessive proliferation of non-functional leukocytes (dotted lines) or blasts in the bone marrow and then in the blood* (Losson et al., 2020).⁴

2.2.3 Symptoms and signs

Most patients are diagnosed with CML during the CP of the disease, often through routine physical examinations or blood tests. Interestingly, approximately 30-50% of newly diagnosed patients display no symptoms at diagnosis. Common presenting symptoms for the remaining CP patients include fatigue, weight loss, malaise, early satiety, and pain in the left upper quadrant. Medical examinations usually reveal elevated white blood cell (WBC) counts and an enlarged spleen (splenomegaly).

The definitive diagnosis of CML typically involves confirming the presence of the Philadelphia chromosome (Ph) through conventional cytogenetics or fluorescent in situ hybridization (FISH). *BCR::ABL1* fusion transcript is also detected using highly sensitive⁶⁵ reverse transcriptase-polymerase chain reaction (RT-PCR) (Jabbour and Kantarjian, 2014b, 2020).

2.2.4. The role of *BCR::ABL1* as the driver of chronic myeloid leukemia

⁶⁶

The constitutively active *BCR::ABL1* tyrosine kinase disrupts a wide range of signal transduction pathways, resulting in the promotion of leukemic cell growth, proliferation, and survival (Figure 2.5) (Cortes et al., 2021; Deininger et

al., 2000; Dell'aversana et al., 2022; Michor et al., 2005). Some key dysregulated pathways include PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT. Dysregulated BCR::ABL1 pathways not only suppress apoptosis and stimulate cell proliferation but also induce an increased production of reactive oxygen species (ROS) within the mitochondria (Bolton-Gillespie et al., 2013; Nieborowska-Skorska et al., 2013). Subsequent elevation in ROS levels results in oxidative DNA damage, characterized by an increased occurrence of DNA point mutations and double-stranded breaks. Due to inefficient and erroneous DNA repair mechanisms, these changes ultimately lead to abnormal alterations in chromosomal structure (Bolton-Gillespie et al., 2013).

Figure 2.5: BCR-ABL1-dependent signaling complexes: Several signal transduction pathways are deregulated by the constitutively active BCR::ABL1 tyrosine kinase, including PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT, ultimately resulting in leukemic cell survival, growth, and proliferation (Mughal et al., 2016).

2.3 Evolution of CML treatment

2.3.1 Initial treatment strategies

Until the end of the 20th century, CML therapy was restricted to nonspecific agents, evolving from the primitive usage of arsenic-containing compounds in the 19th century to the administration of busulfan, hydroxyurea and, later on, interferon-alpha (IFN α) (Silver et al., 1999).

Interferon was used for the first time to treat CML patients in 1983 (Talpaz et al., 1983). In the initial trial, 5 out of 7 patients achieved a complete hematological response (CHR) following treatment with high doses of IFN α , and splenomegaly observed in 3 patients was reversed. From that point onward,

304 IFN α emerged as the preferred medication for CML patients. The remission rate
and survival were improved⁷² compared to the previously used agents, and the 5-
year survival rates were increased⁷³ from 42% to 57% (1997). However, IFN α was
linked⁷⁴ to a broad spectrum of significant side effects that impeded quality of
305 life, making long-term usage impractical for many patients. Many studies in the
306 early 1980s established the curative potential of allogeneic stem cell
transplant (allo-SCT) for CML, and allo-SCT became the treatment of choice for
young patients with CML and a human leukocyte antigen (HLA)- identical donor
(Goldman et al., 1982; Thomas et al., 1982)⁴. Up to the early 1990s, CML CP was
the most common indication for allo-SCT worldwide. It was also the most
effective treatment for patients with advanced stage⁷⁵ CML, though⁷⁶ the results
in CML AP and CML BP were considerably worse than those in the chronic
phase because of increased rates of recurrence and treatment-related
mortality (TRM) (Crawley et al., 2005; Gratwohl et al., 2002; Thomas et al.,
1982)⁴.

2.4.2 Imatinib⁵³ targeted therapy

During the 1990s, a groundbreaking advancement in the management of CML
emerged with the development of the first-generation tyrosine kinase inhibitor
(TKI) targeting BCR::ABL1, known as imatinib⁵³ (STI571, Gleevec®, Glivec®,
formerly CGP 57148B), by biochemist Nicholas Lyndon. Imatinib, a 2-
phenylamino pyrimidine, inhibits the enzymatic action of the constitutive active
BCR::ABL1 tyrosine kinase by occupying the ATP-binding pocket of the
BCR::ABL1 oncoprotein, thereby inhibits phosphorylation⁷⁷ and activate⁷⁸
⁷⁹
downstream effector molecules that cause the leukemic phenotype (Figure 2.6)
(Druker et al., 2001)⁴. With the advent of imatinib⁵³, the treatment and
management of CML patients underwent a revolutionary transformation,
resulting in significantly improved prognosis, response rate, overall survival,

and patient outcomes when compared to previous therapeutic approaches (Druker et al., 2006; Hochhaus et al., 2009; O'Brien et al., 2003).²⁶ The 8-year⁴ overall survival rate saw remarkable improvement, rising from approximately 20% to 80-90% (Jabbour and Kantarjian, 2014b)

About 60% of CML patients maintain on the standard dosages of imatinib⁸⁰ after six years⁸¹ and about 40% required⁸² an alternative treatment or higher doses of imatinib⁵³ (de Lavallade et al., 2008).⁴ The main reasons⁸³ for this are⁸⁴ secondary resistance⁸⁵ which results from the expansion of subclones with point mutations in the BCR::ABL1 kinase domain (KD). This situation often results in disease relapse upon cessation of treatment (Goldman, 2010; Michor et al., 2005).⁴ As a result, most CML patients require ongoing TKI therapy, which brings with it potential risks of toxicity, challenges with adherence, development of drug resistance, recurrence of the disease, and the accompanying financial burden (Senapati et al., 2023).⁴

Figure 2.6: Representation of the mechanism of action of TKI (Lussana et al., 2018).⁴

2.4.4. Second- and third-generation tyrosine kinase inhibitors

The identification of mutations in the BCR::ABL1 kinase domain that confer resistance to imatinib⁵³ and were associated with drug resistance led to the development of the second-generation TKIs, dasatinib, nilotinib, and bosutinib (Golas et al., 2003; Lombardo et al., 2004; Weisberg et al., 2006).⁴

Dasatinib is 350 times more potent than imatinib⁵³ *in vitro* (Tokarski et al., 2006)⁴ and is also known to inhibit the Src family of kinases (Shah et al., 2004)⁴. **Nilotinib**⁸⁶ is a structural analog⁸⁷ of imatinib⁵³ with a 50 times higher affinity to the BCR::ABL1 ATP binding site *in vitro* (Weisberg et al., 2006).⁴ The second-generation TKIs were active against most imatinib-resistant single-mutants, except for the T315I mutation (Weisberg et al., 2007),⁴ leading to the

311 development of the third-generation TKI, ponatinib (²⁶O'Hare et al., 2009)⁴ with activity against the T315I mutation (⁴Cortes et al., 2013). However, the acquisition of multiple mutations can still render BCR::ABL1 resistant to **ponatinib** (Zabriskie et al., 2014).

Four BCR::ABL1 TKIs ⁸⁸are approved⁵³ for frontline therapy: imatinib, dasatinib, bosutinib, and nilotinib. All four of these options offer the potential for a nearly normal quality ⁸⁹of life and life expectancy, provided that patients adhere to the treatment regimen, undergo optimal monitoring with minimal interruptions in therapy, and are managed appropriately at the earliest indication of genuine treatment resistance (⁴Senapati et al., 2023). Furthermore, long-term toxicity events associated with the second-generation ⁹⁰TKIs, have been raising concerns about their use, especially in specific groups of patients.

312 **Asciminib** ⁹¹was approved in 2021 for the third-line therapy of CML and the treatment of T315I-mutated CML. It works through a novel mechanism that involves binding to the myristoyl pocket of the ABL1 and allosterically inhibiting the TK activity (Figure 2.7). Though all grade and ♣ grade 3 AEs were lower with asciminib than bosutinib in the ASCEMBL trial when studied at the higher 200 mg BID dose for T315I-mutated CML, asciminib showed ♣ grade 3 adverse events (AEs) in (56.4% vs. 68.4%) of patients and arterio-occlusive events (AOE) in 8% of patients. However, longer follow-ups in ⁹²larger patient numbers will better define its safety (⁴Hughes et al., 2019).

Figure 4.7: Binding of the Myristoyl Site of the BCR::ABL1 Protein by Asciminib
(⁴Hughes et al., 2019)

Olverembatinib (HQP1351) is a new third-generation TKI that could inhibit both wild-type and T315I-mutated BCR::ABL1 in cell lines. The drug is approved in China for ⁹³the treatment of adults with TKI-resistant CML in CP or AP ⁹⁴harboring the T315I mutation (⁴Jiang et al., 2022).

2.5 Response to Therapy

Response to TKI therapy is highly predictive of long-term outcomes,⁹⁵ and milestones of response by the National Comprehensive Cancer Network (NCCN) and European Leukemia Net (ELN) consensus panels. The response⁹⁶ to treatment with TKI is monitored⁹⁷ through hematologic response (HR), cytogenetic response (CR), and molecular response (MR). HR is evaluated⁹⁸ by observing peripheral blood (PB) blood counts, and CR is assessed⁹⁹ by determining the percentage of BM cells that test positive for the Ph chromosome using Fluorescence in-situ Hybridization (FISH). MR is gauged¹⁰¹ using Quantitative reverse-transcription PCR (QPCR), which measures the reduction in BCR::ABL1 mRNA compared to a standard baseline or the International Scale (Table 2.1 and 2.2).

Table 2.1: Definition of response(Mughal et al., 2016)

*Table 2: European LeukemiaNet and National Comprehensive Cancer Network treatment response milestones for chronic myeloid leukemia*¹⁰² *expressed as BCR::ABL1 on the International Scale. Bold text indicates where the recommendations differ*⁴ *(Branford and Apperley, 2022)*

2.5.1. Treatment-free remission

Despite the efficacy of TKI in improving the survival of CML patients, it results in certain¹⁰³ off-target toxicities. Vascular events and pleural effusions are among the potential adverse effects. Additionally, interactions with other drugs can occur, and discontinuation of TKI therapy is recommended¹⁰⁴ for young women planning to become pregnant. The financial burden and lifelong therapy¹⁰⁵ can also be substantial (Efficace et al., 2013; Force et al., 2007; Heiblig et al., 2018)⁴.

The Stop Imatinib (STIM) trial focused on discontinuing imatinib treatment in CML patients who had achieved complete molecular response (CMR) for at

least two years. Over half (58%) of these patients experienced CML relapse within six months of stopping imatinib.⁵³ However, the positive outcome was that all relapsed patients responded well to the reintroduction of TKI therapy,¹⁰⁶ suggesting a similarity between the relapsed disease and the initial CML (Bordeaux et al., 2010).⁴

The Australian TWISTER study, with selection criteria similar to STIM, demonstrated that less than half (47.1%) of the patients remained relapse-free at 24 months after discontinuation of TKI therapy. No patients with molecular relapse after discontinuation have progressed or developed *BCR-ABL* mutations (median follow-up, 42 months). Patients relapsed¹⁰⁷ remained sensitive to imatinib re-treatment (Ross et al., 2013)⁴ (Figure 2.8) (Table 2.3).¹⁰⁸ The factors contributing to sustained Treatment-Free Remission (TFR) in some patients have often been attributed to the immune system's²⁶ anti-leukemic role, particularly within the bone marrow microenvironment (BMM), implying that immune response plays a critical role in preventing CML relapse after discontinuation of TKI therapy (Illander et al., 2017).⁴ Further research is needed to better understand¹⁰⁹ the complex interplay between TKIs, the immune system, and the persistence of leukemia¹¹⁰ stem cells to improve treatment strategies and outcomes for CML patients.

*Figure 2.8: Pathobiology and treatment of chronic myeloid leukemia*¹¹¹ (Park, C.S., and Lacorazza, H.D., 2020)

*Table 2.3. Key*¹¹² *characteristics of selected studies of discontinuation of tyrosine kinase inhibitor therapy.*

2.6 CML LSCs

Although TKIs have revolutionized CML treatment, transforming it from a fatal to a manageable disease, they cannot eliminate the quiescent LSC population, which remains a potential source of relapse (Figure 2.9).

CML LSCs are not addicted to BCR::ABL1 activity and, therefore, are not eliminated by TKI therapy. Corbin et al. demonstrated CML LSCs were able to survive TKI therapy despite inhibition of BCR::ABL1 activity in both differentiated and primitive cells. Moreover, quiescent primitive CML cells survive imatinib concentration 10times higher inhibitory concentration of differentiated cells(Corbin et al., 2011). Additionally, Hamilton et al. reported prolonged treatment with the potent ABL kinase inhibitor dasatinib, combined with the absence of growth factors, is insufficient to target CML LSCs despite complete inhibition of BCR::ABL1 tyrosine kinase activity(Hamilton et al., 2012).

These studies demonstrated that CML LSCs are resistant to TKIs, and thus targeting BCR::ABL1 independent signaling pathways may be needed to eliminate them effectively.

2.6.1 Origin and Evolution of CML LSCs

In 1999, Tessa Holyoake presented the first direct evidence of a primitive, CD34+lin- quiescent subset of leukemic cells in CML patients with *in vitro* and *in vivo* self-renewal properties (Holyoake et al., 1999). Later, it was found that these cells were unaffected by TKI. Moreover, BCR::ABL1-positive LSCs were identified in CML patients who achieved long-term remission after imatinib treatment. The BCR::ABL1-positive CD34+CD38- cells isolated from these patients demonstrated long-term repopulation capacity in immunocompromised mice(Hamilton et al., 2012). Further investigations revealed that these cells are not vulnerable to TKIs since their survival is independent of BCR::ABL1 kinase activity. BCR::ABL1 is either inactivated or expressed at very low levels in these cells(Kumari et al., 2012).

Figure 2.9: Model of CML disease persistence with imatinib treatment (Corbin et al., 2011).⁴

2.6.2 Surface Markers in CML LSCs

Although CML LSCs share many features with their ¹²³normal counterparts, like self-renewal capacity, multilineage differentiation ability, and the expression of similar surface markers (they are CD34+CD38-Lin- cells) there are some differences that have¹²⁴ allowed their *in vitro* and *in vivo* characterization.

Extensive screening in CML LSC has been performed¹²⁵ to find surface molecules that distinguish between LSC BCR::ABL+ and normal HSC.

2.6.2.1 IL-1RAP

The first molecule proposed for the identification of LSC BCR::ABL1+ was Interleukin 1 Receptor Accessory Protein (IL-1RAP), which is selectively up-regulated^{126,127}

in CML LSC. It has been shown¹²⁸ that IL-1RAP is a good¹²⁹ biomarker activated by Interleukin 1 (IL-1), which plays an essential role in maintaining LSCs(Landberg et al., 2016; Warda et al., 2019).⁴

2.6.2.2 Dipeptidyl peptidase IV (DPP4; CD26)

CD26 is selectively upregulated¹²⁷ in CML LSC, becoming a marker of LSC BCR::ABL1. CD26 disrupts the CXCL12-CXCR4 axis by cleaving the CXCL12 molecule and is implicated in releasing CML LSCs from the BMM into the blood(Bocchia et al., 2018, 2020; Raspadori et al., 2019; Sharma et al., 2022).⁴

2.6.2.3 IL-2 receptor (CD25)

BCR::ABL1+ LSC express Interleukin 2 receptor alfa chain (IL-2RA; CD25), a molecule that depends on STAT5 activity. CD25 serves as a negative regulator of

the growth of CML LSC, acting as a ¹³⁰tumor suppressor in CML(Sadovnik et al., 2017).⁴

2.5.2.4 CD93

CD93 ¹³¹is ¹³²selectively expressed on a CD34+CD38–CD90+ subpopulation that engrafts patient-derived cells in an immunocompromised model (in contrast to the CD93– counterpart that fails to engraft) and displays a stem cell-like signature of gene expression. CD93-expressing cells ¹³³were not eliminated by TKIs and persisted in patients even after prolonged treatment. Comparison between patients in TFR who did or did not experience molecular recurrence showed that the former ¹³⁴harbored detectable CD93+ cells within the CD34+CD38–CD90+ fraction, whereas the latter did not. CD93 ¹³⁵is expressed in CML LSCs and platelets, endothelial cells, and other cell types; it is ¹³⁶highly unlikely that CD93 may serve as a therapeutic target. However, further studies are warranted to investigate whether CD93 may be used as ¹³⁷predictive biomarkers ¹³⁸to distinguish those CML patients at high risk of molecular recurrence after discontinuation(Kinstrie et al., 2015, 2020).

2.6.2.5 CD33

¹³⁹the sialic acid receptor CD33 ¹⁴⁰is expressed in CML CP LSC but only variably in BP LSC(Herrmann et al., 2012).⁴

2.5.2.6 CD36

Primitive CML cells expressing CD36 showed decreased imatinib sensitivity ¹⁴¹and on BC LSC in proximity to adipose tissue, whereby it mediates fatty acid uptake and oxidation(Landberg et al., 2018).⁴

Over the last decade, researchers have successfully identified and validated additional aberrant surface markers that offer improved discrimination between residual normal HSCs and CML LSCs. The discovery of highly specific, robust, and reproducible biomarkers holds significant potential for enhancing

diagnosis and facilitating the isolation and biomolecular/functional characterization of LSCs.

Moreover, precise estimation of LSCs may have multiple implications beyond predicting treatment response. It could enable the tracking and quantification of residual LSCs during and after therapy as a predictor of TFR success.

Additionally, identifying specific targets exclusively expressed on the surface of CML ¹⁴²LSCs, but not on normal HSCs or other cell types could pave the way for highly selective therapeutic targeting of LSCs.

2.7 Factors influencing CML LSC survival

CML LSC has shown several intrinsic mechanisms of resistance to therapy, including those dependent or independent of the presence of BCR::ABL1 fusion protein. In addition, several reports have suggested that LSC could remain in specific protective niches within the BMM, where they have an optimal microenvironment conducive to developing resistance to therapy and inducing relapse(Zhang et al., 2012).⁴

2.7.1 Intrinsic mechanism of resistance

Several pathways specific to LSCs have ¹⁴³been recognized, primarily transcription factors such as Signal Transducer and Activator of Transcription 3/5 (STAT3/5), Forkhead transcription factors of the class O 3A (FOXO3A), β -catenin, p53, c-MYC, NOTCH, BMI-1, and others, these master regulators govern crucial cellular processes like apoptosis, self-renewal, determination of cell fate, and senescence(Abraham et al., 2016; Kuepper et al., 2019).⁴

2.7.1.1 Sonic Hedgehog (Hh) pathway

¹⁴⁴Hh is a crucial regulator of both HSCs and LSCs by controlling self-renewal processes. When the Hh ligand binds to the Patched (PTCH) receptor, it relieves inhibition of the G protein-coupled receptor Smoothened (SMO). As a result, the transcription factor GLI1 ¹⁴⁵is activated, translocating into the nucleus. Within the

¹⁴⁶nucleus, GLI1 influences the transcription of various target genes involved in cell cycle regulation and apoptosis (CCND1, MYC, etc.) while also promoting MDM2-dependent degradation of p53. In CML LSCs, this pathway becomes hyperactivated due to the increased expression of SMO(Dierks et al., 2008; Zhao et al., 2009).⁴

2.7.1.2 Wnt/ β -catenin

Nuclear β -catenin is crucial for the self-renewal and survival of both normal HSCs and LSCs. It activates essential genes like NOTCH and is ¹⁴⁷key to LSC survival. Both canonical and noncanonical Wnt pathways influence β -catenin localization and activity. Noncanonical Wnt ¹⁴⁸signaling activates NFAT, leading to increased autocrine cytokine production that provides compensatory signals to LSCs during TKI exposure(Gregory et al., 2010; Wang et al., 2010).⁴

2.7.1.3 PI3K/AKT

The PI3K/AKT pathway mediates proliferation and becomes activated by binding growth factors to receptor tyrosine kinases. In the context of CML LSCs, BCR::ABL1 activates the PI3K/AKT ¹⁴⁹signaling, contributing to the maintenance of these cells. This activation leads to the phosphorylation and cytosolic retention of FOXO by AKT, which subsequently hinders the transcription of FOXO target genes involved in apoptosis (Sykes et al., 2011).⁴

In addition to other pathways, the ¹⁵⁰signaling of transforming growth factor β (TGF- β) also plays a role in influencing FOXO nuclear translocation through the ¹⁵¹PI3K/AKT signaling pathway(Naka et al., 2010a).⁴

2.7.1.4 Janus kinases (JAK)

In CML, JAK2 ¹⁵²signaling has ¹⁵³been observed to converge on activating STAT3 and ³¹⁷STAT5. The phosphorylation and translocation of STAT5 into the nucleus are well-established as critical contributors to CML pathogenesis. Additionally,

STAT3 ¹⁵⁴signaling ¹⁵⁵has ¹⁵⁶been linked to the survival of CMLLSCs. Both JAK2 and JAK1 have been implicated in the activation of ¹⁵⁶STAT3 ⁴(Eiring et al., 2015).

2.7.1.5 Protein phosphatase 2A (PP2A)

The ¹⁵⁷tumor suppressor PP2A gene encodes a multimeric serine/threonine phosphatase. In the context of CML, PP2A becomes inactivated due to the overexpression of its endogenous inhibitors (SET Nuclear Proto-Oncogene) SET and CIP2A (Cellular Inhibitor of PP2A). The loss of PP2A, resulting from BCR::ABL1-dependent expression of SET, has been linked to the maintenance of quiescent LSCs. Moreover, it has ¹⁵⁸been observed that BCR::ABL1 expression plays a vital role in the recruitment of JAK2 and the activation of a JAK2/β-catenin pathway, which is crucial for LSC survival and self-renewal ⁴(Neviani et al., 2013).

2.7.1.6 Promyelocytic leukemia ¹⁵⁹(PML)

³¹⁸ | ¹⁶⁰PML gene is involved in the t(15;17) of acute promyelocytic leukemia, ¹⁶¹encodes a protein localizing to nuclear bodies that act as a ¹⁶²tumor suppressor, controlling apoptosis, cellular proliferation, senescence, and negatively regulating *mTOR*. PML has ¹⁶³been shown to play an indispensable role in maintaining LSC quiescence in CML ⁴(Bernardi et al., 2006; Ito et al., 2008).

2.7.1.7 Dual Specificity Tyrosine Phosphorylation Regulated Kinase 2 (DYRK2)

KLF4 (Kruppel family of transcription factors) transcription factor has ¹⁶⁴been shown to sustain CML LSCs via downregulation of the dual-specificity kinase *DYRK2*, which mediates the stabilization of *p53* and the proteasomal degradation of *c-MYC* (*MYC proto-oncogene*). This results in reduced apoptosis and increased self-renewal. The central role of *p53* and *c-MYC* in LSC maintenance had already ¹⁶⁵been elegantly demonstrated by a seminal proteomic study conducted by Tessa ²⁶Holyoake's group ⁴(Park et al., 2019).

2.7.2 Epigenetic mechanisms

319 Enhancer of Zeste Homolog 2 (*EZH2*) is Polycomb Repressive Complex 2 (*PRC2*) that trimethylates histone H3 at lysine 27 but may also directly regulate gene expression. It is highly expressed in CML LSCs and contributes to their survival(Scott et al., 2016).⁴ Targeting histone deacetylases was also explored against CML LSCs,¹⁶⁶ in light of the reported efficacy of such a strategy in inducing apoptosis in non-proliferating cells. It gave promising results when tested in combination¹⁶⁷ with TKIs in cellular and mouse models. Sirtuin 1 (*SIRT1*) deacetylase is a multifunctional protein implicated in many cellular pathways, including energy metabolism and stress response, and *TP53* and *FOXOs* are its targets(Abraham et al., 2019).⁴ *SIRT1* is overexpressed in CML LSCs and contributes to LSC resistance to TKIs. Later, *SIRT1* was found¹⁶⁸ to mediate increased mitochondrial oxidative phosphorylation in CML, a vital survival mechanism of LSCs. The histone acetyltransferase CREB binding protein (*CBP*) has also been implicated¹⁶⁹ in LSC self-renewal, as opposed to p300, which promotes differentiation and senescence. Both can be recruited¹⁷⁰ by b-catenin upon activation of the Wnt pathway. Inhibition of *CBP* avored¹⁷¹ the formation of b-catenin/p300 complexes, switching the balance from cell proliferation to differentiation(Li et al., 2012).⁴

2.7.3 Micro RNA

microRNAs play a significant role in CML LSCs'²⁶ self-renewal, maintenance, and potentially developing resistance to TKIs. The regulatory function of miR-126 in the dormancy of HSCs and CML LSCs has been well-documented. The BCR::ABL1 protein kinase triggers the phosphorylation of the SPRED1 protein, leading to the suppression of miR-126. This molecular event results in a decrease in the stemness state of the cells. In contrast, BM endosteal Sca-1+ endothelial cells have been observed¹⁷² to release miR-126 through extracellular vesicles. Treatment with TKIs can counteract the inhibitory impact of miR-126

by raising its levels via TKI therapy. The sensitization of CML LSCs to TKIs becomes evident in reduced miR-126 levels(B Zhang et al., 2018).

Enhanced JAK-STAT ¹⁷³signaling, facilitated by BCR::ABL1, promotes an increase in ADAR adenosine deaminase RNA specific (*ADAR1*) expression, thereby ¹⁷⁴enhancing the conversion of adenosine to inosine. This enzymatic process plays a crucial role in miRNA stability control. The ADAR1 protein has been seen to impede the ²⁴generation of mir-let7, a precursor molecule for miRNA, ultimately boosting the self-renewal capacity of CML LSCs(⁴Zipeto et al., 2016).

Upon exposure to ⁵³imatinib, CML LSCs show elevated miR-21 expression, leading to resistance against TKIs. Depletion of miR-21 in CML LSCs can be ¹⁷⁶achieved by disrupting the PI3K/AKT ¹⁷⁷signaling pathway, which results in the upregulation of programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN), thereby effectively restoring sensitivity to TKIs in CML LSCs.

The transcription factor E2F Transcription Factor 1(E2F1) drives the proliferation of CML LSCs by ¹²⁷upregulating miR-183. Furthermore, imatinib therapy reduces miR-30a levels by involving Beclin1 and Autophagy-related gene 5 (ATG5), promoting LSC resistance to TKIs(⁴Wang et al., 2015).

CML CD34+CD38- subset ¹⁷⁸displays an increase in miR-29a-3p and miR-660-5p expression, independent of BCR::ABL1 kinase activity. This upregulation correlates with the downregulation of their respective targets, ten-eleven translocation (TET2) and endothelial PAS domain protein 1 (EPAS1). This molecular mechanism has ¹⁷⁹been shown to confer resistance to TKI treatment in vitro(⁴Salati et al., 2017).

Pellicano et al. have demonstrated that BCR::ABL1 kinase-dependent upregulation of ¹⁸⁰hsa-mir-183 leads to the downregulation of its direct target, Early Growth Response 1 (EGR1) transcription factor. This, in turn, results in the upregulation of the cell cycle regulator E2F1, governing cell proliferation and

p53-dependent/independent apoptosis. Inhibiting E2F1 activity reduces the ability of LSCs to form colonies, halts their cell cycle progression, and initiates programmed cell death through the p53 pathway, affecting LSC survival and proliferation while sparing HSCs(Pellicano et al., 2018).⁴

Recent findings indicate increased expression of miR-196a-5p in the CD26+CD34+CD38- subset compared to the CD26-CD34+CD38- subset.

However, ¹⁸¹the precise functions and implications ¹⁸¹of this specific microRNA in the context of CML LSCs are not fully understood(Ruiz et al., 2021).⁴ Top of Form

2.7.4 Autocrine factors

BCR::ABL1-induced transformation relies on various mechanisms, including the autocrine production of cytokines like Interleukin (IL)-3 and Granulocyte-Colony Stimulating Factor (G-CSF) by LSCs, leading to STAT5 activation and growth factor-independent ¹⁸²signaling. Additionally, recent reports have highlighted kinase-independent autocrine loops that contribute to LSC persistence. Notably, the BCR::ABL1 kinase-independent autocrine production of Tumor Necrosis ¹⁸³Factor alpha (TNF α) supports CML LSC survival through the nuclear factor κ B (NF κ B)/p65 pathway, resulting in the expression of the IL-3 and granulocyte/macrophage-colony stimulating factor common β -chain receptor(Gallipoli et al., 2013).⁴

Moreover, an autocrine loop has ¹⁸⁴been identified, which increases the concentration of bone morphogenetic proteins (BMP) 2 and 4 in the BMM.

These BMPs, typically produced by mesenchymal cells and involved in regulating the proliferation and fate of normal HSCs, have been implicated in LSC resistance to TKIs(Laperrousaz et al., 2013).⁴

Recent findings have also revealed that CML LSCs ¹²⁷upregulate pleiotrophin (PTN), a heparin-binding growth factor usually produced by BM stromal cells and endothelial cells (ECs). PTN has ¹⁸⁵been shown to promote CML LSC survival

and confer TKI resistance. These autocrine loops play a critical role in supporting the survival and persistence of CML LSCs and can potentially serve as targets for therapeutic intervention (Himburg et al., 2019).

2.7.5 Autophagy

Autophagy, an evolutionarily conserved catabolic process, involves the lysosomal degradation and recycling unnecessary cellular components to generate adenosine triphosphate (ATP) and essential building blocks during nutrient and oxygen deprivation (Mortensen et al., 2011; Rubinsztein et al., 2012). While autophagy plays a role in maintaining HSCs, it also functions as a pro-survival pathway that enables tumor cells to withstand metabolic stress and evade apoptosis triggered by anti-cancer agents. In the context of CML, Autophagy is a crucial mechanism LSCs employ to enhance their survival against therapeutic challenges (Baquero et al., 2019). Treatment with TKIs promotes autophagy, and inhibiting autophagy intensifies the effectiveness of TKIs in targeting and eliminating both stem and progenitor cells (Bellodi et al., 2009; Mortensen et al., 2011). In summary, autophagy serves dual roles, essential for normal HSC maintenance but also exploited by both tumor cells and CML LSCs to bolster survival and counteract the effects of therapeutic interventions. The intricate regulation of autophagy, as influenced by BCR::ABL1 and therapeutic agents like TKIs, underscores its potential as a therapeutic target in leukemia treatment.

2.7.6 Energy metabolism

Since energy metabolism plays an essential role in regulating HSCs, investigating its role in LSCs becomes particularly pertinent. Kuntz et al. demonstrated that CML CD34+CD38- cells exhibit heightened TCA cycle flux and mitochondrial respiration compared to their CD34- counterparts. Interestingly, the more primitive CD34+CD38- LSCs depend considerably more

on OXPHOS than non-leukemic HSCs, aligning with the glycolytic metabolic profile typical of HSCs(Kuntz et al., 2017)⁴. An intriguing prospect emerges as integrin-linked kinase, a protein situated in focal adhesions with primarily a scaffolding function in cell-matrix interactions. It appears to influence mitochondrial metabolism and holds promise as a therapeutic target for quiescent CML LSCs that remain unresponsive to TKI treatment(Rothe et al., 2020)⁴.

Utilizing single-cell RNA sequencing, Giustacchini et al. elegantly revealed that BCR::ABL1+ LSCs within the same patient-derived CML sample exhibit overexpressed genes associated with OXPHOS in contrast to non-malignant HSCs(Giustacchini et al., 2017)⁴. This observation supports the previous global mRNA microarray analyses, where CP-CML¹⁹⁵ LSCs showed enhanced oxidative phenotype marked by elevated expression of mitochondrial respiratory chain (MRC) genes compared to non-leukemic HSCs. Furthermore, transcriptome profiling identified deregulated miRNAs in TKI-nonresponsive CML LSCs compared to TKI-responsive ones, with dysregulation of miR-185. Intriguingly, the restoration of miR-185 function impeded drug-resistant CML LSCs. Notably, the target gene of miR-185, PAK6, was found to enhance¹⁹⁶ drug resistance in TKI-nonresponsive LSCs. Coupled with the detection of increased OXPHOS and ROS expression in TKI-nonresponsive LSCs, these findings suggest that miRNAs and their target genes play pivotal roles in mitochondrial metabolism regulation within CML, including drug-resistant LSCs(Lin et al., 2020)⁴.

Abraham et al. uncovered a partial¹⁹⁷ regulation of increased OXPHOS in CML LSCs through the NAD-dependent deacetylase sirtuin-1 (SIRT1). Their work demonstrated that SIRT1 expression activates the transcription factor PGC-1 α , either directly or in an AMPK-dependent manner. Subsequently, PGC-1 α

stimulates the expression of mitochondrially encoded genes associated with mitochondrial respiration and enhances electron transport chain (ETC) activity. Direct inhibition of PGC-1 α only partially curbed CML pathogenesis *in vivo*, unlike SIRT1 inhibition. A prior study by the same group underscored the significance of SIRT1 in CML LSCs by inhibiting p53, highlighting the multifaceted role of this enzyme (Abraham et al., 2019; Li et al., 2012).

2.7.7 Immune response

Several studies have focused on the role of natural killer (NK) cell populations in TFR. In the EURO-SKI trial, longer relapse-free survival ¹⁹⁸ was associated with increased NK cells. Patients with more immature mature NK cell phenotype (CD56^{bright}) were less likely to sustain TFR (Burchert et al., 2015). ⁴ The levels of ¹⁹⁹ tumor necrosis factor α and interferon γ secretion were associated with the success of ²⁰⁰ discontinuation of TKIs (Ilander et al., 2017). ⁴ Another study revealed that patients who stopped IM treatment displayed an increased number of NK cells and a reduction in CD3+CD8+ CD62L+ cells compared to those still receiving IM. Among patients who maintained TFR, a higher proportion of activating NK cell receptors like NKG2D were present in NK cells. However, there was reduced expression of inhibitory NK cell receptors like KIR2DL2/DL3/DS236 and a lower percentage of regulatory T cells marked by FoxP3 expression and monocytic myeloid-derived suppressor cells (Irani et al., 2020; Ohyashiki et al., 2012). ⁴ These observations suggest the potential utility of these factors in forming a predictive scoring system for TFR. In another prospective analysis, the expression of CD86 (B7.2), a ligand for the inhibitory receptor CTLA-4 on plasmacytoid dendritic cells (pDCs), was identified as predictive of TFR. A higher count (>95) of CD86+ pDCs per 105 lymphocytes correlated with a reduced rate of relapse-free survival (30%), while a lower count (<95) of CD86+ pDCs per 105 lymphocytes was associated with an

increased TFR rate (70%) (Schütz et al., 2017).⁴ A more comprehensive study on the immune system and potential inflammatory influences, as well as their interactions with medications, holds promise for enhancing TFR rates in the future (Chen et al., 2021).⁴

²⁶It's worth considering that many of these studies primarily assessed peripheral blood (PB) NK cells, potentially missing distinctions present in BM-derived NK cells. In the BM of CML patients, a mature CD57+ subset ²⁰¹was found to be significantly higher than in healthy controls, suggesting an influence of the BMM on the differentiation status of NK cells in CML. However, the specific importance of CD57+ NK cells in TFR remains uncertain (Yao et al., 2020).⁴ Expanding NK cell immunotherapy is being explored to eliminate minimal residual disease (MRD) in CML, potentially serving as adjuvant therapy for patients attempting TFR. Other innovative NK-based treatments include anti-leukemic monoclonal antibodies that trigger targeted NK antibody-mediated responses and CAR-NK cell therapy, ²⁰²although these have not yet ²⁰³been described for CML but rather for other malignancies (Allison et al., 2022).⁴

³²³Below is the list of studies on TKIs with other agents targeting survival pathways of CML LSCs.

Zhang ²⁰⁴B et al. exhibited that by combining imatinib ⁵³with inhibitors of histone deacetylases, they could effectively target quiescent CML cells in vitro and in vivo humanized mice ²⁰⁵model(Zhang et al., 2010).⁴

The inhibition of ALOX5, an enzyme responsible for converting fatty acids into leukotrienes ²⁰⁶prolonged the lifespan of mice with leukemia ²⁰⁷suggesting that it ²⁰⁸played a crucial role in sustaining the survival of CML LSCs(Chen et al., 2009).⁴

Disrupting the Hedgehog pathway through the loss of Smoothened (SMO) ²⁰⁹was proven to eradicate CML LSCs. Consequently, a phase I clinical trial ²¹⁰was launched to assess the viability and safety of combining imatinib ⁵³with an SMO

inhibitor alongside dasatinib in patients with chronic phase CML (CP-CML). However, the adverse effects reported with the SMO inhibitor, ²¹¹coupled with its ²¹¹limited clinical effectiveness, ²¹¹discouraged further investigation and its ⁴application in CP-CML(Irvine et al., 2016).

CML LSCs demonstrated sensitivity to pioglitazone-induced activation of PPAR- α . Interestingly, ²¹²the administration of pioglitazone in conjunction with ⁵³imatinib resulted in sustained Complete Molecular Response (CMR) in some CML patients, though this ²¹³was observed in a small subset. Notably, among a group of twenty-four CP-CML patients treated with a combination of pioglitazone and ⁵³imatinib, 56% achieved MR (4.5), compared to fewer than half (21%) who ²¹⁴were projected to ²¹⁵achieve the same outcome with ⁵³imatinib alone. More recently, Holyoake and colleagues showcased the potential of eliminating CML LSCs through the activation of Tumour Protein 53 (TP53) combined with the inhibition of MYC. However, as this treatment regimen lacks a TKI, which is the established standard care for managing CML, this innovative approach might not find application in CP-CML patients who respond well to TKIs ⁴(Pagnano et al., 2020; Rousselot et al., 2017).

Figure 2.9: Factors influencing the persistence of CML LSC and TFR(Kumar and Krause, 2021b)

While numerous in vitro and preclinical investigations have uncovered novel approaches for addressing CML LSCs, their transition to clinical applications has been limited. The imperative need of the moment is to develop a successful strategy for eradicating CML LSCs.

2.8 The BM Microenvironment in chronic myeloid ²¹⁶leukemia

Like their ²¹⁷normal hematopoietic stem cell counterparts, LSCs inhabit a distinct microenvironment that governs their activities (Figure 2.10).

LSCs are known to coopt or hijack BMM that protect and nurture them at the expense of normal hematopoiesis. In light of this complex interplay, it is ²¹⁸believed that the study of the BMM is at least as important as the study of LSCs, as a deep understanding of the role of BMM may provide essential clues on how to eliminate LSCs selectively (Magalhães-Gama et al., 2022).⁴

The crosstalk between LSCs and BMM is mediated by

- i) Physiological factor- Hypoxic microenvironment
- (ii) cell-cell direct interactions via surface molecules and involves a broad array of cell types on the BM side, like mesenchymal stromal, endothelial, perivascular, ²¹⁹osteolineage, and immune cells.
- (iii) soluble or exosome-incapsulated factors and

2.8.1 Hypoxic microenvironment

Low oxygen tension of BMM profoundly influences LSC features, most importantly, their metabolism. Quiescent and self-renewing HSCs rely on glycolysis for energy production, which is an adaptation ²²⁰to the hypoxic environment of the BMM and reflects the low energetic demands of HSCs. A metabolic rewiring to oxidative phosphorylation (OxPHOS) occurs upon differentiation and lineage commitment. Cancer stem cells ²²¹are dependent on OxPHOS or glycolysis, depending on the cancer type, for growth and maintenance. In CML LSCs, OxPHOS is crucial for producing energy and anabolic precursors, thus providing an attractive metabolic vulnerability that ²²²might be exploited for their selective targeting. Moreover, hypoxia stabilizes ³²⁴Hypoxia-Inducible Factors 1 (HIF1), a transcription factor that plays a crucial role in regulating the proliferation, maintenance, and survival of LSCs (Ng et al., 2014).⁴

In CML, physiologic hypoxia has been shown to promote the maintenance of CML ²²³LSCs, despite effective inhibition of the BCR::ABL1 protein. Hypoxia has

been found to potentiate the survival of quiescent CML leukemia-initiating²²⁴ cells (Ng et al., 2014).⁴

Several miRNAs have been reported to be differentially expressed²²⁵ in response²²⁶ to hypoxia in cancer. For instance, a 14-miRNA hypoxia signature was derived²²⁷ as a prognostic marker for poorer overall survival in invasive bladder cancer (Khan et al., 2021).⁴ These are²²⁹ hypoxia-responsive miRNA (HRMs) capable of simultaneously regulating numerous genes and influencing multiple components of a signaling²²⁸ pathway (Licausi et al., 2011).⁴ Future studies on HRMs will aid in understanding their function and regulatory basis in response to hypoxia.

There are no studies exploring²³⁰ **the hypoxia-regulated miRNA in mediating the survival of LSCs.**

Figure 2.10. CML stem cells and the bone marrow microenvironment for CML LSCs. CD26 disrupts the CXCL12/CXCR4 axis by cleaving CXCL12 to promote LSCs'²⁶ mobilization into the blood. LSCs bind to VCAM-1 and Selectin on their bone marrow niche. Secretion of LSCs demands a tightly regulated metabolism. BMP2/4 and other chemokines/cytokines through auto- or paracrine mechanisms also support quiescence, cellular growth, and drug resistance of CML LSCs. LSC, leukemia stem cell; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen 4; CXCL12, C-X-C. (Ito et al., 2022)⁴

2.8.2 Direct cell-cell contact interactions between CML LSCs and the BMM

2.8.2.1 Cell adhesion

One of the mechanisms mediating LSC survival is the interaction with the BMM (Schepers et al., 2015).⁴ The interaction²³¹ of LSCs with the BMM is pivotal for the growth of leukemic cells, as it can confer resistance to treatment (Houshmand

et al., 2019)⁴. This phenomenon has been elucidated through in vitro studies, where the growth inhibition of malignant cells by either JAK2 or ALOX5 inhibitors is observed solely in the absence of stromal cells (Manshouri et al., 2011)^{4 232}, and does not manifest in primary CML LSCs co-cultured with stromal cells (Dolinska et al., 2017)⁴ (Figure 4.11).

CD44: Elevation in the ²³³expression of the cell surface glycoprotein CD44 was observed in CML cells, facilitating their homing and engraftment through binding to extracellular matrix proteins and osteopontin. Furthermore, treatment with a CD44 antibody inhibits CML development in wild-type recipients in the ²³⁴*in-vivo* model (Krause et al., 2006)⁴.

E-Selectin: Various studies highlight the significance of E-selectins, crucial for the engraftment of CML cells (Krause et al., 2014)⁴ which ²³⁵are also prominently expressed on endothelial cells (ECs) within the AML BM environment, promoting chemoresistance within the vascular niche (Stucki et al., 2001)⁴

Integrins: Integrins are essential in regulating leukemic cell functions both *in vitro* and *in vivo*. Integrin β 1, for instance, has been shown to modulate the proliferation of CML cells through adhesion to BM stromal cells and extracellular matrix components like fibronectin (Bhatia et al., 2001)⁴

Fibronectin: Fibronectin mediates ²³⁶downregulation of proapoptotic factor *Bim1* and leads to treatment resistance of LSCs (Sengupta et al., 2009)⁴.

Galectin-3: Interaction with BM stromal cells induces the Galectin-3 (Gal-3) expression in CML cell lines, promoting leukemic cell proliferation and consequent TKI resistance in vitro. Consistent with these findings, in vivo overexpression of Gal-3 encourages the lodgment of CML cells in the BMM (Yamamoto-Sugitani et al., 2011)⁴. Similarly, AML cells trigger the expression of a growth arrest-specific gene (Gas6) in the BMM, further enhancing their

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proliferation. This same regulatory axis supports the self-renewal of CML LSCs (Jin et al., 2017).⁴

N-cadherin: N-cadherin, responsible for mediating the adhesion of CML cells to the BMM, may induce TKI insensitivity in leukemic cells through the activation of β -catenin and heightened Wnt signaling²³⁷ (Zhang et al., 2013).⁴

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Figure 2.11. This schematic demonstrates the effects of E-selectin inhibition within the bone marrow microenvironment and the impact upon chronic myeloid leukemia²³⁸ stem cells and mechanistically how this is controlled²³⁹ by the SCL/TAL1 – CD44 axis. HPC: hematopoietic progenitor cell; HSC²⁴⁰; hematopoietic stem cell; LSC: leukemic stem cell. <https://www.haematologica.org/article/view/9468>

2.8.2.2 Cytokines and Chemokines:

The aberrant production of cytokines and chemokines within the BMM can drive the progression of the disease (Camacho et al., 2017).⁴

An altered level of pro-inflammatory cytokines (including IL-1, IL-1b, IL 6, macrophage inflammatory protein-1 a (MIP-1a), MIP-1b, and TNF) or chemokines has been reported²⁴¹ in CML BMM, and this was found²⁴² not fully corrected upon TKI-induced remission. This²⁴³ differentially affects CML LSC and normal HSC in terms of maintenance and growth, providing a competitive advantage to LSCs (Zhang et al., 2012).⁴

CXCL12: Levels of C-X-C motif chemokine ligand 12 (CXCL12), implicated in HSC maintenance, have been shown to be reduced²⁴⁴ in CML BMM and are detrimental to HSCs but have been found to promote LSC expansion by increasing their self-renewing divisions (Agarwal et al., 2019).²⁴⁵⁴

CXCR4: In CML, CXCR4 is upregulated^{127,246}, leading to heightened survival of quiescent LSCs *in vitro* by inducing the migration of leukemic cells towards BM

stromal cells²⁴⁷. Consequently, inhibition of the CXCL12-CXCR4 axis restores the sensitivity of LSCs to TKIs *in vitro* (Jin et al., 2008)⁴.

Interlukin-1: Elevated expression of the IL-1 receptor in CML LSCs implies a contribution of IL-1 signaling²⁴⁸ to their maintenance. Correspondingly, treatment with IL-1 receptor antagonists curtails the growth of CML LSCs and exhibits an additional with TKI treatment (Zhang et al., 2016)⁴.

TNF α : tumor²⁴⁹ necrosis factor α (TNF α), an inflammatory cytokine often elevated in myeloid malignancies, fosters the survival²⁵⁰ of malignant cells (Gallipoli et al., 2013)⁴.

BMP: In CML BMM, there is an abnormal abundance of BMP2 and BMP4. Together with overexpression of their receptor BMPR1B, this leads to a strong upregulation of the BMP pathway in CML LSCs that has been implicated²⁵¹ in their survival and expansion (Laperrousaz et al., 2013)⁴.

MSTNpp: High throughput screening of 313 human cytokines on the growth of CD34+CD38- cells isolated from CML patients has confirmed the previously reported regulatory effects of IL-3, IL-1a/b, GM-CSF, and IFN γ but has also identified myostatin propeptide (MSTNpp) as a novel positive regulator. It was found²⁵² that MSTNpp can indeed be detected²⁵³ in the plasma of CML patients and that it may be produced²⁵⁴ by mesenchymal stromal cells. By binding to a yet unknown receptor on the surface of CML cells, MSTNpp was confirmed²⁵⁵ to expand primitive cells via STAT5 and SMAD2/3 (von Palffy et al., 2020)⁴.

Figure 2.12: Malignant cells interact with their BMM through multiple pathways

2.8.2.3 Remodeling of BMM in CML

LSCs exhibit numerous shared biological characteristics with regular HSCs (Kreso and Dick, 2014)⁴. Considering the compromised nature of normal hematopoiesis in individuals with hematological²⁵⁶ malignancies, it has been hypothesized²⁵⁷ that LSCs possess the ability to²⁵⁸ outcompete HSCs and transform

BMM into a self-reinforcing malignant environment (. As a result, the BMM might play a pivotal role in both leukemic relapse and the progression of the disease (Figure 2.12)

327 In a transgenic mouse model of CML, the aberrant BMM produces a significantly higher quantity of cytokines that preferentially support the growth of malignant cells over healthy HSCs (Zhang et al., 2012)⁴. For instance, CML cells exhibit elevated production of G-CSF, which subsequently leads to decreased expression of CXCL12 by BMM cells. Furthermore, treating leukemic cells with imatinib⁵³ partially²⁵⁹ restores the cytokine expression pattern, implying that leukemia²⁶⁰ induced the remodeling²⁶¹ of the BMM (Zhang et al., 2012)²⁶²(Zhang et al., 2012)⁴. Similarly, GM-CSF, IL-6, and IL-1 α can be detected²⁶³ in the serum of CML mice are increas²⁶⁴ (Welner et al., 2015)⁴.

The role of IL-6 is noteworthy, as exposing normal HSCs to CML cells heightens myeloid differentiation capacity, diminishes self-renewal potential, and induces altered gene expression in hematopoietic progenitor cells. Remarkably, treatment with an anti-IL-6 blocking antibody mitigates the onset of CML, indicating that BCR::ABL1+ leukemic cells facilitate the disease and influence normal hematopoiesis through IL-6 signaling²⁶⁵. It is crucial to highlight that patient-derived CML cells evoke similar changes in normal HSCs and hematopoietic progenitors in culture, further substantiating the capacity of both mouse and human LSCs to modify the surrounding cells within the BMM (Welner et al., 2015)⁴.

Moreover, CML cells induce the secretion of PIGF by BM stromal cells during co-culture. This elevated PIGF level stimulates angiogenesis²⁶⁶ and proliferation of leukemic cells. Consequently, treatment with a PIGF antagonist prolongs the survival of CML mice and exhibits²⁶⁷ additional therapeutic efficacy when

combined with imatinib⁵³, underscoring the ability of CML leukemic cells to reconfigure the BMM to promote disease progression (Schmidt et al., 2011)^{4 268}. Schepers et al. demonstrate that CML cells remodel the BM endosteal niche, leading to an increased fraction of osteoblasts with an altered gene expression pattern and compromised ability to support normal HSCs (Schepers et al., 2013)⁴. Moreover, the removal of osteoblasts²⁶⁹ significantly accelerates leukemia²⁷ development and diminishes the survival of CML mice. In this context, the CML BMM impacts the cycling of hematopoietic cells by upregulating¹²⁷ the expression of the Notch ligand, Jagged-1 (Bowers et al., 2015)⁴.

2.8.2.4 Remodeling of BMM by exosomes.

Furthermore, the BMM can undergo reprogramming by malignant cells by releasing exosomes (Boyiadzis and Whiteside, 2015)⁴. Exosomes, the vesicles derived from the cell membrane containing protein and RNA, are potential mediators in intercellular communication (Mostafazadeh et al., 2021)^{4 271}. The uptake of these exosomes induces changes in the secretion profile and modifies the proliferation and migration capabilities of BM stromal cells²⁷² (Roccaro et al., 2013)⁴.

miR-126: In CML²⁷³ blast crisis cell²⁷⁴ line transfers exosomes carrying miR-126 to endothelial cells (ECs) during co-culture. This transfer results in decreased expression of VCAM-1 and CXCL12 in ECs and a reduction in the adhesion capacity of the CML cell line (Taverna et al., 2014)⁴.

These investigations indicate that malignant cells establish a self-reinforcing niche by transmitting signals to neighboring²⁷⁵ BM cells. Consequently, the reshaped BMM becomes detrimental to HSCs while supporting leukemic cells, thereby accelerating the progression of malignancy. There are²⁷⁶ no therapies targeting the altered CML BM microenvironment have²⁷⁷ been implemented in clinical practice.

2.9 iPSCs for disease ²⁷⁸modeling

2.9.1 Application of iPSCs in disease ²⁷⁹modeling

³²⁸ Reprogramming human somatic cells results in the ²⁴generation of induced pluripotent stem cells (iPSCs) that behave similarly into embryonic stem cells (ESCs), i.e., they are capable of self-renewal, large-scale expansion, and

³²⁹ differentiation into three germ layers, including blood (Choi et al., 2009; Park et al., 2008; Takahashi et al., 2007a; Yu et al., 2009).⁴ Because iPSCs ²⁸⁰recapitulate the entire genome of diseased cells, they are already used in ²⁸¹modeling human diseases (Grskovic et al., 2011).⁴ iPSCs provide a unique opportunity to study human diseases in vitro, thus helping to unravel the complicated genotype-phenotype relationship in several monogenic disorders. iPSCs generated from patients with blood diseases can ²⁸²be used to ²⁸³generate blood cells carrying disease-specific genetic traits at the cellular level to recapitulate the disease phenotype under in vitro conditions and provide insights into the pathophysiology and emergence of the disease(Papapetrou, 2019a; Park et al., 2008).⁴

Figure 2.13: Progress in therapies based on iPSCs.

2.9.2 iPSCs to study LSCs

Due to the rarity of the LSC population, it is challenging to obtain sufficient cells for research to understand the mechanisms of TKI resistance of these cells and screen drugs to eradicate them. iPSCs derived from patients with genetic diseases or cells with genetic alterations can be differentiated into scalable quantities of disease-relevant differentiated cells(Hosoi et al., 2014; Kumano et al., 2012).⁴ Thus, they are a powerful tool for studying disease pathogenesis and drug screening. iPSCs generated from patients with leukemias help understand how oncogenes and patient-specific chromosomal abnormalities influence the development of leukemia-like phenotypes ²⁸in-vitro.

Previous studies have demonstrated the successful ²⁴generation of CML-iPSCs from peripheral blood mononuclear cells and CD34+ hematopoietic stem and progenitor cells (HSPCs) from CML patients(Bedel et al., 2013; Charaf et al., 2017; Hosoi et al., 2014; Imeri et al., 2023; Kumano et al., 2012; Miyauchi et al., 2018; Telliam et al., 2016; Turhan et al., 2019)⁴. CML-iPSCs exhibit resistance to IM like CML-LSCs, so they are considered suitable for investigating the cellular mechanisms underlying IM resistance. CML-iPSCs could ²⁸⁵be differentiated into hematopoietic progenitors that recapitulated some of the pathophysiologic features of the disease. ***However, it remains unclear how BCR::ABL1 expression and TKI treatment impact the efficiency and quality of iPSC generation from CML cells. CML-iPSCs generated from the same patient at various stages of the disease and from patients with different responses to TKI therapy are valuable resources for investigating molecular and epigenetic mechanisms of CML progression and drug resistance, leading to the identification of novel therapeutic targets and drug candidates for the treatment of CML. Moreover, iPSCs can be efficiently genetically manipulated by gene editing methods to identify specific genes involved in disease pathogenesis.***

2.10 Lacunae in research

v Although several studies have compared transcriptomics in CML patients with ²⁸⁶good or poor responses to TKI, very few reports on transcriptomics using CML CD34+ cells; some of these studies have addressed only specific pathways in mediating CML LSC survival. There ²⁸⁸is no report on global transcriptome profiling in primary CML CD34+ cells and validation of identified targets in ²⁸⁷a large number of samples.

v Several studies have addressed the role of miRNAs in CML LSC maintenance, and modulating them could eliminate these LSCs. However, the role of hypoxia-

associated miRNA in regulating primary CML cell survival and maintenance is unknown.

v Primary CML MSCs have a limited lifespan and undergo senescence at the early passage. Currently, ²⁹⁰there is no CML patient-derived MSC line ²⁹⁰that can be ²⁸⁹used as a model to study the LSC-stromal interaction.

v As CML LSCs are a rare population of cells, several groups have generated induced pluripotent stem cells (iPSCs). However, it remains unclear how *BCR::ABL1* expression and TKI treatment could impact the efficiency and quality of iPSC generation from CML cells.

1.	leukemia → leukaemia	Mixed dialects of English	Correctness
2.	<i>In 1960, Nowell and Hungerford made a groundbreaking revelation in blood samples obtained from individuals affected with chronic myeloid leukemia (CML), uncovering a tiny chromosome known as a minute chromosome (Peter C. Nowell and David A. Hungerford, 1960).</i>	Unclear sentences	Clarity
3.	hematology → haematology	Mixed dialects of English	Correctness
4.	<i>(Deininger et al., 2000; Nowicki et al., 2003); (Hehlmann et al., 1993); (Galton, 1953); (Allan et al., 1995); (Druker et al., 2001); (Soverini et al., 2019); (Kumar and Krause, 2021a); (Camacho et al., 2017; Jin et al., 2008; Manshouri et al., 2011; Zhang et al., 2013, 2019; Zhao et al., 2009, 202...</i>	Citation style options	Correctness
5.	arseniate → arsenic	Confused words	Correctness
6.	<i>Busulfan; busulfan</i>	Text inconsistencies	Correctness
7.	<i>was then replaced</i>	Passive voice misuse	Clarity
8.	the development of → developing	Wordy sentences	Clarity
9.	favorable → favourable	Mixed dialects of English	Correctness
10.	closely	Wordy sentences	Clarity
11.	normal → average	Word choice	Engagement
12.	and precluding	Conjunction use	Correctness
13.	treatment → therapy	Word choice	Engagement
14.	<i>is focused</i>	Passive voice misuse	Clarity

15.	, and upon → . Upon	Hard-to-read text	Clarity
16.	signaling → signalling	Mixed dialects of English	Correctness
17.	<i>being tested</i>	Passive voice misuse	Clarity
18.	signaling → signalling	Mixed dialects of English	Correctness
19.		Tone suggestions	Delivery
20.	<i>be characterized</i>	Passive voice misuse	Clarity
21.	option → options	Incorrect noun number	Correctness
22.		Tone suggestions	Delivery
23.	<i>been implemented</i>	Passive voice misuse	Clarity
24.	<i>generation; Generation</i>	Text inconsistencies	Correctness
25.	leukaemia-stromal	Mixed dialects of English	Correctness
26.	<i>patient's; individual's; " ; " ; O'Dwyer; Dell'aversana; O'Brien; O'Hare; system's; Holyoake's; LSCs'; It's</i>	Text inconsistencies	Correctness
27.	<i>CML patient-derived iPSCs will be an ideal model for drug and CRISPR library screening, as it can retain the patient's pathophysiology without altering the genetic makeup.</i>	Unclear sentences	Clarity
28.	modeling → modelling	Mixed dialects of English	Correctness
29.	signaling → signalling	Mixed dialects of English	Correctness
30.	<i>are also described</i>	Passive voice misuse	Clarity
31.	<i>are listed</i>	Passive voice misuse	Clarity
32.	precisely regulating	Wordy sentences	Clarity

33.	a sequence of	Wordy sentences	Clarity
34.	In contrast → ¶ In contrast	Intricate text	Clarity
35.	<i>These intrinsic fluctuations are then followed by a selective step driven by cytokines.</i>	Passive voice misuse	Clarity
36.	<i>MPP-multipotent progenitor; CMP-common myeloid progenitor; CLP-common lymphoid progenitor; MEP-megakaryocyte-erythroid progenitor; GMP - granulocyte-macrophage progenitor (Olson et al., 2020).</i>	Incomplete sentences	Delivery
37.	<i>are regulated</i>	Passive voice misuse	Clarity
38.	leukemia → leukaemia	Mixed dialects of English	Correctness
39.	. Subsequently	Punctuation in compound/complex sentences	Correctness
40.	leukaemia-initiating	Mixed dialects of English	Correctness
41.	leukemia → leukaemia	Mixed dialects of English	Correctness
42.	tumor → tumour	Mixed dialects of English	Correctness
43.	Geardon → Gordon	Misspelled words	Correctness
44.	<i>are believed</i>	Passive voice misuse	Clarity
45.	leukemia → leukaemia	Mixed dialects of English	Correctness
46.	leukemia → leukaemia	Mixed dialects of English	Correctness
47.	leukemia → leukaemia	Mixed dialects of English	Correctness
48.	<i>be linked</i>	Passive voice misuse	Clarity
49.	the "Philadelphia	Determiner use (a/an/the/this, etc.)	Correctness

50.	leukemia → leukaemia	Mixed dialects of English	Correctness
51.	leukemia → leukaemia	Mixed dialects of English	Correctness
52.	). .	Improper formatting	Correctness
53.	imatinib; Imatinib	Text inconsistencies	Correctness
54.	The introduction of imatinib in the year 2000 significantly contributed to a decrease in annual mortality rates, reducing them from 10%-20% to 1%-2%(Goldman, 2010).	Unclear sentences	Clarity
55.	mostly → primarily, mainly	Word choice	Engagement
56.	is mostly diagnosed	Passive voice misuse	Clarity
57.	, but if → . Still, if	Hard-to-read text	Clarity
58.	phase → stage	Word choice	Engagement
59.	phase → stage	Word choice	Engagement
60.	leukemia → leukaemia	Mixed dialects of English	Correctness
61.	It was observed	Passive voice misuse	Clarity
62.	an accelerated	Determiner use (a/an/the/this, etc.)	Correctness
63.	leukemia → leukaemia	Mixed dialects of English	Correctness
64.	is characterized	Passive voice misuse	Clarity
65.	highly sensitive → empathetic, compassionate, susceptible	Word choice	Engagement
66.	leukemia → leukaemia	Mixed dialects of English	Correctness
67.	signaling → signalling	Mixed dialects of English	Correctness

68.	<i>are deregulated</i>	Passive voice misuse	Clarity
69.	Treatment Strategies	Confused words	Correctness
70.	hematological → haematological	Mixed dialects of English	Correctness
71.	<i>was reversed</i>	Passive voice misuse	Clarity
72.	<i>The remission rate and survival were improved</i>	Passive voice misuse	Clarity
73.	<i>were increased</i>	Passive voice misuse	Clarity
74.	<i>was linked</i>	Passive voice misuse	Clarity
75.	advanced-stage → advanced-stage	Misspelled words	Correctness
76.	, though → . However,	Hard-to-read text	Clarity
77.	inhibits → inhibiting	Incorrect verb forms	Correctness
78.	inhibits phosphorylation	Improper formatting	Correctness
79.	activate → activating	Incorrect verb forms	Correctness
80.	on	Wrong or missing prepositions	Correctness
81.	, and	Punctuation in compound/complex sentences	Correctness
82.	required → require	Incorrect verb forms	Correctness
83.	reasons → reason	Incorrect noun number	Correctness
84.	are → is	Faulty subject-verb agreement	Correctness
85.	, which	Punctuation in compound/complex sentences	Correctness
86.	. Nilotinib	Improper formatting	Correctness
87.	analog → analogue	Mixed dialects of English	Correctness

88.	<i>are approved</i>	Passive voice misuse	Clarity
89.	of life → <i>of life</i>	Misspelled words	Correctness
90.	<i>TKIs,</i>	Punctuation in compound/complex sentences	Correctness
91.	<i>was approved</i>	Passive voice misuse	Clarity
92.	larger → <i>more significant</i>	Word choice	Engagement
93.	the treatment of → <i>treating</i>	Wordy sentences	Clarity
94.	harboring → <i>harbouring</i>	Mixed dialects of English	Correctness
95.	<i>outcomes,</i>	Punctuation in compound/complex sentences	Correctness
96.	response → <i>answer</i>	Word choice	Engagement
97.	<i>is monitored</i>	Passive voice misuse	Clarity
98.	<i>is evaluated</i>	Passive voice misuse	Clarity
99.	, and CR → <i>. CR</i>	Hard-to-read text	Clarity
100.	<i>is assessed</i>	Passive voice misuse	Clarity
101.	<i>is gauged</i>	Passive voice misuse	Clarity
102.	leukemia → <i>leukaemia</i>	Mixed dialects of English	Correctness
103.	certain → <i>specific</i>	Word choice	Engagement
104.	<i>is recommended</i>	Passive voice misuse	Clarity
105.	therapy → <i>treatment</i>	Word choice	Engagement
106.	<i>reintroducing</i>	Wordy sentences	Clarity

107.	who relapsed	Pronoun use	Correctness
108.	Table2.3 → Table 2.3	Misspelled words	Correctness
109.	to understand better	Inappropriate colloquialisms	Delivery
110.	leukemia → leukaemia	Mixed dialects of English	Correctness
111.	leukemia → leukaemia	Mixed dialects of English	Correctness
112.	Key → Critical	Word choice	Engagement
113.	were able to → could	Wordy sentences	Clarity
114.	at imatinib	Wrong or missing prepositions	Correctness
115.	10times → 10 times	Misspelled words	Correctness
116.	than the inhibitory	Incorrect phrasing	Correctness
117.	signaling → signalling	Mixed dialects of English	Correctness
118.	CD34+lin-quiescent	Misspelled words	Correctness
119.	it was found	Passive voice misuse	Clarity
120.	BCR::ABL1-positive LSCs were identified	Passive voice misuse	Clarity
121.	↓ → is	Incorrect phrasing	Correctness
122.	very low → deficient, shallow	Word choice	Engagement
123.	normal → regular	Word choice	Engagement
124.	some differences have	Wordy sentences	Clarity
125.	been performed	Passive voice misuse	Clarity
126.	is selectively up-regulated	Passive voice misuse	Clarity
127.	up-regulated; upregulated;	Text inconsistencies	Correctness

	<i>upregulating; upregulate</i>		
128.	<i>been shown</i>	Passive voice misuse	Clarity
129.	good → promising	Word choice	Engagement
130.	tumor → tumour	Mixed dialects of English	Correctness
131.	is selectively → is selectively	Improper formatting	Correctness
132.	<i>is selectively expressed</i>	Passive voice misuse	Clarity
133.	<i>were not eliminated</i>	Passive voice misuse	Clarity
134.	harbored → harboured	Mixed dialects of English	Correctness
135.	<i>is expressed</i>	Passive voice misuse	Clarity
136.	highly unlikely → doubtful, improbable, implausible	Word choice	Engagement
137.	a predictive	Determiner use (a/an/the/this, etc.)	Correctness
138.	biomarkers → biomarker	Incorrect noun number	Correctness
139.	the sialic → The sialic	Confused words	Correctness
140.	<i>is expressed</i>	Passive voice misuse	Clarity
141.	and	Conjunction use	Correctness
142.	LSCs,	Punctuation in compound/complex sentences	Correctness
143.	<i>been recognized</i>	Passive voice misuse	Clarity
144.	Hh → He	Confused words	Correctness
145.	<i>is activated</i>	Passive voice misuse	Clarity
146.	nucleus → heart, core, seat, middle	Word choice	Engagement

147.	key → critical, vital	Word choice	Engagement
148.	signaling → signalling	Mixed dialects of English	Correctness
149.	signaling → signalling	Mixed dialects of English	Correctness
150.	signaling → signalling	Mixed dialects of English	Correctness
151.	signaling → signalling	Mixed dialects of English	Correctness
152.	signaling → signalling	Mixed dialects of English	Correctness
153.	<i>been observed</i>	Passive voice misuse	Clarity
154.	signaling → signalling	Mixed dialects of English	Correctness
155.	<i>been linked</i>	Passive voice misuse	Clarity
156.	<i>Both JAK2 and JAK1 have been implicated in the activation of STAT3(Eiring et al., 2015).</i>	Unclear sentences	Clarity
157.	tumor → tumour	Mixed dialects of English	Correctness
158.	<i>been observed</i>	Passive voice misuse	Clarity
159.	leukemia → leukaemia	Mixed dialects of English	Correctness
160.	leukemia → leukaemia	Mixed dialects of English	Correctness
161.	and encodes	Conjunction use	Correctness
162.	tumor → tumour	Mixed dialects of English	Correctness
163.	<i>been shown</i>	Passive voice misuse	Clarity
164.	<i>been shown</i>	Passive voice misuse	Clarity
165.	<i>been elegantly demonstrated</i>	Passive voice misuse	Clarity
166.	LSCs,	Punctuation in	Correctness

		compound/complex sentences	
167.	in combination	Wordy sentences	Clarity
168.	<i>was found</i>	Passive voice misuse	Clarity
169.	<i>been implicated</i>	Passive voice misuse	Clarity
170.	<i>be recruited</i>	Passive voice misuse	Clarity
171.	favorer → favoured	Mixed dialects of English	Correctness
172.	<i>been observed</i>	Passive voice misuse	Clarity
173.	signaling → signalling	Mixed dialects of English	Correctness
174.	enhancing → improving	Word choice	Engagement
175.	<i>been seen</i>	Passive voice misuse	Clarity
176.	<i>be achieved</i>	Passive voice misuse	Clarity
177.	signaling → signalling	Mixed dialects of English	Correctness
178.	increases	Wordy sentences	Clarity
179.	<i>been shown</i>	Passive voice misuse	Clarity
180.	<i>hsa-mir-183</i>	Unknown words	Correctness
181.	<i>However, the precise functions and implications of this specific microRNA in the context of CML LSCs are not fully understood(Ruiz et al., 2021).</i>	Unclear sentences	Clarity
182.	signaling → signalling	Mixed dialects of English	Correctness
183.	Factor-alpha → Factor-alpha	Misspelled words	Correctness
184.	<i>been identified</i>	Passive voice misuse	Clarity

185.	<i>been shown</i>	Passive voice misuse	Clarity
186.		Tone suggestions	Delivery
187.	<i>Autophagy; autophagy</i>	Text inconsistencies	Correctness
188.	<i>of unnecessary</i>	Wrong or missing prepositions	Correctness
189.	tumor → <i>tumour</i>	Mixed dialects of English	Correctness
190.	<i>. Treatment</i>	Improper formatting	Correctness
191.	normal → <i>regular, routine</i>	Word choice	Engagement
192.	tumor → <i>tumour</i>	Mixed dialects of English	Correctness
193.	leukemia → <i>leukaemia</i>	Mixed dialects of English	Correctness
194.	<i>is essential</i>	Wordy sentences	Clarity
195.	, where CP-CML → <i>. CP-CML</i>	Hard-to-read text	Clarity
196.	was found to enhance → <i>enhanced</i>	Wordy sentences	Clarity
197.	a partial → <i>partially</i>	Wordy sentences	Clarity
198.	<i>was associated</i>	Passive voice misuse	Clarity
199.	tumor → <i>tumour</i>	Mixed dialects of English	Correctness
200.	<i>The levels of tumor necrosis factor α and interferon γ secretion were associated with the success of discontinuation of TKIs (Ilander et al., 2017).</i>	Unclear sentences	Clarity
201.	<i>was found</i>	Passive voice misuse	Clarity
202.	, although → <i>. However,</i>	Hard-to-read text	Clarity

203.	<i>been described</i>	Passive voice misuse	Clarity
204.	B → B.	Confused words	Correctness
205.	model → models	Incorrect noun number	Correctness
206.	leukotrienes,	Punctuation in compound/complex sentences	Correctness
207.	leukemia → leukaemia	Mixed dialects of English	Correctness
208.	, suggesting	Punctuation in compound/complex sentences	Correctness
209.	was proven → proved	Wordy sentences	Clarity
210.	<i>I clinical trial was launched</i>	Passive voice misuse	Clarity
211.	<i>However, the adverse effects reported with the SMO inhibitor, coupled with its limited clinical effectiveness, discouraged further investigation and its application in CP-CML(Irvine et al., 2016).</i>	Unclear sentences	Clarity
212.	administering	Wordy sentences	Clarity
213.	<i>was observed</i>	Passive voice misuse	Clarity
214.	<i>were projected</i>	Passive voice misuse	Clarity
215.	achieve → reach	Word choice	Engagement
216.	leukemia → leukaemia	Mixed dialects of English	Correctness
217.	normal → regular	Word choice	Engagement
218.	<i>is believed</i>	Passive voice misuse	Clarity
219.	osteolineage	Unknown words	Correctness
220.	is an adaptation → adapts	Wordy sentences	Clarity

221.	are dependent → depend	Wordy sentences	Clarity
222.	<i>might be exploited</i>	Passive voice misuse	Clarity
223.	LSCs,	Punctuation in compound/complex sentences	Correctness
224.	leukaemia-initiating	Mixed dialects of English	Correctness
225.	<i>been reported</i>	Passive voice misuse	Clarity
226.	<i>be differentially expressed</i>	Passive voice misuse	Clarity
227.	<i>was derived</i>	Passive voice misuse	Clarity
228.	signaling → signalling	Mixed dialects of English	Correctness
229.	<i>These are hypoxia-responsive miRNA (HRMs) capable of simultaneously regulating numerous genes and influencing multiple components of a signaling pathway(Licausi et al., 2011).</i>	Unclear sentences	Clarity
230.	No studies are exploring	Wordy sentences	Clarity
231.	interaction → exchange	Word choice	Engagement
232.	),	Punctuation in compound/complex sentences	Correctness
233.	<i>CD44: Elevation in the expression of the cell surface glycoprotein CD44 was observed in CML cells, facilitating their homing and engraftment through binding to extracellular matrix proteins and osteopontin.</i>	Unclear sentences	Clarity
234.	in-vivo → in-vivo	Misspelled words	Correctness
235.	, which	Punctuation in compound/complex sentences	Correctness

236.	the downregulation	Determiner use (a/an/the/this, etc.)	Correctness
237.	signaling → signalling	Mixed dialects of English	Correctness
238.	leukemia → leukaemia	Mixed dialects of English	Correctness
239.	is controlled	Passive voice misuse	Clarity
240.	HSC; → HSC:	Incorrect punctuation	Correctness
241.	been reported	Passive voice misuse	Clarity
242.	was found	Passive voice misuse	Clarity
243.	This	Intricate text	Clarity
244.	have been shown to be → are	Wordy sentences	Clarity
245.	self-renewing-divisions-(-Agarwal	Misspelled words	Correctness
246.	is upregulated	Passive voice misuse	Clarity
247.	cells .	Improper formatting	Correctness
248.	signaling → signalling	Mixed dialects of English	Correctness
249.	tumor → tumour	Mixed dialects of English	Correctness
250.	the survival → the survival	Improper formatting	Correctness
251.	been implicated	Passive voice misuse	Clarity
252.	was found	Passive voice misuse	Clarity
253.	be detected	Passive voice misuse	Clarity
254.	be produced	Passive voice misuse	Clarity
255.	was confirmed	Passive voice misuse	Clarity

256.	hematological → haematological	Mixed dialects of English	Correctness
257.	<i>been hypothesized</i>	Passive voice misuse	Clarity
258.	possess the ability to → can	Wordy sentences	Clarity
259.	<i>partially</i>	Misplaced words or phrases	Correctness
260.	leukemia → leukaemia	Mixed dialects of English	Correctness
261.	remodeling → remodelling	Mixed dialects of English	Correctness
262.	al.,	Punctuation in compound/complex sentences	Correctness
263.	<i>be detected</i>	Passive voice misuse	Clarity
264.	ineres → increase, inches	Misspelled words	Correctness
265.	signaling → signalling	Mixed dialects of English	Correctness
266.	the angiogenesis	Determiner use (a/an/the/this, etc.)	Correctness
267.	and exhibits → . It exhibits	Hard-to-read text	Clarity
268.	). .	Improper formatting	Correctness
269.	the removal of → removing	Wordy sentences	Clarity
270.	leukemia → leukaemia	Mixed dialects of English	Correctness
271.	. The	Improper formatting	Correctness
272.	<i>The uptake of these exosomes induces changes in the secretion profile and modifies the proliferation and migration capabilities of BM stromal cells(Roccaro et al., 2013).</i>	Unclear sentences	Clarity
273.	a CML	Determiner use (a/an/the/this,	Correctness

		etc.)	
274.	, the cell	Incorrect phrasing	Correctness
275.	neighboring → neighbouring	Mixed dialects of English	Correctness
276.	There are no	Wordy sentences	Clarity
277.	that have	Pronoun use	Correctness
278.	modeling → modelling	Mixed dialects of English	Correctness
279.	modeling → modelling	Mixed dialects of English	Correctness
280.	recapitulate → recapitulate	Misspelled words	Correctness
281.	modeling → modelling	Mixed dialects of English	Correctness
282.	be used	Passive voice misuse	Clarity
283.	generate → create	Word choice	Engagement
284.	in-vitro → in vitro	Misspelled words	Correctness
285.	be differentiated	Passive voice misuse	Clarity
286.	good → excellent	Word choice	Engagement
287.	a large number of → many	Wordy sentences	Clarity
288.		Tone suggestions	Delivery
289.	be used	Passive voice misuse	Clarity
290.	Currently, there is no CML patient-derived MSC line that can be used as a model to study the LSC-stromal interaction.	Unclear sentences	Clarity
291.	a balanced translocation of chromosomes 9 and 22 t(9;22)	Old disease, new targets. Part-II, haematological malignancies	Originality

	(q34;q11		
292.	<i>Chapter 2 is the literature review, which elaborates</i>	Super-Flexible Sensors and Advanced 3D Morphing Actuators based on Elastic Instability	Originality
293.	<i>Results and Discussion The results section is divided into four parts</i>	A machine learning approach for viral genome classification BMC Bioinformatics Full Text https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-017-1602-3	Originality
294.	<i>for self-renewal (Eppert et al., 2011; Goardon et al., 2011; Jamieson et al., 2004;</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
295.	<i>LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; MPP, multi-potent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP,</i>	Molecular Insights into Major Peripheral T-cell Lymphoma Entities with Advances in a Representative Model System	Originality
296.	<i>Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder characterized by</i>	Epigenetic reprogramming and emerging epigenetic therapies in CML	Originality
297.	<i>The origin of the Philadelphia chromosome. The Philadelphia chromosome results from the translocation between the Abelson murine leukemia (ABL1) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22.</i>	What have we learned so far?	Originality
298.	<i>conventional cytogenetics or fluorescent in situ hybridization (FISH</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
299.	<i>highly sensitive reverse transcriptase-polymerase chain</i>	Exploring combined treatment strategies to target quiescent	Originality

	<i>reaction (RT-PCR)(Jabbour and Kantarjian,</i>	chronic myeloid leukemia (CML) stem cells	
300.	<i>an increased production of reactive oxygen species (ROS) within the mitochondria</i>	Antioxidants battle infrared A radiation-induced skin damage https://www.dermatologytimes.com/view/antioxidants-battle-infrared-radiation-induced-skin-damage	Originality
301.	<i>tyrosine kinase, including PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT, ultimately resulting in leukemic cell survival, growth, and proliferation</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
302.	<i>CML therapy was restricted to nonspecific agents, evolving from the primitive usage of</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
303.	<i>compounds in the 19th century to the administration of busulfan, hydroxyurea and, later on, interferon-alpha (IFNα) (Silver et al., 1999). Interferon was used for the first time to treat CML patients in 1983(Talpaz et al., 1983). In the</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
304.	<i>compared to the previously used agents, and the 5-year survival rates were increased from 42% to 57% (1997). However, IFNα was</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
305.	<i>established the curative potential of allogeneic stem cell transplant (allo-SCT) for CML,</i>	Update on the management of Philadelphia chromosome positive chronic myelogenous leukemia: role of nilotinib	Originality
306.	<i>became the treatment of choice for young patients with CML and a</i>	iCMLf - Stem Cell Transplantation - News and Views International CML Foundation https://www.cml-foundation.org/news/15-cell-transplantation.html	Originality

307.	<i>kinase domain that confer resistance to imatinib and were associated with</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
308.	<i>led to the development of the second-generation TKIs, dasatinib, nilotinib, and bosutinib (Golas et al., 2003; Lombardo et al., 2004; Weisberg et al.</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
309.	<i>Dasatinib is 350 times more potent than imatinib in vitro</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
310.	<i>to inhibit the Src family of kinases (Shah et al., 2004). Nilotinib is a structural analog of imatinib with a 50 times higher affinity to the</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
311.	<i>Cortes et al., 2013). However, the acquisition of multiple mutations can still render</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
312.	<i>Furthermore, long-term toxicity events associated with the second-generation TKIs, have been raising concerns</i>	Exploring combined treatment strategies to target quiescent chronic myeloid leukemia (CML) stem cells	Originality
313.	<i>National Comprehensive Cancer Network (NCCN) and European Leukemia Net (ELN</i>	An Analysis of Treatment Patterns, Receipt of Guideline-Concordant Care, and Survival Outcomes among Elderly Women with Non-Metastatic Breast Cancer Using the SEER-Medicare Linked Dataset	Originality
314.	<i>prolonged treatment with the potent ABL kinase inhibitor dasatinib, combined with</i>	An investigation of metabolic vulnerabilities in chronic myeloid leukaemic stem cells	Originality
315.	<i>endothelial cells, and other cell types; it is</i>	Angiogenesis and vascular leakage in diabetic retinopathy	Originality

316.	<i>Over the last decade, researchers have successfully identified</i>	Molecular Epidemiology of Inflammation: Link with Type 2 Diabetes and Coronary Heart Disease	Originality
317.	<i>The phosphorylation and translocation of STAT5 into the nucleus</i>	Extracellular Matrix, Nuclear and Chromatin Structure and Gene Expression in Normal Tissues and Malignant Tumors: A Work in Progress	Originality
318.	<i>PML gene is involved in the t(15;17</i>	[Promyelocytic leukaemia protein and defect in transforming growth factor-beta signal pathway in acute promyelocytic leukaemia].	Originality
319.	<i>Polycarb Repressive Complex 2 (PRC2) that trimethylates histone H3 at lysine 27</i>	Regulation of EZH2 in Triple-Negative Breast Cancer	Originality
320.	<i>programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN), thereby</i>	IJMS Free Full-Text The Role of Noncoding RNAs in the Regulation of Anoikis and Anchorage-Independent Growth in Cancer https://www.mdpi.com/1422-0067/22/2/627	Originality
321.	<i>survival through the nuclear factor κB (NFκB</i>	Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice	Originality
322.	<i>produced by BM stromal cells and endothelial cells</i>	Bone marrow-derived cells or C-X-C motif chemokine 12 (CXCL12) treatment improve thin endometrium in a mouse model†	Originality
323.	<i>TKIs with other agents targeting survival pathways of CML LSCs.</i>	An investigation of metabolic vulnerabilities in chronic myeloid leukaemic stem cells	Originality
324.	<i>a transcription factor that plays a crucial role in</i>	Roles of NF- κ B in Cancer and Inflammatory Diseases and Their Therapeutic Approaches	Originality

325.	<i>to BM stromal cells and extracellular matrix components</i>	Scholars@Duke publication: Viable bone marrow stromal cells are required for the in vitro survival of B-cell precursor acute lymphoblastic leukemic cells. https://scholars.duke.edu/display/pub1439463	Originality
326.	<i>This schematic demonstrates the effects of E-selectin inhibition within the bone marrow microenvironment and the impact upon chronic myeloid leukemia stem cells and mechanistically how this is controlled by the SCL/TAL1 – CD44 axis. HPC: hematopoietic progenitor cell; HSC; hematopoietic stem cell; ...</i>	Defining niche interactions to target chronic myeloid leukemia stem cells	Originality
327.	<i>In a transgenic mouse model of CML, the</i>	Effective Targeting of Quiescent Chronic Myelogenous Leukemia Stem Cells by Histone Deacetylase Inhibitors in Combination with Imatinib Mesylate<!-- --> - refine.bio https://www.refine.bio/experiments/GSE20876/effective-targeting-of-quiescent-chronic-myelogenous-leukemia-stem-cells-by-histone-deacetylase-inhibitors-in-combination-with-imatinib-mesylate	Originality
328.	<i>the generation of induced pluripotent stem cells (iPSCs) that behave</i>	Discovery of survival factor for primitive chronic myeloid leukemia cells using induced pluripotent stem cells	Originality
329.	<i>et al., 2009; Park et al., 2008; Takahashi et al.</i>	Extending Image Processing Strain Measurement System to Evaluate Fracture Behavior of Wall-Thinned Pipes	Originality

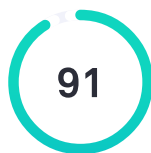
Untitled

by issac nelson

General metrics

52,568	7,490	547	29 min 57 sec	57 min 36 sec
characters	words	sentences	reading time	speaking time

Score



This text scores better than 91% of all texts checked by Grammarly

Writing Issues

265	94	171
Issues left	Critical	Advanced

Plagiarism



34
sources

4% of your text matches 34 sources on the web or in archives of academic publications

Writing Issues

128

Clarity

109

Passive voice misuse



10

Unclear sentences



8

Wordy sentences



1

Hard-to-read text



115

Correctness

3

Text inconsistencies



3

Incorrect phrasing



18

Incorrect verb forms



1

Incomplete sentences



8

Determiner use (a/an/the/this, etc.)



3

Wrong or missing prepositions



4

Confused words



20

Improper formatting



2

Conjunction use



26

Mixed dialects of english



1

Citation style options



11

Misspelled words



1

Misplaced words or phrases



1

Misuse of modifiers



3

Punctuation in compound/complex sentences



2

Faulty subject-verb agreement



3

Comma misuse within clauses



1

Incorrect punctuation



1

Unknown words



1	Incorrect noun number	
2	Closing punctuation	
14	Engagement	
14	Word choice	
8	Delivery	
4	Incomplete sentences	
2	Inappropriate colloquialisms	
2	Tone suggestions	

Unique Words

23%

Measures vocabulary diversity by calculating the percentage of words used only once in your document

unique words

Rare Words

48%

Measures depth of vocabulary by identifying words that are not among the 5,000 most common English words.

rare words

Word Length

5.1

Measures average word length

characters per word

Sentence Length

13.7

Measures average sentence length

words per sentence

Untitled

3.2 Samples

3.2.1 Cell lines

EM-2, KU812, Lama84, KCL22, KYO, MEG01, JURL MK1, and K562 cell lines

266

¹were procured from DZMS, German Collection of Microorganisms and Cell Cultures. ²Cell lines were maintained in RPMI supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin and streptomycin (Gibco).

3.2.2 Primary cells

After obtaining written informed consent, Peripheral blood / Bone marrow (BM) aspirates ³were collected from patients with chronic phase CML at diagnosis, post-treatment, and those with suspected lymphoma diagnosis (without bone marrow involvement- to be used as a source for normal MSCs). ²The IRB (Min No. 14754 dated 27.07.2022) approved this study.

3.3 Cell culture methods

3.3.1 Isolation of mononuclear cells

Briefly, Mononuclear cells (MNCs) were isolated using Lymphoprep (STEMCELL Technologies) by density gradient separation, and CD34+ cells ⁴were enriched immunomagnetically using an EasySep CD34 Positive Selection Kit (STEMCELL Technologies) following the manufacturer's protocol. ²Purity was verified by re-staining isolated cells with an allophycocyanin-labeled (APC) anti-CD34 antibody (Biolegend) and ⁵analyzing cells on a FACS Accuri C6 (BD) machine.

3.3.2 Culture and Expansion of CD34+ Cells

Cryopreserved CD34+ cells of the CML patients and the ⁶normal donor were thawed and cultured in CD34+ cell expansion medium constituting StemSpan™ SFEM II medium (Stem Cell Technologies) supplemented with CD34+ Expansion

Supplement (Stem Cell Technologies) and UM729 (Stem Cell Technologies). The² cells were cultured for 6-7 days with half medium change every alternative day to assess the cell proliferation.

3.4 RNA extraction

3.4.1 RNA Extraction from mononuclear cells

Total RNA was extracted from PB/BM Cells using TRI reagent (Sigma, St. Louis, USA) per the manufacturer's protocol. Briefly², 10X10⁶ cells were lysed in 1ml of Trizol reagent, followed by adding 0.2ml of chloroform and incubated for 8 minutes. The² mixture was centrifuged⁷ at 12000rpm for 15 minutes (4°C). To the² aqueous phase equal⁸ volume of isopropanol added⁹ and incubated¹⁰ for 10¹¹ minutes, centrifuged for 5 minutes at 12000rpm (4°C).² The¹¹ RNA pellet formed was gently washed with 75% ethanol (prepared using nuclease-free water) twice and centrifuged at 12000rpm (4°C). The² pellet was air-dried for 2-3 minutes, resuspended in RNase-free water, and immediately stored at -80°C in aliquots.

3.4.2 RNA extraction protocol for low number of cells

Total RNA from the low number of cells was extracted using Total¹² RNA extraction purification plus kit (Norgen Biotek) per the manufacturer's protocol. Briefly², cells were lysed using Buffer RL buffer and then passed through the genomic DNA Removal column to remove the DNA contamination. Ethanol² was added to the flowthrough and loaded onto an RNA purification column. The² bound RNA is then washed¹³ with Wash Solution A, and the purified total RNA is¹⁴ eluted¹⁴ with the Elution Solution A. RNA concentrations were determined with a Nanodrop instrument (Thermo Fisher Scientific).

3.4.3 RNA Quantity and Quality

RNA concentration was determined¹⁵ by NanoDrop® ND-1000UV, which measures the absorbance at 260nm. Additionally², RNA quality was assessed in

all the samples through electrophoresis in a 1% agarose gel in TAE buffer. High-quality², intact RNA appeared as two distinct bright bands 28S¹⁶ and 18S ribosomal RNAs in the gel.

3.4.4 cDNA synthesis

268 Following manufacturer's¹⁷ instructions, 2µg total RNA converted¹⁸ to cDNA using a High-Capacity Reverse Transcription Kit (ThermoFisher Scientific) and stored for further downstream applications. Confirmation² of cDNA conversion was confirmed through RT-PCR by evaluating the housekeeping gene βglucuronidase (*GUS*) (Table 3.2).

3.5 Strand-specific RNA-sequencing

3.5.1 Samples

RNA was extracted from CML CD34+ (N=10) and mobilized⁵ peripheral blood (mPB) CD34+ (N=4) cells using Total RNA Purification Plus Kit (Norgen Biotek) as described earlier and then subjected to Strand-specific transcriptomics.

3.5.2 RNA QC

RNA QC was evaluated¹⁹ using Agilent Bioanalyzer 2100 and Qubit High sensitivity RNA assay.

3.5.3 RNA Sequencing

269 The transcriptome profile of CML CD34+ cells was compared²⁰ to the mPB CD34+ cells, as well as publicly available datasets of normal CD34+ CD45RA- and BM CD34+ (GEO ID: GSE48846 and GSM1937399). The² raw reads were filtered using Trimmomatic for quality scores and adapters. The² filtered reads were aligned to the Human genome (hg19) using splice aware^{21,22} aligner like HISAT2 to quantify reads mapped to each transcript. The² alignment percentage of the reads was in the range of 5.5-97.5% for all the samples. The² total number of uniquely mapped reads was counted²³ using feature counts. The² uniquely

mapped reads were then subjected to differential gene expression using Deseq2 (Figure 1).

3.5.4 NanoString nCounter Plexset custom panel

With the differentially expressed gene list obtained from the transcriptomic data, we made a custom gene panel consisting of 21 target genes associated with cell surface markers, transporters, cell adhesion, and immune regulation based on previous publications. The expression of these 21 genes was then examined in an independent cohort of CML CD34+ (N=59) and mPB CD34+ (N=9) cells. Read counts were normalized using a positive control and three housekeeping genes (*ACTB*, *GAPDH*, and *GUSB*).

Figure 3.1: Schematic representation of the work outline of whole transcriptomics.

3.6 Establishment of hypoxic culture condition

3.6.1 Cell viability

270 | CML cells were cultured under normoxic and hypoxic conditions. Total cell counts and cell viability were assessed with the trypan blue exclusion method. For cell lines, 0.5-1x10⁵ cells were seeded in 24 well plates in 500 mL medium, and then cell viability was evaluated by culturing under normoxia and hypoxia inhibitors at 24, 48, 72, and 96h.

3.6.2 Cell proliferation

1x10⁶ CML cells were stained with 10 mM CFSE (Carboxyfluorescein succinimidyl ester) for 15 minutes at 37°C in the dark. The cells were washed with a complete medium and maintained under normoxia and hypoxia conditions. Fluorescence intensity was measured using flow cytometry every 24 hours.

3.6.3 Cell cycle analysis

EM-2/ KU812 cells (1×10⁶ cells/ml) were cultured in normoxia and hypoxic conditions for three days and followed by cell cycle analysis. The cell cycle analysis performed using propidium iodide (PI) staining. The cells were collected appropriately and washed with PBS. The cells fixed with ice-cold 70% ethanol. The cells were incubated at 4°C for 30 minutes and washed twice with PBS by centrifugation at 2000rpm to pellet the cells. RNase A (50ul of 100ug/ml) added and incubated at 37°C for 30 minutes. PI (200ul of 50ug/ml) was added incubated on ice for 20 minutes. The cells were analyzed with FACS Calibur to detect PI fluorescence, which shows the cell cycle profile.

3.6.4 Immunofluorescence

1x10⁵ cells were cytospin and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% TritonX-100 (Sigma) in phosphate buffer saline (PBS) for 15 min and blocking using a buffer containing 1% bovine serum albumin (BSA) and 5% Fetal bovine serum (FBS) in PBS for 20 min at room temperature. The cells were incubated with anti-HIF1α primary antibodies (Table 3.8) in the blocking buffer overnight (16 hours) at 4°C, followed by incubation with suitable labeled secondary antibodies for 2 hours at room temperature. For nuclear staining, the cells incubated with 4',6-diamidino-2- phenylindole (DAPI) for 10 min at room temperature and visualized under a fluorescence microscope (DS6000, Leica Microsystems) with appropriate filters(Manian et al., 2018).

3.7 miRNA Profiling by Nano-string

3.7.1 Experimental design

CML primary cells were cultured under normoxic and hypoxic conditions for 48h, and then RNA was extracted using the Trizol Method (Sigma-Aldrich). CML cells showing increased GLUT1 expression were subjected to miRNA profiling (Table 3.2). NanoString miRNA pre-designed panel simultaneously detects >800

human miRNAs, including five housekeeping transcripts, and was used to evaluate the dysregulated miRNA in hypoxic conditions(Jet et al., 2021).

3.7.2 Data analysis

All data analysis was performed using nSolver software. miRNA expression was assessed to identify differential expression between CML cells maintained under normoxia vs. hypoxia conditions.

3.8 Reverse transcriptase polymerase chain reaction

3.8.1 Analysis of BCR::ABL1 fusion transcripts

cDNA was subjected to 2 rounds of nested PCR as reported previously, and agarose gel electrophoresis was performed to identify the type of *BCR::ABL1* fusion transcript [e13a2 or b2a2/e14a2 or e1a2 or b3a2] (Table 3.2)(Markose et al., 2009).

3.8.2 BCR::ABL1 kinase domain mutation screening

As reported previously, mutations in the BCR::ABL1 kinase domain were screened using Sanger sequencing. The sequences were aligned using SeqScape (Applied Biosystems), and the type of the mutation was predicted using Mutation Taster (<http://www.mutationtaster.org/>)(Table 3.2)(Markose et al., 2009).

3.8.3 Real-time Quantitative PCR for mRNA

Quantitative RT-PCR was set up with SYBR Premix Ex Taq II (Takara Bio) using specific primers (Table 3.2) and analyzed with QuantStudio 6 Flex (Applied Biosystems, Carlsbad, CA, USA).

3.8.4 Quantitative reverse transcription PCR for miRNA

RNA will be extracted and reverse transcribed using miR-X Single step CDNA Kit (Takara Bio). qRT-PCR will be performed in an Applied Biosystems 7500 instrument using the SYBR Green Advantage Real-Time Master Mix Kit (Takara Bio) manufacturer's instructions.

3.8.5 Droplet ⁵⁶digital ⁵⁷PCR

Droplet digital PCR performed as described by Datari et al. ²Briefly, a duplex ddPCR reaction mix ⁵⁷was prepared comprising of 1 X ddPCR Supermix for Probes (Bio-Rad, München, Germany), primers, probe, and template cDNA (2000 ng of reverse transcribed RNA). ²After ^{58,59}sealing, the ⁶⁰samples transferred to the QX200 Droplet Generator (Bio-Rad) and approximately 20,000 droplets ⁶⁰per ^{61,62}well ²generated. ⁶³PCR was carried out at 95°C for 10 mins, 94°C for 30 secs, ⁶⁴and annealing and extension at 60°C for 90 seconds, 1st cycle at 98°C for 10 minutes and holding at 12°C. ²Positive and negative droplets ⁶⁴counted by employing QX200 Droplet Reader (Bio-Rad) and assessed by Poisson statistics ⁵utilising QuantaSoft software version 1.7.4.0917 (Bio-Rad, München, Germany). (Maier et al., n.d.).

3.9 Immunoblot

⁶⁶Protein lysates prepared by using radioimmunoprecipitation assay buffer supplemented with a protease inhibitor mixture and phenylmethylsulfonyl ⁶⁷fluoride. ²The cell lysate was loaded in 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. ²The membrane ⁶⁸blocked with non-fat dry milk powder in tris-buffered saline containing Tween 20 and incubated with primary antibodies (Table 3.7). ²The proteins ⁶⁹detected using the ECL chemiluminescence kit and imaged using the FluorChem E system ⁴⁷(Bedel et al., 2013).

3.10 Molecular inhibition

3.10.1. Preparation of lentiviruses

For the generation of lentiviruses, the gRNA and Cas9 expression plasmids were co-transfected with pMD2.G plasmid ⁴⁷(Addgene 12259) and psPAX2 plasmid ⁴⁷(Addgene 12260) (gifts from Didier Trono). ²The virus supernatants were collected after 48, 60, and 72 hours, pooled together, and concentrated 100

times using the Lenti-X Concentrator (Takara Bio) and stored at -80°C as aliquots.

3.9.2 Lentiviral-mediated CTSG knockdown in CML cells

For stable CTSG suppression, EM-2 cells were seeded at 2x10⁵ cells/24-well and infected with 20ul of the concentrated lentiviral vector containing shRNA constructs targeting human CTSG or a non-targeting sequence (Tet-pLKO-puro (Addgene: 21915))(Figure 3.2) in the presence of polybrene for 24 hours. The expression of the shRNA was induced⁷⁰ with doxycycline (Dox) (1µg/ml) for 5⁷¹ days; subsequently, western blotting and *in vitro* assays were performed.⁷²

Figure 3.2: Vector map for Tet-pLKO-puro

3.9.3 Generation of CRISPR-based dual-guide RNA knockout of miR-30b

3.9.3.1 Generation of dgRNA expression lentiviral vectors: The dgRNAs were cloned⁷³ in the pKLV2.2-h7SKgRNA5(SapI)-hU6gRNA5(BbsI)-PGKpuroBFP-W (Addgene plasmid # 72666; a gift from Kosuke Yusa)(Figure 3.3), to generate pKLV2.2 dgRNA expression lentiviral vectors. The² plasmid was first digested with SapI for cloning the first gRNA and then with BbsI for cloning the second gRNA. The² oligos for gRNA1 and gRNA2 were synthesized^{5,74} with specific 5' overhangs for cloning at the SapI and BbsI sites of the plasmid.

Figure 3.3: Vector map for PLKO2.2

3.9.3.2.Generation of Cas9 expressing cell lines: The K562 cell line was transduced⁷⁵ with pCLIP-Cas9-Nuclease-EFS-Blasticidin (Transomic, Cat No. V085) lentiviral vector, and then the selected with 2⁷⁶µg/mL of blasticidin starting five days after transduction, for ten days.

3.9.3.3.Transduction of dgRNA lentiviral vectors in K562 cell lines: K562-Cas9 cell lines were transduced with pKLV2.2 dgRNA viruses in the presence of 8µg/mL polybrene by spinfection⁷⁷ at 2250 rpm for 1.5 hours at room temperature. After² six days, BFP+ cells were flow sorted and further cultured

to estimate mutations caused by the dual gRNAs at the targeted genomic region.

3.9.3.4. Capillary Electrophoresis: The first round of PCR ⁷⁸was carried out with target genomic locus-specific primers (**Table**) that flank the gRNA binding regions. ²The second round of PCRs ⁷⁹was performed using a 5' FAM ⁸⁰labeled forward primer and the same reverse primer used in the first round of PCR. ²The second round PCR products (~2µl) were denatured in ⁵deionized formaldehyde and ⁵analyzed by capillary electrophoresis using an ABI 3500 Genetic Analyzer (Thermo Fischer Scientific). ²The peak sizes and heights of the amplified products were determined using the Peak Scanner software (Thermo Fisher Scientific).

3.10 Colony forming unit – assay

Colony-forming cell assays ⁸¹were performed to investigate the proliferation and differentiation of hematopoietic and leukemic progenitor cells by their ability to form colonies of mature blood cells in a semisolid medium. ²Briefly, 1,000 cells ⁸²were mixed in 1 mL MethoCult (STEMCELL Technologies), and then ⁸³the number of colonies was scored on day 14 following the manufacturer's protocol.

3.11 Xenotransplantation model

EM-2 cells ⁸⁴were transduced with lentivirus containing the Dox inducible shCTSG followed by Dox induction for 48h. ²These cells induced with and without dox were transplanted into sub-lethally irradiated (2 Gy) 8–10 weeks ⁸⁵old NSG mice (The Jackson Laboratory, Bar Harbor, ME, USA) via tail vein. ²The median survival of mice was evaluated ⁴⁷(Rothe et al., 2020).

3.12 Generation and ⁵characterization of CML MSC

3.12.1 Isolation and culture of bone marrow-derived MSC

BM-MNCs from healthy and CML patients resuspended in α-MEM containing 20% FBS and 1% penicillin-streptomycin and plated at an initial 5×10^5

cells/cm² density. After² three days, the cells were washed with PBS (Media Tech Inc.), and the remaining adherent cells were cultured⁸⁶ until the cells reached approximately 80% confluency. MSCs² from passage one were cryopreserved and stored for RNA/DNA(Zhang et al., 2019).⁴⁷

3.12.2 Immunophenotyping of CML MSCs

Healthy donor and CML patient-derived MSCs were subjected to trypsinization⁵ and washed once with PBS. The cells² incubated⁸⁷ for 20 minutes with 1µl of fluorochrome-conjugated antibodies (all purchased from BioLegend) (Table 3.5). Flow² cytometric data analyzed^{5,88} using FlowJo software (BD Biosciences).

3.12.3 Generation of Immortalised⁵ CML MSC lines (iCML MSC)

Cryopreserved MSCs from the first passage were revived and used for immortalization.⁵ 0.2X10⁶² cells were transduced⁸⁹ with TRE-HEE-UbC-hKO1-rtTA lentiviruses encoding HPV16-E6/E7 (HEE) genes (a kind gift from Yukio Nakamura) by spinfection⁹⁰ at 2250 rpm for 1.5 h. They² were cultured⁹¹ in 20% MEM, with half-medium change on alternate days. The medium was² supplemented⁹² with 1 µg/mL doxycycline (dox) (Sigma-Aldrich, St. Louis, MO, USA) from the fourth day. The cells were maintained⁹³ throughout the culture at a seeding density of 5–6x10⁵ cells/mL, medium was changed^{94,95} on alternative days. Single² cells were sorted and characterized⁵ immunophenotypically for the MSC surface markers(Bagchi et al., 2021).⁴⁷

Figure 3.4 Experiment outline⁹⁷ immortalisation⁵ of CML MSCs

3.12.4 Multilineage differentiation

Trilineage differentiation for osteogenic, adipogenic, and chondrogenic lineage was evaluated⁹⁸ in the primary CML MSCs, iCML MSC line, and HS-5 (Healthy donor iMSCs) as control. Cells² seeded⁹⁹ in 24 well¹⁰⁰ culture dishes at 5000 cells/cm² for adipogenic differentiation¹⁰¹, expanded to sub-confluence¹⁰², and replaced with the adipogenic differentiation media (Stem Cell Technologies).

Undifferentiated² MSCs cultured on a stromal medium¹⁰³ were used² as a control. After² 21 days, cells¹⁰⁴ fixed² with 10% formalin, washed, and stained using Oil Red O. All images^{5,105} were visualized² using the Olympus virtual slide system. The² Oil Red O staining was eluted with 100% isopropanol for 15 minutes at room temperature, and dye concentration was quantified by absorbance measurement at 510nm(Vinod et al., 2023).⁴⁷

3.12.4.1 Adipogenic differentiation

Following adipogenic differentiation, the cells¹⁰⁶ fixed² with 10% formaldehyde, washed, and stained with Oil Red O (Sigma). All² images were acqy¹⁰⁷ using the Olympus virtual slide system. The² Oil Red O staining¹⁰⁸ was eluted² with 100% isopropanol for 15 min at room temperature, and dye concentration¹⁰⁹ was quantified² by absorbance measurement at 510 nm.

3.12.4.2 Osteogenic differentiation

After 28 days of differentiation, the pellets were subjected to fixation using 4% paraformaldehyde, paraffin-embedded, sectioned (4µm).

Alizarin Red: After⁵ deparaffinization¹¹⁰ and hydration, the sections were stained with Alizarin red¹¹⁰ solution², followed by dehydration, and mounted using a DPX mount.

²⁷⁸ **Von Kossa stain:** Sections were incubated with 1% silver nitrate under UV light for 20 minutes. After² distilled water rinse, the slides were treated with sodium thiosulphate for 5 minutes and then counterstained with nuclear fast red. Images² were acquired using the Olympus virtual slide system.

3.12.4.3 Chondrogenic differentiation

After 28 days of differentiation, the pellets were subjected to fixation using 4% paraformaldehyde, paraffin-embedded, sectioned (4 µm), and subjected to confirmatory staining.

The following protocols¹¹¹ were used² for staining the individual samples:

Alcian blue stain: Alcian blue ¹¹²stain ¹¹³was used to assess glycosaminoglycan accumulation. ²Sections were treated with Alcian blue (pH: 2.5, Cat no: J60122, Alfa Aesar, US) as described

Safranin O fast green: First, the sections were stained with Wiegert's Iron Hematoxylin, followed by ¹¹⁴acid ¹¹⁵alcohol, Fast green solution, acetic acid (, and finally with Safranin O solution (1%).

Toluidine blue: 0.1% dye solution for 5 minutes (CI 52040 Qualigens).

PicroSirius red: 0.1% dye concentration counterstained with Hematoxylin

3.12.5 Immunohistochemistry

3.12.5.1 Collagen type II (Chondrogenic differentiated pellet). ²After chondrogenic differentiation, the sections underwent antigen retrieval by treating the ¹¹⁶sections with pronase (1mg/mL) and hyaluronidase (2.5mg/mL). ²The histopathology sections were then incubated with mouse monoclonal anti-collagen type II antibody (5µg/mL) (DSHB, II-II6B3) at 4°C. ²Subsequently, they were ¹¹⁷subjected to a 30-minute incubation with a secondary antibody (HRP ¹¹⁸labeled goat anti-mouse immunoglobulin (31430, Pierce, Wisconsin, USA)). ²The stained sections were ¹¹⁹then treated with a 3, 3-diaminobenzidine ¹²⁰solution, and then counterstained with Hematoxylin. ²Histological imaging of the stained ¹²¹sections ¹²²was performed using an Olympus BX43F microscope.

3.12.5.2 Collagen type X

The chondrogenic differentiated paraffin pellet sections ¹²³first treated with chondroitinase ABC (1mg/mL) and pepsin (2.5mg/mL). ²The ¹²⁴sections incubated with primary antibody for mouse monoclonal anti-collagen type X antibodies (5µg/mL) (DSHB, II-II6B3) at 4°C, followed by a 30-minute incubation with HRP ¹²⁵labeled goat anti-mouse immunoglobulin antibody (31430, Pierce, Wisconsin, USA) (1:250 dilution) then treated with DAB solution followed by counterstained

with Hematoxylin. Histological ²imaging was carried out ¹²⁶using an Olympus BX43F microscope.

3.12.6 iCML MSCs functional analysis

For coculture experiments to show the interaction between CML CD34+ cells and the MSC stromal layer, the stromal cells were seeded ¹²⁷in tissue culture dishes at 20,000 cells/cm² concentration. After ²12h, CML CD34+ cells were ¹²⁸added to the stromal layer at a ratio of 1:10 (stromal cell: leukemic cells). The ¹²⁹protective effect was evaluated by measuring apoptosis after 48h with and without imatinib treatment using Annexin V.

Apoptosis analysis was performed ¹³⁰by ¹³¹using Annexin-V and PI (Annexin-V staining kit, Invitrogen – ThermoFisher Scientific) using the manufacturer's instructions and assessed using flow cytometry. Briefly ², the suspension cells ²⁷⁹were collected and washed with ice-cold PBS. The ²cells were suspended ¹³²in 1X Annexin V binding buffer at a concentration of 1x10⁵ cells/100µl buffer, ¹³³ anti ¹³⁷annexin V antibody ¹³⁴added ¹³⁵to the cells and incubated in dark ¹³⁶for 15 min at room temperature ²⁸⁰(Zhang et al., 2013). ⁴⁷

3.12.7 Collection of conditioning media from the MSC:

MSCs were seeded in tissue culture-treated plates in standard growth media and allowed to grow to 60-70% confluency. After ²the cells reached confluency, fresh, complete media was added ¹³⁸to the cells. The conditioned media (CM) ²containing secreted factors was collected ¹³⁹after 24 hrs, centrifuged and filtered through a 0.2µm filter. CM ²was aliquoted and stored at -200C until further use ⁴⁷(Zhang et al., 2013).

3.13 Cytokine secretion profiling by cytokine antibody array

²⁸¹Human Cytokine Antibody Array C Series 1000 (RayBiotech, Norcross, GA), which allows us to detect 274 cytokines, was used per the manufacturer's instructions. Membranes ²with prespotted ¹⁴⁰capture antibodies were blocked

282 using 1X blocking buffer for two hours and then incubated with a conditioning
283 medium for 3 hours at 25°C. Chemokines were detected by incubation with
biotinylated detection antibodies for 2 hours at 25°C. Following the addition of
horseradish peroxidase-conjugated streptavidin and overnight incubation at 4
°C, signals were revealed by enhanced chemiluminescence (Cyanogen). Signals
were captured by a 3-5sec exposure on a Chemidoc system (Biorad)(Xin et al.,
2015).

3.13.1 Semi-Quantification of cytokine antibody array data

Chemiluminescent cytokine array data were semi-quantified with ImageJ
software. The intensity of each cytokine spot was measured based on spot
signal density. For every spot, the net density gray level was determined by
normalizing with the negative controls from the measured density gray levels.
The density gray level of six positive controls was used to normalize the results
from different membranes. The density for each cytokine was then averaged
over duplicated spot signal.

3.14 Generation of iPSC from CML and healthy donor CD34+

3.14.1 Reprogramming of CML and normal CD34+ cells

The CD34+ cells were cultured in the expansion medium for 3-days and then
nucleofected with episomal reprogramming plasmids (pCXLE-hOCT3/4-shp53
(Addgene: 27077), pCXLE-hSK (Addgene 27078) and pCXLE-hUL (Addgene:
27080) (kind gift from Shinya Yamanaka), as described previously.

Electroporation was carried out using a Neon Transfection System
(ThermoFisher) at 1600V, 10ms, and 3 pulses. After five days of culture, the
nucleofected cells were transferred to Matrigel-coated plates containing
Essential-8 (E8) medium (ThermoFisher Scientific). Individual colonies were
hand-picked between day 21 and day 32 and were passaged and maintained in

mTeSR Plus medium (StemCell Technologies) supplemented with 10µM IM (Sigma Aldrich) to generate stable iPSC clones(Miyauchi et al., 2018).

3.14.2 Characterisation of iPSCS

The colonies were dissociated using TrypLE (ThermoFisher Scientific) and then stained using a PE-conjugated TRA-1-60 antibody (ThermoFisher Scientific) for 20 mins To analyze the expression of TRA-1-60 in iPSCs. The cells were washed with mTeSR Plus (Stem Cell Technologies) containing Revitacell (ThermoFisher Scientific), and the expression was analyzed using Navios (Beckman Coulter) or FACS Aria (BD Biosciences) flow cytometers. The extent of purity of CD34+ cells after their magnetic separation from PBMNCs was measured by flow cytometry using APC-conjugated anti-CD34 antibody (BioLegend)(Manian et al., 2018).

3.14.3 Alkaline phosphatase staining

Alkaline Phosphatase staining performed using the Alkaline Phosphatase Detection Kit (Sigma-Aldrich) following the manufacturer's instructions(Bedel et al., 2013) .

3.14.4 immunofluorescence

Immunofluorescence analysis of the pluripotency marker expression was evaluated as described before. Briefly, iPSC colonies were fixed, permeabilized , and incubated with primary antibodies in the blocking buffer overnight (16 hours) at 4°C, followed by incubation with suitable labeled secondary antibodies for 2 hours at room temperature (Table 3.8)(Manian et al., 2018).

3.14.5 Trilineage differentiation

Trilineage differentiation of iPSCs to ectoderm, endoderm, and mesoderm lineages was carried out using STEMdiff tri-lineage differentiation Kit (Stem Cell Technologies) per the manufacturer's protocols. Briefly, iPSCs were dissociated using TrypLE™ (Gibco) and cultured in mTeSR + medium (Stem Cell Technologies) supplemented with 10 µM revitacell (ThermoFisher Scientific).

On day 1, pre-warmed STEMdiff™ Trilineage medium was added to each well to promote ectoderm, endoderm, and mesoderm differentiation, and the plates were incubated at 37°C for 24 hours. The medium was changed periodically until day 5 (mesoderm and endoderm lineages) or day 7 (ectoderm lineage), after which immunofluorescence was performed according to established protocols (Table 3.8) (Manian et al., 2018).

3.14.6 Hematopoietic Differentiation of iPSCs

For the hematopoietic differentiation of iPSCs, we used the STEMdiff™ Hematopoietic Kit (Stem Cell Technologies). Briefly, iPSCs were either single-cell sorted or seeded as small aggregates in mTeSR Plus (Stem Cell Technologies). Subsequently, the culture medium was changed to STEMdiff™ A medium, with or without IM. After three days, 70,000 cells were reseeded in STEMdiff™ A medium and maintained for another three days. Following this, the medium was switched to STEMdiff™ B. After 12 days of culture, the cells present in suspension were harvested and analyzed by flow cytometry for the expression of hematopoietic surface markers (Miyachi et al., 2018).

3.14.7 Colony forming assay

In each experiment, 7,000 hematopoietic progenitors generated from iPSCs were plated onto the MethoCult GF H4636 medium (Stem Cell Technologies). The number of colonies in each experiment was scored on day 14 following the manufacturer's instructions.

3.15 Quantification and Statistical Analysis

Two-tailed Student's t-test for unpaired samples with unequal variance or 1-way ANOVA with Tukey's correction for multiple comparisons using GraphPad Prism version 8 (GraphPad Software). Differences between various treatment groups were analyzed by 2-way ANOVA, with Tukey's correction for multiple comparisons, using GraphPad Prism version 8 (GraphPad Software). p values <

0.05 were considered statistically significant. Survival² curves of mice were compared¹⁷⁸ with the log-rank (Mantel-Cox) test.

Table 3.1 – Reagents used in this study.

Reagents

Company

Cat. No.²

HISEPTM LSM 1077

Himedia

LS001

TRIzol

Sigma

T9424-200ML

Cell Lysis

Qiagen

158908

Protein Precipitate

158912

DNA Hydration

158914

1XRBC lysis buffer

52304

EasySep™ Human Whole Blood CD34 Positive Selection Kit II

Stem Cell Technologies

17879

StemSpan™ SFEM II

09655

StemSpan™ CD34+ Expansion Supplement (10X)

02691

UM729

72332

Imatinib mesylate

Sigma

SML 1027

Dasatinib

SML2589

Nilotinib

PHR2833

Ponatinib

TA9H9876F4A8

1X Polybrene

H9268

Doxycycline Hyclate

D9891-1G

Lenti-X-Concentrator

Takara Bio

631231

miRNA First-Strand Synthesis

KIT

638313

miRNA TB green Advantage kit

638316

Sybergreen RT PCR Sybergreen RT PCR

4448890

RNA LATER

ThermoFisher Scientific

AM7024

CFSE Kit

C34554

Blasticidin

A1113903

High-Capacity cDNA Reverse Transcription Kit

4368814

Table 3.2 – List of oligos used in this study

Gene/mRNA

Primers/Probe

Primers (5'-3')

GLUT1

Forward Primer

TGCGCCCAACTTCGTCATGTCG

Reverse Primer

CGTAGCCGTTTTCGAAGATGCG

VEGF

Forward Primer

GATGAGCTCATTGTAATATGAG

Reverse Primer

ATATTACCATTAGCTCATCTTT

CTSG

Forward Primer

TCTGCTGGCCTTTCTCCTACC

Reverse Primer

CACATCTGCTCTGACCTGCTG

SUCNR1

Forward Primer

GGGAGTTGGAAGCAGTATCAG

Reverse Primer

GCATGTCCCTGAAGTGATCTC

GUS

Forward Primer

CCTGTGACCTTTGTGAGCAA

Reverse Primer

GTCTGCCGTGAACAGTCCA

BCR::ABL1 RQ-PCR primers and primer probes

BCR - e1a2

Forward Primer

CTG GCC CAA CGA TGG CGA

ABL

Reverse Primer

CAC TCA GAC CCT GAG GCT CAA

BCR b3a2

Forward Primer

TCC GCT GAC CAT CAA YAA GGA

ABL

Forward Primer

GAT ACG AAG GGA GGG TGT ACC A

ABL

Reverse Primer

CTC GGC CAG GGT GTT GAA

ABL

Probe

6-FAM TGC TTC TGA TGG CAA GCT CTA CGT CTC CT -TAMRA

BCR::ABL1

Probe

6-FAM - CCC TTC AGC GGC CAG TAG CAT CTG A - TAMRA

BCR::ABL1 Nested PCR primers

BCR-e1-A

GACTGCAGCTCCAATGAGAAC

ABL-a3-B

GTTTGGGCTTCACACCATTCC

BCR-e1-C

CAGAACTCGCAACAGTCCTTC

ABL-a3-D

TTCCCCATTGTGATTATAGCCTA

ABL-a3-E3

TGACTGGCGTGATGTAGTTGCTT

BCR-b1-A

GAAGTGTTTCAGAAGCTTCTCC

BCR-b2-C

CAGATGCTGACCAACTCGTGT

Table 3.3 List of shRNAs and gRNAs

Gene

5'-3'

shCTSG

GTGCAGAGGGATAGGCAGTCTCGAGACTGCCTATCCCTCTGCAC

miR 30b dgRNA2 F

ACTGAAACTTGGTTTAAAGC

miR dgRNA2 R

GCTTTAAACCAAGTTTCAGT

mir30b del PCR FP

CAAGTAGATCCCTGCCAGCT

mir30b del PCR RP

TGTGAATGCTGTGCCTGTTC

Table 3.4 List of Restrictions enzymes used for cloning

Restriction enzymes

Company

Cat. No.

Sap I

New England Biolabs

R0569S

Bbs I

R0539S

*Hind*III

R0104S

Table 3.5 List of flow cytometry antibodies

Flow cytometry reagents

Company

Cat. No.

CD73 APC

Biolegend

344006

CD45 pacific blue

368540

CD34 APC

343608

CD90- PE CY7

328124

CD38 FITC¹⁷⁹

356610

TRA-1-60

ThermoFisher Scientific

MA1-023

Table 3.6 – List of western blot reagents

Western blot Reagents

Company

Cat. No.²

Ladder

Gene Direx

PM0010500

Westar supernova

Cyanagen

xls30100

Stacking Gel Buffer -1ltr

BioRad

161-0799

Resolving Gel Buffer-1ltr

BioRad

161-0798

Blotting grade blocker, 300gm

BioRad

1706404

Acryl/BIS 19:1, 40% SOLN 500ml

BioRad

1610144

Laemmli Sample Buffer 30ml

BioRad

38220090

Immobilon P 26.5x3.75m roll PVDF 45 μ M

Merck

IPVH00010

PHOSSTOP

Sigma

4906837001

Complete™Ultra Tablets, Mini, Easy protease inhibitor

Sigma

5892970001

TWEEN 20

MP Biomedicals

11TWEEN201

Bovine serum albumin

HiMedia

MB083 100G

Bio-Rad Protein Assay Dye Reagent

BioRad

5000006

Table 3.7

List of Western blot antibodies

Antibodies

Company

Cat. No.

Anti-CTSG

Cell Signalling Technology

63665S

Anti-p-CRKL

3181S

Anti-Cas9

14697s

β-actin

Sigma

A5441

Table 3.8

List of immunofluorescence antibodies

Antibody

Company

Cat. No.

Anti-TRA-1-81

StemLight Pluripotency Antibody Kit (Cell Signaling Technology)

9656S

Anti-SSEA-4

Anti-NANOG

Anti-OCT-4

Anti-HIF1a

Abclonal

A7684

Anti-PAX6

Cell Signaling Technology

60433

Anti-Brachyury

81694

Anti-SOX17

81778

4.1 Differential gene expression of CML CD34+ vs. healthy donor CD34+ hematopoietic progenitor cells.

290 Imatinib has revolutionized⁵ the management of chronic myeloid leukemia¹⁸⁰ (CML) by significantly improving treatment outcomes. However², a significant

291 challenge in CML therapy arises from the development of resistance to TKIs, often attributed to the presence of leukemic stem cells (LSCs). These² LSCs can evade standard treatment and are responsible for disease recurrence and progression. They² exhibit a remarkable ability to survive through BCR::ABL1 independent mechanisms, suggesting the involvement of alternative signaling¹⁸ pathways in their survival and maintenance. Therefore², it becomes crucial to identify specific gene signatures that are selectively expressed¹⁸² in LSCs. Understanding the molecular mechanisms underlying LSC survival may help develop targeted therapeutic strategies to selectively eliminate LSCs and improve treatment outcomes in CML patients^{183 2}. One¹⁸⁵ way to address this would be to compare the transcriptomic profiles between healthy and CML stem cells and to¹⁸⁵ identify specific gene signatures that are selectively expressed¹⁸⁴ in CML

stem cells. Although ² *several studies over the years have addressed the differences between CML cells vs. normal HSCs to identify the signatures of these LSCs,* ¹⁸⁷ very few reports ^{186,187} exists ¹⁸⁸ using CML CD34+ cells.

In the absence of a clear cell surface marker discriminating CML LSC from normal healthy HSCs, CD34+ cells are considered ^{188,189} as ¹⁹⁰ LSPCs and ¹⁹¹ are studied by many groups. ² CML CD34+ cells have ¹⁹¹ been reported to exhibit distinct metabolic differences, showcasing enhanced oxidative phosphorylation (OXPHOS) when compared to healthy HSCs ⁴⁷ (Kuntz et al., 2017). ² Tigecycline, ¹⁹² an antibiotic, also effectively inhibited OXPHOS in treatment-resistant CML LSCs, both in vitro and in vivo, while sparing HSCs. ² In a Phase I trial involving relapsed/refractory AML patients, tigecycline demonstrated a satisfactory safety profile but failed to yield significant clinical benefits. ² This ¹⁹³ outcome was attributed to the shorter half-life of tigecycline (Reed GA et al., 2016).

Previous reports on transcriptomic signatures in CML have highlighted an increased expression of focal ¹⁹⁴ adhesion related genes in CML CD34+ cells and have identified ILK as a crucial focal adhesion component required for LSC self-renewal in vivo. ² The ⁵ study predominantly utilized in vitro and in vivo models to assess the effects of ILK inhibition. ² Zhao et al. (2022) reported the role of MS4A3 as a functional ¹⁹⁵ tumor suppressor in CML, where its downregulation resulted in the retention of ¹⁹⁶ leukemia stem/progenitor cells (LSPCs) in a state of differentiation blockage that rendered them insensitive to TKIs. ² In ¹⁹⁷ this study exogenous MS4A3 protein was introduced using a targeted delivery strategy, although the feasibility of clinical translation for this approach remains uncertain. ² Thus, further investigations are imperative to devise more pragmatic methods for inducing MS4A3 reexpression or facilitating the delivery of ectopic MS4A3 ⁴⁷ (Zhao et al., 2022). ² Overall, ⁵ these studies collectively emphasize the complexity of CML and the importance of targeting specific cellular processes

and molecules for therapeutic intervention. ²They also highlight the significance of considering metabolic differences and alternative strategies in addressing treatment-resistant cells while acknowledging the practical challenges in translating laboratory findings into clinical applications.

In the present study, using ¹⁹⁸global transcriptomics approach, we attempted to identify selective gene signatures and pathways differentially expressed in CML CD34+ cells ¹⁹⁹to comprehensively understand the mechanisms contributing to CML LSC survival.

4.1.1 Global transcriptome profiling of CML CD34+ and normal CD34+ cells by RNA-Sequencing

To identify the unique gene signature of CML LSCs, we compared the transcriptomics profiles of CML CD34+ and healthy donor CD34+ ⁵(mobilized peripheral blood CD34+ (mPB CD34+) cells by NGS in an Illumina NGS platform. ²⁰Global transcriptome profiling of differentially expressed genes was evaluated by filtering based on the reads per kilobase million (RPKM) > 1 and a minimum of a 2-fold difference (CuffDiff, FDR-adjusted $p < 0.05$) between CML CD34+ (n = 10) and mPB CD34+ cells (n = 4). ²⁹²²Principal Component Analysis (PCA) showed a distinct separation between the two groups, revealing ²⁰¹a clear clustering of samples aligning with their respective groups. ²(Figure 4.1.1A). ²We observed 1563 genes upregulated and 1400 genes downregulated in CML CD34+ cells compared to mPB CD34+ (Figure 4.1.1B). ²Top upregulated genes include *GSTM1* (Glutathione S-transferase mu 1), *AZU1* ⁴⁷(Azurocidin 1), *LTF* (Lactotransferrin), *CTSG* (Cathepsin G), *HIST14C* (histone proteins) and downregulated gene include *CXCL13* (C-X-C ligand 13), *ITGA3* (Integrin Subunit Alpha 3), *CDH4* ⁴⁷(Cadherin 4), *PRMD16* (protein arginine methyltransferase 16) (Figure 4.1.1C). ²²⁰²Both the up and downregulated genes are involved in immune response, cytokine ²⁰³signaling, and cell adhesion.

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Figure 4.1.1 Global transcriptomics analysis of CML and mPB CD34+ cells. (A) Principal component analysis (PCA) plot of CML CD34+ and mPB CD34+ cells. (B) Volcano plot showing the significant upregulated and downregulated genes in the CML CD34+ cells. Red dots indicate upregulated genes, and Blue dots indicate downregulated genes between CML and mPB CD34+ cells (C) Heatmap visualizing the expression of the significantly differentially expressed genes showing top-up and downregulated genes with a 2-fold difference (FDR-adjusted $p < 0.05$) between CML and normal CD34+.

Pathway enrichment analysis of the top differentially expressed genes ($\log_2 F > 1$, $p < 0.05$) was done using Enrichr (Rothe et al., 2020) (https://maayanlab.cloud/Enrichr/). Gene ontology analysis using GO biological process, GO cellular component, and GO molecular function revealed genes associated with mitotic sister chromatid segregation spindle, single strand helicase activity, and mitochondrial matrix to be dysregulated in CML CD34+ (Figure 4.1.2A-C). Based on Reactome, KEGG, and MSigDB datasets, genes associated with cell cycle, Fanconi anemia pathway, and Metabolism were significantly dysregulated in CML CD34+ cells (Figure 4.1.2D-F). Similar to the previous studies, we observed enhanced OxPHOS in CML CD34+ cells compared to the mPB CD34+ (Kuntz et al., 2017). Genes such as *FANCI*, *FANCM*, *FANCB*, *FANCG*, and *FANCD2* associated with the Fanconi anemia pathway were upregulated, which could lead to enhanced DNA repair. In Metabolism, genes related to the ETC-complex I, such as NADH: ubiquinone oxidoreductase subunits and fatty acid β oxidation such as *ACADVL*, *NDUFAB1*, *ACADM*, and *DECR* were significantly upregulated in CML CD34+ cells. These results suggest CML CD34+ cells have altered Metabolism and enhanced DNA repair mechanisms. As reported earlier, dysregulation of the Fanconi anemia

pathway was observed in CML CD34+ cells (Chakraborty et al., 2012), leading to chromosome instability. For instance, human cord blood CD34+ cells transduced with BCR::ABL1 retroviral vectors showed impaired FANCD2 foci formation, whereas FANCD2 monoubiquitination in these cells was unaffected. The mechanism by which c-NHEJ factors disrupt the Fanconi anemia pathway in CML CD34+ cells is not yet fully understood, and further research is needed to elucidate this process. Similar to previous studies showing the alteration in the genes related to the mitochondrial respiratory complex (MRC)(Flis et al., 2012)(Flis et al., 2012)(Flis et al., 2012), we observed 30 genes associated with ETC-I to be significantly upregulated in CML CD34+ cells. Metabolic profiling of CML CD34+ vs. normal CD34+ has revealed enhanced OXPHOS in CML CD34+ (Kuntz et al., 2017). Dysregulation of mitochondrial metabolism can increase oxidative stress and DNA damage, leading to genomic instability (Flis et al., 2012). All these studies, including ours, suggest that mitochondrial metabolism can be a potential therapeutic target in CML.

Gene ontology analysis of significantly downregulated genes revealed the genes associated with positive regulation of cytokine production, Focal adhesion, and inhibitory MHCI receptor activity to be downregulated (Figure 4.1.3A-C). Pathway enrichment of significantly downregulated genes revealed the genes associated with cytokine signaling, inflammatory response, and TNFα signaling to be downregulated in CML CD34+ cells (Figure 4.1.3D-F). Together, these results suggest that in CML LSPCs, immune response, inflammation, and cytokine signaling are downregulated.

Figure 4.1.2 Pathways dysregulated in significantly upregulated genes:

Enrichment analysis was performed using Enrichr A) Reactome 2021 B) MsigDB Hallmark 2020 C) KEGG 2021 D) GO Biological process E) GO cellular components and F) GO molecular functions of significantly enriched pathways

was manually curated based on the smallest p-values obtained across all the databases.

4.1.2 Altered cell surface markers:

We then evaluated alterations in cell surface markers in CML CD34+ cells, as these changes can serve as biomarkers and help distinguish between LSPCs and HSCs. ²Our analysis revealed that in CML CD34+ cells, the expressions of CD200R1, CD276, CD96, and CD36 ²²⁰were increased, while CD79A, CD37, and CD22 were decreased compared to mPB donor CD34+ cells (Figure 4.1.4).

Figure 4.1.3 Pathways dysregulated in significantly downregulated genes:

Enrichment analysis ²²¹was performed using Enrichr A) Reactome 2021 B) MsigDB Hallmark 2020 C) KEGG 2021 D) GO Biological process D) Go cellular components and F) GO molecular functions of significantly enriched pathways was manually curated based on the smallest p-values obtained across all the databases.

CD36 is a receptor for ⁵oxidized low-density lipoprotein (ox-LDL) in macrophages and a fatty acid transporter in adipocytes. ²Interestingly, CD36 ²²has been shown ²²³to be ²²⁴upregulated in CD34+CD38^{low} CML cells, and the CD36-expressing subset within this population demonstrates reduced responsiveness to imatinib but heightened vulnerability to antibody-based therapeutic targeting (Landberg et al., 2018).⁴⁷CD276 plays a role in ²²⁵the regulation of proliferation, apoptosis, invasion, cell cycle progression, cell differentiation, cell autophagy, and epithelial-mesenchymal transition across various cancers ⁴(Liu et al., 2021). CD96 functions as an immune checkpoint that governs the antitumor activity of CD8+ T-cells. ²Overcoming chemoresistance mediated by PD-1 blockade by enhancing CD8+ T-cell function in cervical cancer has been achieved through CD96 targeting⁴⁷(Feng et al., 2023). ²CD96 ²²⁶is also reported as an LSC marker in AML⁴⁷(Hosen et al., 2007). ²Further research is warranted to elucidate the precise

roles of these targets in normal hematopoiesis and determine their potential as therapeutic targets in CML.

CD79A, CD37, and CD22 are expressed on the surface of B-cells and play a crucial role in B-cell signaling and activation (Bobrowicz et al., 2020; Mason et al., 1995; Tedder et al., 2005)(Bobrowicz et al., 2020; Mason et al., 1995; Tedder et al., 2005)(Bobrowicz et al., 2020; Mason et al., 1995; Tedder et al., 2005). The downregulation of these markers in CML CD34+ cells suggests altered B-cell signaling and activation in the disease, leading to selective accumulation of myeloid cells and reduced lymphoid lineage. Even though previous studies have shown elevated expression of CD26 and CD25 in CML LSCs, we did not observe any difference(Herrmann et al., 2014; Sadovnik et al., 2017)(Herrmann et al., 2014; Sadovnik et al., 2017)(Herrmann et al., 2014; Sadovnik et al., 2017). One possible reason could be that transcriptomics was carried out using CML CD34+ (leukemic stem/progenitor cells) instead of CML CD34+CD38-, which is considered more primitive.

4.1.3 Aberrant expression of serine proteases in CML CD34+ cells:

Among the significantly upregulated genes in CML CD34+ cells, one of the top targets was serine proteases. Notably, genes-associated serine proteases such as Cathepsin G (*CTSG*), Cathepsin L (*CTSL*), neutrophil elastase (*ELANE*), and ribonucleases (*RNASE*) were significantly upregulated in CML CD34+ cells (Figure 4.1.5). In normal hematopoiesis, serine proteases are expressed from common myeloid progenitors (CMPs) to myelocytes/neutrophils. Serine proteases have been implicated in the development and progression of solid and hematological cancers. In addition, proteinase 3 (*P3*), a serine protease expressed by myeloid cells, has been shown to inhibit T-cell proliferation. Furthermore, neutrophil elastase (*ELANE*) and proteinase 3 (*PR3*), which are

serine proteases, are aberrantly expressed in human myeloid leukemias. Serine proteases may play a role in the CML LSCs survival.

4.1.4 Aberrant expression of Cathepsin G, calcium-binding protein, and cell adhesion:

The validation of targets acquired from the gene expression data can pose a substantial challenge, mainly due to the low proportion of CD34+ cells in both CML and mPB donors. However, we have successfully addressed this issue by utilizing NanoString technology, which could generate good-quality gene expression results even from 100ng of RNA. Based on Bloodspot (<https://servers.binf.ku.dk/bloodspot/>) and previous publications (Rothe K et al., 2013; Zhang et al., 2013; Bühner, E.D. et al., 2023), a 21-custom gene panel was designed. The expression of these 21 genes was evaluated in an independent cohort of CML-CD34+ (N=59) and mPB- CD34+ (N=9) samples. We observed significant upregulation of *CTSG* and *ELANE* (Figure 4.1.6A) and considerable downregulation of *CDH2*, *S100A8*, *S100A9*, and *RXRA* in CML CD34+ cells compared to the normal (healthy volunteer) CD34+ (Figure 4.1.6B). *CTSG* and *ELANE* are primary granules, and *S100A8/A9* are secondary granules released into the environment in response to infection. *CTSG* is a multifunctional protease that plays a role in the immune response and can regulate inflammatory responses by stimulating the production of cytokines and chemokines. *CTSG* is also involved in other processes, such as the transmigration of leukocytes, platelet activation, and conversion of angiotensin I to angiotensin II (Rawat et al., 2021). Avilés-Vázquez S et al. have shown high expression of *CTSG* and *ELANE* comparing CML CD34+CD38- vs. normal CD34+CD38-cells (Avilés-Vázquez S et al., 2017). Downregulation of *S100A9*, *S100A8*, and *CDH2* was also previously reported to be downregulated in CML CD34+ cells (Bühner et al., 2023; Zhang et al., 2013).

Figure 4.1.4: Altered Cell surface markers in CML CD34+ cells compared with mPB CD34+ cells. Y-axis is in log10 scale

Figure 4.1.5: Aberrant expression serine proteases in CML CD34+ cells. Y-axis is in log10 scale

Figure 4.1.6: Validation of differentially expressed gene in CML vs. normal CD34+ cells. A) Bar graph showing log fold change of selected target genes in CML CD34+ normalized with the mPB CD34+ A) Upregulated B) Downregulated. The target gene expression was normalized with the housekeeping gene (GUSB, GAPDH, and ACTB). Y-axis is in log10 scale

4.1.5 CTSG expression is higher in CML CD34+ cells than in mPB CD34+ cells.

CTSG, a serine protease, is synthesized and expressed during the promyelocyte stage of hematopoietic development and is stored in the azurophil (primary) granules. It plays a multifaceted role in normal hematopoiesis, encompassing cell differentiation, immune response, antimicrobial activity, and platelet activation (Zhang et al., 2013). However, the precise functions and mechanisms of CTSG in hematopoiesis remain unknown. Elevated levels of CTSG have been linked to certain inflammatory conditions, autoimmune diseases, and hematological malignancies (Alatrash et al., 2017; Gao et al., 2018).

In the acute myeloid leukemia (AML) context, CTSG has been identified as a novel immunotherapeutic target. CTSG levels have been assessed in AML patients at diagnosis and relapse and found to be a potential biomarker for disease progression (Alatrash et al., 2017). As CML CD34+ cells were found to have high expression of CTSG, we aimed to assess it in CML primary samples using q-RT-PCR. There was significant upregulation of CTSG in treatment-naïve CML patients compared to healthy donor granulocytes, as depicted in Figure

4.1.7A. However,² no significant difference in *CTSG* expression levels was²⁵¹ observed between CML patients who achieved major²⁵² molecular response (MMR) and those who did not (**Figure 4.1.7B**), suggesting that *CTSG* expression may not directly correlate with response to TKI therapy or disease prognosis in CML.

Further, the expression of *CTSG* was investigated in CML patients during blast crisis (BC) compared to those in the chronic phase (CP). *CTSG*² expression was markedly reduced in CML blast crisis patients (**Figure 4.1.7C**). This² result was further corroborated²⁵³ by an immunoblotting experiment showing increased *CTSG* protein expression in CML CD34+ cells compared to mPB CD34+ cells (**Figure 4.1.7D**).

Top of Form

Bottom of Form

4.1.6 Molecular inhibition of *CTSG* in the CML cell line reduced the colony-forming capacity *in vitro*

To evaluate the functional significance of *CTSG* in CML, we carried out *CTSG* knockdown (*CTSG* KD) in the CML cell line. The² experimental design is outlined²⁵ in **Figure 4.1.8A**. Our² initial screening of CML cell lines revealed that the EM-2 cell line to express highest²⁵⁵ level of *CTSG* protein expression (**Figure 4.1.8B**). Hence², we transduced EM-2 cells with doxycycline (dox)-inducible *CTSG* shRNAs (*CTSG* KD). After² five days of dox induction, *CTSG* KD cells showed a marked reduction in the *CTSG* protein expression in *CTSG* KD cells compared with the scramble, confirming knockdown (**Figure 4.1.8C**).

We first assessed the difference in the colony-forming capacity after *CTSG* KD *in-vitro*. EM2² *CTSG* KD cells exhibited a reduced capacity for colony formation *in-vitro*²⁵⁶, supporting the possible role of *CTSG* in the maintenance of CML stemness (**Figure 4.1.8D**).

Figure 4.1.7: CTSG expression is high in CML patients. ²**A)** qRT-PCR analysis of transcript levels of CTSG in **A)** Treatment naïve CP- CML (n = 12) vs. Healthy donor (HD) granulocytes (n = 5) **B)** CML patients who achieved MMR (n=6) vs. no MMR with TKI (n = 7) **C)** CML patients at chronic phase(CP)(n=11), accelerated phase (AP)(n=8) and Blast crisis (BC)(n=5) ⁵normalized with β -ACTIN (housekeeping gene) **D)** Western blot analysis of CTSG levels in CML CD34+(n=2) and normal CD34+(n=2). ² β -ACTIN ²⁵⁷was used as ²⁵⁸loading control.

4.1.7 CTSG KD reduces the leukemogenic potential *in vivo*

To investigate the impact of CTSG suppression on CML cell persistence and leukemogenic potential *in-vivo*, we transplanted EM2- CTSG KD cells, with and without doxycycline induction, into sublethally irradiated NSG mice (**Figure 4.1.9A**). ²One week after transplantation, doxycycline hydrate ²⁵⁹was added to the drinking water provided to the mice to induce CTSG knockdown ²⁶⁰*in-vivo*.

CTSG knockdown significantly increased the overall survival of the mice compared to the control group (**Figure 4.1.9B**), suggesting that the suppression of CTSG could potentially reduce CML cell survival and engraftment *in vivo*.

²These findings suggest ²⁶¹CTSG ²⁶²as a potential target for therapeutic intervention in CML. ²Strategies aimed at suppressing CTSG expression or activity could ²⁶³hold promise in reducing CML cell survival and disease burden.

²⁹⁹ Further investigations are warranted to elucidate the precise mechanisms by which CTSG influences leukemogenesis and to determine the specific downstream pathways or cellular processes affected by its suppression. ²Understanding the molecular and cellular consequences of CTSG knockdown in the context of CML will provide valuable insights for developing targeted therapies to disrupt the leukemogenic potential of CML cells.

Figure 4.1.8: CTSG knockdown reduces stemness in CML cell line: A)

Experimental outline of CTSG KD in EM-2 cell line; **B.** Western blot analysis of CTSG protein expression across CML cell lines **C)** CTSG protein expression before and after KD in EM2 cells. β -actin ² was used ²⁶⁴ as the loading control. **D)** ² Colony formation assay in EM-2 CTSG KD and scrambled.

Figure 4.1.9 CTSG knockdown increased survival in NSG mice: A) Work outline of

Cell line derived xenotransplant model. Dox-induced ² shCTSG KD cells were injected into NSG mice kept on dox or normal water and monitored survival. **B)** ² NSG mice injected with CTSG KD cells showed significantly improved survival compared scrambled. ²⁶⁵

1.	<i>EM-2, KU812, Lama84, KCL22, KYO, MEG01, JURL MK1, and K562 cell lines were procured</i>	Passive voice misuse	Clarity
2.	<i>. Cell; . The; . Purity; . Briefly; . To; . Ethanol; . Additionally; . High-quality; . Confirmation; . Read; . Total; . For; . Fluorescence; . The; . RNase; . PI; . CML; . NanoString; . miRNA; . qRT-PCR; . After; . PCR; . Positive; . These; . MSCs; . Flow; . 0.2X106; . They; . Single; . Cells;</i>	Text inconsistencies	Correctness
3.	<i>were collected</i>	Passive voice misuse	Clarity
4.	<i>were enriched</i>	Passive voice misuse	Clarity
5.	<i>analyzing; mobilized; normalized; analyzed; permeabilization; visualized; utilising; synthesized; deionized; characterization; trypsinization; Immortalised; immortalization; characterized; immortalisation; deparaffinization; normalizing; normalize; Characterisation; analyze; permeabilized; revoluti...</i>	Text inconsistencies	Correctness
6.	normal → average, regular, typical	Word choice	Engagement
7.	<i>was centrifuged</i>	Passive voice misuse	Clarity
8.	<i>, an equal</i>	Incorrect phrasing	Correctness
9.	<i>was added</i>	Incorrect verb forms	Correctness
10.	incubated → set	Word choice	Engagement
11.	<i>To the aqueous phase equal volume of isopropanol added and incubated for 10 minutes, centrifuged for 5 minutes at 12000rpm (4°C).</i>	Incomplete sentences	Correctness
12.	<i>a Total</i>	Determiner use (a/an/the/this,	Correctness

		etc.)	
13.	<i>is then washed</i>	Passive voice misuse	Clarity
14.	<i>is eluted</i>	Passive voice misuse	Clarity
15.	<i>was determined</i>	Passive voice misuse	Clarity
16.	<i>of 28S</i>	Wrong or missing prepositions	Correctness
17.	<i>the manufacturer's</i>	Determiner use (a/an/the/this, etc.)	Correctness
18.	<i>was converted</i>	Incorrect verb forms	Correctness
19.	<i>was evaluated</i>	Passive voice misuse	Clarity
20.	<i>was compared</i>	Passive voice misuse	Clarity
21.	splice-aware → <i>a aware</i>	Incorrect phrasing	Correctness
22.	aware → <i>splice-aware</i>	Confused words	Correctness
23.	<i>was counted</i>	Passive voice misuse	Clarity
24.	<i>was then examined</i>	Passive voice misuse	Clarity
25.	<i>CML cells were cultured</i>	Passive voice misuse	Clarity
26.	<i>Total cell counts and cell viability were assessed</i>	Passive voice misuse	Clarity
27.	<i>Total cell counts and cell viability were assessed with the trypan blue exclusion method.</i>	Unclear sentences	Clarity
28.	<i>were seeded</i>	Passive voice misuse	Clarity
29.	well → <i>healthy</i>	Word choice	Engagement
30.	<i>cell viability was evaluated</i>	Passive voice misuse	Clarity

31.	10 → ten	Improper formatting	Correctness
32.	EM-2/ KU812 cells (1×10 ⁶ cells/ml) were cultured	Passive voice misuse	Clarity
33.	analysis performed	Improper formatting	Correctness
34.	was performed	Incorrect verb forms	Correctness
35.	cells fixed → cells fixed	Improper formatting	Correctness
36.	were fixed	Incorrect verb forms	Correctness
37.	was added	Incorrect verb forms	Correctness
38.	was added	Passive voice misuse	Clarity
39.	added incubated	Improper formatting	Correctness
40.	incubated → set	Word choice	Engagement
41.	and incubated	Conjunction use	Correctness
42.	were analyzed	Passive voice misuse	Clarity
43.	and fixed → and fixed	Improper formatting	Correctness
44.	4 → four	Improper formatting	Correctness
45.	labeled → labelled	Mixed dialects of English	Correctness
46.	4 → four	Improper formatting	Correctness
47.	(Manian et al., 2018); (Jet et al., 2021); (Markose et al., 2009); (Bedel et al., 2013); (Addgene 12259); (Addgene 12260); (Rothe et al., 2020); (Zhang et al., 2019); (Bagchi et al., 2021); (Vinod et al., 2023); (Zhang et al., 2013); (Xin et al., 2015); (Addgene 27078); (Miyauchi et al., 2018); (Ku...	Citation style options	Correctness

48.	<i>CML primary cells were cultured</i>	Passive voice misuse	Clarity
49.	<i>were subjected</i>	Passive voice misuse	Clarity
50.	<i>was performed</i>	Passive voice misuse	Clarity
51.	nSolver → solver	Misspelled words	Correctness
52.	<i>was assessed</i>	Passive voice misuse	Clarity
53.	<i>was performed</i>	Passive voice misuse	Clarity
54.	<i>were screened</i>	Passive voice misuse	Clarity
55.	<i>was predicted</i>	Passive voice misuse	Clarity
56.	digital → Digital	Confused words	Correctness
57.	<i>was prepared</i>	Passive voice misuse	Clarity
58.	samples transferred	Improper formatting	Correctness
59.	were transferred	Incorrect verb forms	Correctness
60.	were per	Incorrect verb forms	Correctness
61.	generated per well	Misplaced words or phrases	Correctness
62.	<i>After sealing, the samples transferred to the QX200 Droplet Generator (Bio-Rad) and approximately 20,000 droplets per well generated.</i>	Unclear sentences	Clarity
63.	and	Wordy sentences	Clarity
64.	were counted	Incorrect verb forms	Correctness
65.	<i>Positive and negative droplets counted by employing QX200 Droplet Reader (Bio-Rad) and assessed by Poisson statistics utilising QuantaSoft software version</i>	Incomplete sentences	Delivery

1.7.4.0917 (Bio-Rad, München, Germany).

66.	are prepared	Incorrect verb forms	Correctness
67.	Protein lysates prepared by using radioimmunoprecipitation assay buffer supplemented with a protease inhibitor mixture and phenylmethylsulfonyl fluoride.	Incomplete sentences	Delivery
68.	was blocked	Incorrect verb forms	Correctness
69.	were detected	Incorrect verb forms	Correctness
70.	The expression of the shRNA was induced	Passive voice misuse	Clarity
71.	5 → five	Improper formatting	Correctness
72.	were performed	Passive voice misuse	Clarity
73.	were cloned	Passive voice misuse	Clarity
74.	were synthesized	Passive voice misuse	Clarity
75.	was transduced	Passive voice misuse	Clarity
76.	2 → two	Improper formatting	Correctness
77.	epinfection → infection	Misspelled words	Correctness
78.	was carried out	Passive voice misuse	Clarity
79.	was performed	Passive voice misuse	Clarity
80.	labeled → labelled	Mixed dialects of English	Correctness
81.	were performed	Passive voice misuse	Clarity
82.	were mixed	Passive voice misuse	Clarity

83.	<i>the number of colonies was scored</i>	Passive voice misuse	Clarity
84.	<i>were transduced</i>	Passive voice misuse	Clarity
85.	old	Misuse of modifiers	Correctness
86.	<i>the remaining adherent cells were cultured</i>	Passive voice misuse	Clarity
87.	were incubated	Incorrect verb forms	Correctness
88.	was analyzed	Incorrect verb forms	Correctness
89.	<i>were transduced</i>	Passive voice misuse	Clarity
90.	epinfection → infection	Misspelled words	Correctness
91.	<i>were cultured</i>	Passive voice misuse	Clarity
92.	<i>was supplemented</i>	Passive voice misuse	Clarity
93.	<i>The cells were maintained</i>	Passive voice misuse	Clarity
94.	, medium → ; medium, , and medium, . Medium	Punctuation in compound/complex sentences	Correctness
95.	the medium	Determiner use (a/an/the/this, etc.)	Correctness
96.	<i>was changed</i>	Passive voice misuse	Clarity
97.	outline → outlines	Faulty subject-verb agreement	Correctness
98.	<i>was evaluated</i>	Passive voice misuse	Clarity
99.	Cells seeded → Cells seeded	Improper formatting	Correctness
100.	well → healthy, sound, okay, OK	Word choice	Engagement
101.	differentiation,	Punctuation in compound/complex sentences	Correctness

102.	sub-confluence,	Comma misuse within clauses	Correctness
103.	were used	Passive voice misuse	Clarity
104.	were fixed	Incorrect verb forms	Correctness
105.	were visualized	Passive voice misuse	Clarity
106.	were fixed	Incorrect verb forms	Correctness
107.	away → away	Misspelled words	Correctness
108.	was eluted	Passive voice misuse	Clarity
109.	was quantified	Passive voice misuse	Clarity
110.	solution ,	Improper formatting	Correctness
111.	were used	Passive voice misuse	Clarity
112.	stain → colour, paint, dye, tint	Word choice	Engagement
113.	was used	Passive voice misuse	Clarity
114.	by acid → by acid	Improper formatting	Correctness
115.	alcohol ,	Improper formatting	Correctness
116.	sections → cells, areas, teams	Word choice	Engagement
117.	were subjected	Passive voice misuse	Clarity
118.	labeled → labelled	Mixed dialects of English	Correctness
119.	then	Wordy sentences	Clarity
120.	solution,	Punctuation in compound/complex sentences	Correctness
121.	sections → cells, areas	Word choice	Engagement
122.	was performed	Passive voice misuse	Clarity

123.	were first	Incorrect verb forms	Correctness
124.	sections → cells	Word choice	Engagement
125.	labeled → labelled	Mixed dialects of English	Correctness
126.	was carried out	Passive voice misuse	Clarity
127.	were seeded	Passive voice misuse	Clarity
128.	CML CD34+ cells were added	Passive voice misuse	Clarity
129.	The protective effect was evaluated	Passive voice misuse	Clarity
130.	Apoptosis analysis was performed	Passive voice misuse	Clarity
131.	by	Wordy sentences	Clarity
132.	were suspended	Passive voice misuse	Clarity
133.	buffer, → buffer.	Incorrect punctuation	Correctness
134.	anti-annexin → An annexin	Incorrect phrasing	Correctness
135.	was added	Incorrect verb forms	Correctness
136.	the dark	Determiner use (a/an/the/this, etc.)	Correctness
137.	The cells were suspended in 1X Annexin V binding buffer at a concentration of 1x10 ⁵ cells/100µl buffer, anti annexin V antibody added to the cells and incubated in dark for 15 min at room temperature (Zhang et al., 2013).	Unclear sentences	Clarity
138.	complete media was added	Passive voice misuse	Clarity
139.	The conditioned media (CM) containing secreted factors was collected	Passive voice misuse	Clarity

140.	prespotted → repotted	Misspelled words	Correctness
141.	<i>were detected</i>	Passive voice misuse	Clarity
142.	4 → four	Improper formatting	Correctness
143.	<i>were revealed</i>	Passive voice misuse	Clarity
144.	<i>were captured</i>	Passive voice misuse	Clarity
145.	Cytokine Antibody Array Data	Confused words	Correctness
146.	spot → area, site, place, location	Word choice	Engagement
147.	gray → grey	Mixed dialects of English	Correctness
148.	<i>the net density gray level was determined</i>	Passive voice misuse	Clarity
149.	gray → grey	Mixed dialects of English	Correctness
150.	gray → grey	Mixed dialects of English	Correctness
151.	<i>The density gray level of six positive controls was used</i>	Passive voice misuse	Clarity
152.	<i>was then averaged</i>	Passive voice misuse	Clarity
153.	a duplicated	Determiner use (a/an/the/this, etc.)	Correctness
154.	<i>The CD34+ cells were cultured</i>	Passive voice misuse	Clarity
155.	<i>was carried out</i>	Passive voice misuse	Clarity
156.	3 → three	Improper formatting	Correctness
157.	<i>were transferred</i>	Passive voice misuse	Clarity
158.	and were → . They were	Hard-to-read text	Clarity

159.	<i>were washed</i>	Passive voice misuse	Clarity
160.	expression → word, term, presentation, face	Word choice	Engagement
161.	staining performed	Improper formatting	Correctness
162.	was performed	Incorrect verb forms	Correctness
163.	<i>Alkaline Phosphatase staining performed using the Alkaline Phosphatase Detection Kit (Sigma-Aldrich) following the manufacturer's instructions(Bedel et al., 2013).</i>	Incomplete sentences	Delivery
164.	<i>was evaluated</i>	Passive voice misuse	Clarity
165.	<i>were fixed</i>	Passive voice misuse	Clarity
166.	4 → four	Improper formatting	Correctness
167.	labeled → labelled	Mixed dialects of English	Correctness
168.	<i>were dissociated</i>	Passive voice misuse	Clarity
169.	10 → ten	Improper formatting	Correctness
170.	<i>revitacell</i>	Unknown words	Correctness
171.	<i>pre-warmed STEMdiff™ Trilineage medium was added</i>	Passive voice misuse	Clarity
172.	<i>were incubated</i>	Passive voice misuse	Clarity
173.	<i>was changed</i>	Passive voice misuse	Clarity
174.	<i>was switched</i>	Passive voice misuse	Clarity
175.	<i>were plated</i>	Passive voice misuse	Clarity
176.	<i>The number of colonies in each experiment was scored</i>	Passive voice misuse	Clarity

177.	<i>Two-tailed Student's t-test for unpaired samples with unequal variance or 1-way ANOVA with Tukey's correction for multiple comparisons using GraphPad Prism version 8 (GraphPad Software).</i>	Incomplete sentences	Delivery
178.	<i>Survival curves of mice were compared</i>	Passive voice misuse	Clarity
179.	FITC → FILE, FIT	Misspelled words	Correctness
180.	leukemia → leukaemia	Mixed dialects of English	Correctness
181.	signaling → signalling	Mixed dialects of English	Correctness
182.	<i>are selectively expressed</i>	Passive voice misuse	Clarity
183.	<i>to eliminate LSCs and improve treatment outcomes in CML patients selectively</i>	Inappropriate colloquialisms	Delivery
184.	<i>are selectively expressed</i>	Passive voice misuse	Clarity
185.	<i>One way to address this would be to compare the transcriptomic profiles between healthy and CML stem cells and to identify specific gene signatures that are selectively expressed in CML stem cells.</i>	Unclear sentences	Clarity
186.	exists → exist	Faulty subject-verb agreement	Correctness
187.		Tone suggestions	Delivery
188.	<i>In the absence of a clear cell surface marker discriminating CML LSC from normal healthy HSCs, CD34+ cells are considered as LSPCs and are studied by many groups.</i>	Unclear sentences	Clarity
189.	as	Wrong or missing prepositions	Correctness
190.	<i>are studied</i>	Passive voice misuse	Clarity

191.	<i>been reported</i>	Passive voice misuse	Clarity
192.	antibiotic → antibiotic	Misspelled words	Correctness
193.	<i>was attributed</i>	Passive voice misuse	Clarity
194.	adhesion-related	Misspelled words	Correctness
195.	tumor → tumour	Mixed dialects of English	Correctness
196.	leukemia → leukaemia	Mixed dialects of English	Correctness
197.	study,	Comma misuse within clauses	Correctness
198.	the global	Determiner use (a/an/the/this, etc.)	Correctness
199.	to understand the mechanisms contributing to CML LSC survival comprehensively	Inappropriate colloquialisms	Delivery
200.	<i>Global transcriptome profiling of differentially expressed genes was evaluated</i>	Passive voice misuse	Clarity
201.	a clear → an apparent	Word choice	Engagement
202.	Both the → The	Wordy sentences	Clarity
203.	signaling → signalling	Mixed dialects of English	Correctness
204.	genes,	Comma misuse within clauses	Correctness
205.	<i>was done</i>	Passive voice misuse	Clarity
206.	anemia → anaemia	Mixed dialects of English	Correctness
207.	<i>Metabolism; metabolism</i>	Text inconsistencies	Correctness
208.	anemia → anaemia	Mixed dialects of English	Correctness
209.			

	<i>were upregulated</i>	Passive voice misuse	Clarity
210.	<i>were significantly upregulated</i>	Passive voice misuse	Clarity
211.	anemia → anaemia	Mixed dialects of English	Correctness
212.	<i>As reported earlier, dysregulation of the Fanconi anemia pathway was observed in CML CD34+ cells (Chakraborty et al., 2012), leading to chromosome instability.</i>	Unclear sentences	Clarity
213.	anemia → anaemia	Mixed dialects of English	Correctness
214.	<i>All these studies, including ours, suggest that mitochondrial metabolism can be a potential therapeutic target in CML.</i>	Unclear sentences	Clarity
215.	signaling → signalling	Mixed dialects of English	Correctness
216.	signaling → signalling	Mixed dialects of English	Correctness
217.	<i>be downregulated</i>	Passive voice misuse	Clarity
218.	signaling → signalling	Mixed dialects of English	Correctness
219.	<i>was performed</i>	Passive voice misuse	Clarity
220.	<i>were increased</i>	Passive voice misuse	Clarity
221.	<i>was performed</i>	Passive voice misuse	Clarity
222.	<i>been shown</i>	Passive voice misuse	Clarity
223.	has been shown to be → is	Wordy sentences	Clarity
224.	<i>be upregulated</i>	Passive voice misuse	Clarity
225.	the regulation of → regulating	Wordy sentences	Clarity

226.	isa-lee → is also	Misspelled words	Correctness
227.	signaling → signalling	Mixed dialects of English	Correctness
228.	signaling → signalling	Mixed dialects of English	Correctness
229.	<i>was carried out</i>	Passive voice misuse	Clarity
230.	was carried out using → used	Wordy sentences	Clarity
231.	<i>Among the significantly upregulated genes in CML CD34+ cells, one of the top targets was serine proteases.</i>	Unclear sentences	Clarity
232.	<i>In normal hematopoiesis, serine proteases are expressed from common myeloid progenitors (CMPs) to myelocytes/neutrophils.</i>	Unclear sentences	Clarity
233.	. Serine	Improper formatting	Correctness
234.	<i>been implicated</i>	Passive voice misuse	Clarity
235.	hematological → haematological	Mixed dialects of English	Correctness
236.	which are	Wordy sentences	Clarity
237.	leukemias → leukaemias	Mixed dialects of English	Correctness
238.	LSCs → LSC's, LSCs'	Incorrect noun number	Correctness
239.	<i>was designed</i>	Passive voice misuse	Clarity
240.	<i>was evaluated</i>	Passive voice misuse	Clarity
241.	normal → regular, average, standard, typical	Word choice	Engagement
242.	scale.	Closing punctuation	Correctness
243.	scale.	Closing punctuation	Correctness

244.	<i>was normalized</i>	Passive voice misuse	Clarity
245.	<i>The target gene expression was normalized with the housekeeping gene (GUSB, GAPDH, and ACTB).</i>	Unclear sentences	Clarity
246.	<i>been linked</i>	Passive voice misuse	Clarity
247.	hematological → haematological	Mixed dialects of English	Correctness
248.	leukemia → leukaemia	Mixed dialects of English	Correctness
249.	<i>been identified</i>	Passive voice misuse	Clarity
250.	<i>were found</i>	Passive voice misuse	Clarity
251.	<i>was observed</i>	Passive voice misuse	Clarity
252.	major → primary, significant	Word choice	Engagement
253.	<i>was further corroborated</i>	Passive voice misuse	Clarity
254.	<i>is outlined</i>	Passive voice misuse	Clarity
255.	the highest	Determiner use (a/an/the/this, etc.)	Correctness
256.	in-vitro → in vitro	Misspelled words	Correctness
257.	<i>was used</i>	Passive voice misuse	Clarity
258.	a loading, or the loading	Determiner use (a/an/the/this, etc.)	Correctness
259.	<i>was added</i>	Passive voice misuse	Clarity
260.	in-vivo → in vivo	Misspelled words	Correctness
261.	that CTSG	Conjunction use	Correctness
262.	as → is	Confused words	Correctness

263.		Tone suggestions	Delivery
264.	<i>was used</i>	Passive voice misuse	Clarity
265.	<i>to scrambled</i>	Wrong or missing prepositions	Correctness
266.	<i>Cell Cultures. Cell lines were maintained in RPMI supplemented with 10% Fetal Bovine Serum</i>	Numb has distinct function in lung adenocarcinoma and squamous cell carcinoma Oncotarget https://www.oncotarget.com/article/25585/text/	Originality
267.	<i>To the aqueous phase equal volume of isopropanol</i>	Cloning and structural analysis of a cDNA clone encoding glycinin (Gly-1) seed storage protein of peanut	Originality
268.	<i>to cDNA using a High-Capacity Reverse Transcription Kit</i>	Type I IFN signature in childhood-onset systemic lupus erythematosus: A conspiracy of DNA- and RNA-sensing receptors?	Originality
269.	<i>The filtered reads were aligned to the Human genome (hg19) using</i>	The non-coding RNA landscape of human hematopoiesis and leukemia Nature Communications https://www.nature.com/articles/s41467-017-00212-4?error=cookies_not_supported&code=cbad39cb-b54f-49f6-b136-d4e04ebffa8e	Originality
270.	<i>Total cell counts and cell viability were assessed</i>	Roflumilast combined with montelukast versus montelukast alone as add-on treatment in patients with moderate-to-severe asthma	Originality
271.	<i>The cells were incubated at 4°C for 30</i>	CYTOTOXIC AND ANTIPROLIFERATIVE STUDIES OF ISOCORDOIN AND SOME DERIVATIVES AGAINST PROSTATE CANCER CELL LINES INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH	Originality

		https://ijpsr.com/bft-article/cytotoxic-and-antiproliferative-studies-of-isocordoin-and-some-derivatives-against-prostate-cancer-cell-lines/?view=fulltext	
272.	<i>and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with</i>	The role of vimentin in the tumor marker Nup88-dependent multinucleated phenotype BMC Cancer Full Text https://bmccancer.biomedcentral.com/articles/10.1186/s12885-018-4454-y	Originality
273.	<i>containing 1% bovine serum albumin (BSA) and 5</i>	Role of MMPs and TIMP-3 in neuronal death and white matter injury following transient global ischemia.	Originality
274.	<i>Fetal bovine serum (FBS) in PBS for 20 min at</i>	Disease modelling with rabbit and human stem cells	Originality
275.	<i>16 hours) at 4°C, followed by incubation with</i>	Development of an in vitro model of acquired resistance to toceranib phosphate (Palladia®) in canine mast cell tumor	Originality
276.	<i>PCR was carried out at 95°C for 10</i>	Establishment and verification of potential biomarkers for cholangiocarcinoma https://www.spandidos-publications.com/10.3892/etm.2022.11483	Originality
277.	<i>Von Kossa stain: Sections were incubated with 1</i>	Bone scaffold architecture modulates the development of mineralized bone matrix by human embryonic stem cells	Originality
278.	<i>silver nitrate under UV light for 20 minutes.</i>	Investigation of the Inductive Influence of a Variety of Dental Extracellular Matrices on Periodontal Ligament Fibroblasts	Originality
279.	<i>The cells were suspended in 1X</i>	A Reduction in B, T, and Natural	Originality

	<i>Annexin V binding buffer</i>	Killer Cells Expressing CD38 by TAK-079 Inhibits the Induction and Progression of Collagen-Induced Arthritis in Cynomolgus Monkeys Journal of Pharmacology and Experimental Therapeutics https://jpet.aspetjournals.org/content/370/2/182	
280.	<i>added to the cells and incubated in dark for 15 min</i>	Activation of the Lectin Pathway of Complement in Pig-to-Human Xenotransplantation Models	Originality
281.	<i>Human Cytokine Antibody Array C Series 1000 (RayBiotech, Norcross, GA</i>	Adult Human Multipotent Neural Cells Could Be Distinguished from Other Cell Types by Proangiogenic Paracrine Effects via MCP-1 and GRO	Originality
282.	<i>Chemokines were detected by incubation with biotinylated detection</i>	A role for the chemokine receptor CCR6 in mammalian sperm motility and chemotaxis	Originality
283.	<i>addition of horseradish peroxidase-conjugated streptavidin and overnight incubation at 4°C, signals were revealed by</i>	A role for the chemokine receptor CCR6 in mammalian sperm motility and chemotaxis	Originality
284.	<i>and incubated with primary antibodies in the blocking buffer overnight</i>	Modulation of A375 human melanoma cell proliferation and apoptosis by nitric oxide	Originality
285.	<i>16 hours) at 4°C, followed by incubation with</i>	Development of an in vitro model of acquired resistance to toceranib phosphate (Palladia®) in canine mast cell tumor	Originality
286.	<i>cultured in mTeSR + medium (Stem Cell Technologies) supplemented with</i>	Fast but not furious: a streamlined selection method for genome-edited cells	Originality
287.	<i>in suspension were harvested and analyzed by flow cytometry for</i>	STEMdiff™ Hematopoietic Kit STEMCELL Technologies https://www.stemcell.com/products/product-types/cell-culture-	Originality

[media-and-supplements/stemdiff-hematopoietic-kit.html](#)

288.	<i>Differences between various treatment groups were analyzed by</i>	Comparative assessment of in vitro BBB tight junction integrity following exposure to cigarette smoke and e-cigarette vapor: a quantitative evaluation of the protective effects of metformin using small-molecular-weight paracellular markers Fluids and Barriers of the CNS Full Text https://fluidsbarrierscns.biomedcentral.com/articles/10.1186/s12987-021-00261-4	Originality
289.	<i>2-way ANOVA, with Tukey's correction for multiple comparisons,</i>	Roles of interleukin-11 during acute bacterial pneumonia	Originality
290.	<i>Imatinib has revolutionized the management of chronic myeloid</i>	<i>PTCH1</i> expression at diagnosis predicts imatinib failure in chronic myeloid leukaemia patients in chronic phase	Originality
291.	<i>often attributed to the presence of leukemic stem cells</i>	Pre-clinical evaluation of Minnelide as a therapy for acute myeloid leukemia Journal of Translational Medicine Full Text https://translational-medicine.biomedcentral.com/articles/10.1186/s12967-019-1901-8	Originality
292.	<i>Principal Component Analysis (PCA) showed a distinct separation</i>	Analysis of gut microbiota and the effect of lauric acid against necrotic enteritis in <i>Clostridium perfringens</i> and <i>Eimeria</i> side-by-side challenge model	Originality
293.	<i>Red dots indicate upregulated genes, and Blue dots</i>	A small RNA-guided PRC2 complex eliminates DNA as an extreme form of transposon silencing.	Originality
294.	<i>retroviral vectors showed impaired FANCD2 foci formation, whereas</i>	Bcr/Abl interferes with the Fanconi anemia/BRCA pathway:	Originality

	<i>FANCD2 monoubiquitination in these cells was unaffected.</i>	implications in the chromosomal instability of chronic myeloid leukemia cells. PLoS One 5(12): e15525	
295.	<i>the genes related to the mitochondrial respiratory complex</i>	Transcriptomic and proteomic studies on longevity induced by over-expression of HSP22 in drosophila melanogaster	Originality
296.	<i>Serine proteases have been implicated in the development and progression of solid and hematological cancers.</i>	Ectopic expression of human airway trypsin-like protease 4 in acute myeloid leukemia promotes cancer cell invasion and tumor growth	Originality
297.	<i>inflammatory responses by stimulating the production of cytokines and chemokines.</i>	Proteins associated with neutrophil degranulation are upregulated in nasopharyngeal swabs from SARS-CoV-2 patients	Originality
298.	<i>The target gene expression was normalized with the</i>	Novel stromal biomarker screening in pancreatic cancer patients using the in vitro cancer-stromal interaction model BMC Gastroenterology Full Text https://bmcgastroenterol.biomedcentral.com/articles/10.1186/s12876-020-01556-w	Originality
299.	<i>Further investigations are warranted to elucidate the precise</i>	A mechanism underlying the neurotoxicity induced by sodium fluoride and its reversal by epigallocatechin gallate in the rat hippocampus: involvement of NrF2/Keap-1 signaling pathway The Journal of Basic and Applied Zoology Full Text https://basicandappliedzoology.springeropen.com/articles/10.1186/s41936-018-0020-z	Originality

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by issac nelson

General metrics

69,587

characters

9,906

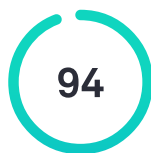
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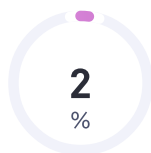
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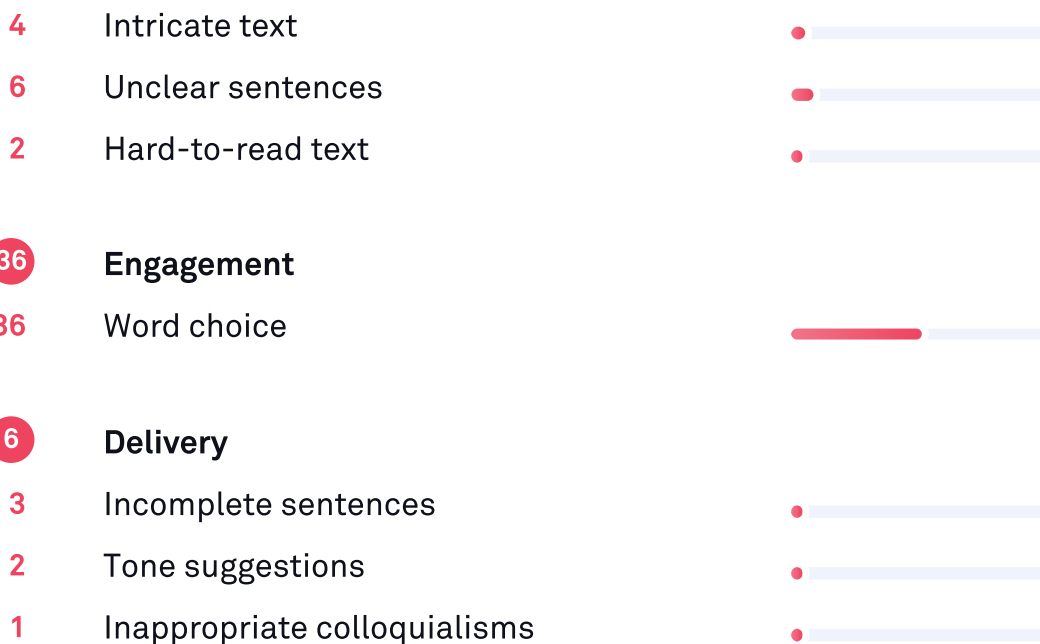
Correctness

30	Mixed dialects of english	
4	Text inconsistencies	
1	Citation style options	
13	Punctuation in compound/complex sentences	
14	Improper formatting	
35	Misspelled words	
2	Incorrect phrasing	
10	Determiner use (a/an/the/this, etc.)	
1	Misplaced words or phrases	
4	Unknown words	
1	Incomplete sentences	
2	Pronoun use	
4	Incorrect noun number	
4	Wrong or missing prepositions	
2	Incorrect verb forms	
1	Misuse of modifiers	
1	Comma misuse within clauses	
2	Incorrect punctuation	
2	Confused words	
1	Faulty tense sequence	
1	Closing punctuation	

118

Clarity

34	Wordy sentences	
72	Passive voice misuse	



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unique words

Rare Words

46%

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rare words

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Measures average word length

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Sentence Length

Measures average sentence length

19.7

words per sentence

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4.2 Role of hypoxia-associated miRNA in CML LSCs

Hypoxia, characterized by reduced oxygen levels, is the defining trait of the bone marrow microenvironment (BMM) where leukemia¹ stem cells (LSCs) reside. Hypoxia-inducible² factor 1 α (HIF-1 α) is a crucial transcription factor that becomes activated and stabilized³ under hypoxic conditions (Simsek et al., 2010).⁴ It² significantly regulates gene expression associated with various cellular processes, including cell proliferation, survival, and stemness(Licausi et al., 2011).⁴

HIFs and hypoxia have diverse tumor-promoting⁵ effects in different types of leukemia⁶ (CML, CLL, ALL, and AML)(Forristal et al., 2015; Gao et al., 2015; Zou et al., 2013).⁴ In the context of CML, leukemic cells cultured under low oxygen conditions demonstrate resistance to IM, heightened clonogenic ability, and greater⁷ engraftment potential(Ng et al., 2014; Singh et al., 2022). Hypoxia² has been shown⁸ to influence the expression of microRNAs (miRNAs) that regulate gene expression post-transcriptionally(Khan et al., 2021; Licausi et al., 2011).⁴ Several² studies have explored the involvement of miRNAs in the maintenance of CML LSCs.² For² instance, miRNAs have been shown⁹ to mediate the abnormal metabolic characteristics observed in CML stem/progenitor cells(Lin et al., 2020; Salati et al., 2017; Wang et al., 2015).⁴ Notably,² miR-300, a tumor suppressor miRNA, can induce quiescence in CML LSCs¹⁰ while miR-126-3p regulates quiescence, self-renewal, and engraftment capacity of CML LSCs (Ruiz et al., 2021).⁴ Low² oxygen tension induces¹¹ a distinct shift in a specific group of miRNAs, termed HypoxamiRs. Emerging² evidence indicates that

hypoxia regulates several facets of HypoxamiR transcription, maturation, and function, influencing miRNA expression and supporting tumor growth (Licausi et al., 2011). Although the role of hypoxia-regulated miRNAs is well-

documented in solid tumors, its role in CML LSCs survival remains unexplored.

The current study aimed to determine the role of dysregulated miRNAs under hypoxic conditions, with the hypothesis that hypoxia-associated miRNAs contribute to the survival and maintenance of CML LSCs.

4.2.1 Establishment of hypoxic culture condition in CML cell line

We cultured CML cell lines (EM-2 and KU812) and primary CML cells under low-oxygen conditions (1% O₂) to replicate a hypoxic microenvironment. Previous studies have shown that cells cultured under hypoxic conditions exhibit reduced proliferation, enter a slow-cycling state, and show induction of HIF-1α expression and its downstream targets. These cellular responses to hypoxia are part of the adaptive mechanisms that allow cells to survive and function under low oxygen conditions (Hubbi and Semenza, 2015). Both CML cell lines under hypoxia showed reduced cell proliferation after 72 hours compared to cells cultured under normal oxygen conditions (21% O₂) (Figure 4.2.1A). Cell cycle analysis revealed an accumulation of cells in the G₀ phase, indicating a slow-cycling or quiescent state similar to stem cells (Figure 4.2.1B). We also observed HIF-1α induction in both the CML cell lines cultured under hypoxic conditions, as evidenced by HIF-1α translocation from cytoplasm to nucleus (Figure 4.2.1C). The expression of HIF-1α downstream target *GLUT1*, responsible for glucose uptake, was upregulated in response to hypoxia (Figure 4.2.1D). Similar to the cell lines, CML primary cells maintained under low oxygen conditions (1% O₂) for 48h also showed induction of HIF-1α protein expression (Figure 4.2.1E) and increased GLUT1 expression (Figure 4.2.1F).

4.2.2 miRNA Profiling of CML Primary Samples

To identify differentially expressed miRNA in CML cells under hypoxia, we performed miRNA profiling using the NanoString platform comprising 827 miRNAs previously reported in solid tumors¹⁶(Armstrong et al., 2015)⁴. Briefly,² RNA extracted from CML primary cells cultured in normoxic and hypoxic conditions¹⁷ were tested¹⁸ for *GLUT1* expression, and those samples with 2 fold¹⁹ increase in *GLUT1* under hypoxic conditions²⁰ were taken²¹ for miRNA profiling. NanoString nCounter technology is a platform that²² can be used²³ for miRNA profiling at cellular levels. The²⁴ technology utilizes molecular barcodes to directly quantify individual RNA molecules^{25,26}, without²⁷ the use of²⁸ reverse transcription or amplification. This²⁹ makes it easier and faster to validate miRNA biomarkers³⁰ as compared to³¹ RNASeq or PCR-based platforms, and the results are highly reproducible. The³² nCounter miRNA Expression Panels³³ are designed³⁴ to provide a sensitive, reliable, and robust platform that works well with multiple sample types. We³⁵ performed miRNA profiling for CML primary samples showed³⁶ high *GLUT1* expression under hypoxic conditions³⁷ as³⁸ described in **Figure 4.2.2A**. The³⁹ data was analyzed using nsolver, and differentially expressed microRNAs⁴⁰ were identified⁴¹ using $P < .05$ and absolute logFC > 2 ⁴² as the cut-off, and *ACTB*, *B2M*, *GAPDH*, *RPL19*, and *RPLP0*⁴³ were used⁴⁴ as reference genes. Principal⁴⁵ component analysis showed⁴⁶ overlapping⁴⁷ signature between normoxia and hypoxia (**Figure 4.2.2B**). This⁴⁸ is probably due to the heterogeneity of the primary CML cells. A⁴⁹ total of 20 miRNAs were significantly dysregulated, including⁵⁰ 5 upregulated and 15 down-regulated in CML cells cultured in hypoxic conditions (**Figure 4.2.2C**). Among⁵¹ the top differentially expressed miRNA, miR-210, known as hypoxia-regulated miRNA, was upregulated considerably under hypoxia (Log2FC-6.19).

Figure 4.2.1: Establishment of

Hypoxic culture conditions in CML cell lines and primary CML cells. CML cell²
lines cultured in normoxia and hypoxia were assessed³⁷ for **A)** Cell proliferation of
EM-2 cells by staining with CFSE, **B)** Cell cycle analysis, **C)** Immunofluorescence
of HIF-1 α , and **D)** qPCR for GLUT1 expression. E)² Immunofluorescence of HIF-1 α
and **F)** qPCR for GLUT1 after culturing CML primary cells under normoxic and
hypoxic conditions for 48h normalized with housekeeping gene GAPDH.

Figure 4.2.2: miRNA Profiling of CML primary cells. A)² Experimental outline **B)**
Principal component analysis **C)** Heatmap showing the differentially expressed
miR in CML primary cells cultured under hypoxic conditions.

4.2.3 Validation³⁸ of differentially regulated miRNAs in CML and healthy donor samples

On further validation³⁸ by RT-qPCR, miR-30b-5p was found to be significantly³⁹
upregulated⁴¹, and miR-362-3p downregulated in CML primary samples cultured
under hypoxic conditions (**Figure 4.2.3A-B**). These² miRNAs have been
extensively evaluated⁴² in solid tumors⁴³ such as breast & hepatocellular cancer;
however, the role of these miRNAs in mediating CML LSCs survival and
maintenance is not clear(Cao et al., 2021; Wu et al., 2020; Xiao et al., 2022).⁴ To²
assess whether this upregulated miRNA was CML cell-specific, we evaluated
the expression of miR 30b-5p and miR 362-5p in healthy donor PBMNCs
maintained under hypoxia vs. normoxia We observed that miR 30b-5p
expression was unaltered or downregulated in healthy donor PBMNCs (**Figure**
4.2.3D-E), suggesting the possible role of miR-30b-5p in maintaining CML cells
under low oxygen tension. Previous² studies reported that miR-30b-5p

functions as a ⁴⁴tumor regulator via target genes. ²For instance, miR-30b-5p regulates cell proliferation by directly targeting MTDH and HOXA1 in glioma and ⁴⁵esophageal cancer (Li et al., 2017; D Zhang et al., 2018). ⁴Also, miR-30b-5p influences cell invasion and migration by modulating CTHRC1 in non-small cell lung cancer (Chen et al., 2014).⁴

Figure 4.2.3: Validation of differentially expressed miRNAs. qPCR results of
A)miR-30b-5p B) miR-363-5p C)miR-660-5p in CML primary cells.²D) miR-30b-5p and E) miR-363-5p in healthy donors cultured in normoxic and hypoxic conditions for 48h.²Relative expression was evaluated⁴⁶by normalizing with U6 (housekeeping gene) expression.

4.2.4 CRISPR-Cas9 Knockout of miR-30b-5p reduces proliferation and colony-forming capacity of K562 cell line in vitro.

4.2.4.1.Screening for miR-30b-5p levels in CML cell lines subjected to hypoxic conditions to identify suitable cell lines for knockout experiment

To understand the functional significance of miR-30b-5p in CML cell lines under hypoxic conditions, we initially screened for miR-30b 5p expression in several CML cell lines EM-2, KU812, K562, KYO1, JURL-MK1, LAMA84, KCL22, MOLM6, and MEG01 cultured under normoxic and hypoxic conditions. ²After 72h, RNA samples ⁴⁷were extracted, cDNA was synthesized and tested for GLUT1 expression. ²Increased GLUT1 expression was observed in MEG01, KYO1, MOLM6, KCL22, K562, and EM-2 cell lines ⁴⁸confirming the induction of hypoxia. ²Next, miR-30b-5p expression ⁴⁹was evaluated in these cell lines. ²Among the CML cell lines tested, K562 showed enhanced miR-30b-5p expression in hypoxic conditions compared to the other CML cell lines (Figure 4.2.4A). ²Hence, we chose the K562 cell line as a model to evaluate the effect of miR-30b-5p KO (Figure 4.2.4B).

4.2.4.2. Generation of K562 cell line expressing Cas9 protein:

We employed CRISPR Cas9 dual guide RNA mediated knockout of miRNA as it results in efficient generation of short deletions within miRNA genomic regions and complete loss of gene function, allowing for precise targeting of specific genes or gene regions and resulting in more specific and controlled gene manipulation.

To generate Cas9 expressing K562 cell line, cells were first transduced with lenti-Cas9. After five days of transduction, cells were subjected to blasticidin selection for two weeks. The cas9-expressing K562 cell line was transduced with lentivirus containing PLKV2.2-BFP cloned with dual guide RNA targeting miR-30b-5p. After 48h, transduction efficiency was found to be 90%, as assessed by the percentage of BFP-expressing cells. BFPBright cells were flow sorted, and then the knockout of miR-30b-5p was confirmed at the genomic level by amplifying the target genomic locus using specific primers flanking the guide RNA binding regions. PCR results showed a ~60 bp reduction in the product size compared to the wild type (320bp vs. 378bp), confirming the deletion (Figure 4.2.4C). To further validate the effect of miR-30b-5p knockout at the mRNA level, the expression levels of miR-30b-5p were quantified using qPCR in Scramble vs. miR-30b-5p KO. The results demonstrated a significant reduction of 70% in miR-30b-5p levels in K562 KO cells compared to K562 scramble cells, as depicted in Figure 4.2.4D.

To assess the functional impact of miR-30b KO, K562 scramble and miR-30b KO cells were cultured in normoxia and hypoxia for 96 hours, and cell proliferation and colony-forming capacity were compared (Figure 4.2.4E-F). K562 miR-30b KO cells cultured under hypoxic conditions showed reduced proliferation compared to those cultured under normoxia (2x10⁵ cells vs. 9x10⁵ cells, P<0.0078) at 96h. miR-30b KO altered the proliferation of K562 cells in

hypoxia, confirming its specific role in hypoxic conditions ⁵⁷altering cell proliferation. ²Further, the colony-forming assay revealed a significant reduction in the colony-forming capacity of K562 miR-30b KO cells maintained in hypoxia vs. normoxia (123 vs. 92 colonies, $P < 0.0022$), suggesting the role of miR-30b-5p in maintaining the stemness of CML cells in low oxygen tension.

Figure 4.2.4: CRISPR-Cas9 dual guide RNA knockout of miR-30b in K562 cell line: **A)** Real-time PCR showing increased miR-30b expression in K562 cells cultured under hypoxic condition **B)** Experimental outline CRISPR-Cas9 dual-guide RNA mediated knockout of miR-30b-5p **C)** GeneScan analysis showing indel confirming KO **D)** qPCR for miR-30b expression in K562 scramble and miR30b KO cells. ²**E)** Cell proliferation by Trypan blue dye exclusion **F)** Colony-forming capacity of K562 scramble and miR-30b-5p KO cells.

4.2.4.3.Target gene prediction of miR-30b-5p

miRNA functions by negatively regulating the mRNA expression at the post-transcriptional level through base-pairing with complementary sequences of the ⁵⁸3'untranslated region (UTR). ²To identify the potential target genes of miR-30b, we used target prediction tools such as miRWalk, TargetScan, miRDB, and miRTarBASE. ²A total of ⁵⁹seventy genes ⁶⁰were predicted to be the target for miR30b, including *PPARGC1B*, *MTDH*, *ELOVL15*, and *EED*. ²These genes were associated with mitochondrial biogenesis, promoting ⁶¹tumor progression, biosynthesis of very long-chain fatty acids, and epigenetic modification. ²While these genes have ⁶²been studied in various contexts, there is currently no clear evidence to suggest that they play a significant role in the pathogenesis or progression of CML. ²Further research is needed to determine whether these genes are involved in the development or treatment of CML. Gene Ontology

analysis revealed genes associated with “positive regulation of T-regulatory cell differentiation” and “regulation of transcription by RNA polymerase II” were dysregulated (Figure 4.2.5A-B). Pathway analysis showed that genes associated with post-transcriptional silencing by small RNAs and TGF-beta Receptor Complex might be a target of miR-30b (Figure 4.2.5C-D). TGF- β (transforming growth factor-beta) signaling plays a complex role in tumor progression, including in hematologic malignancies such as CML. Specifically, TGF- β is known to maintain CML-initiating cells through the Akt-FoxO pathway (Naka et al., 2010b). Further validation of these targets is needed to understand the role of TGF-beta signaling and mir-30b in maintaining CML cells under hypoxic conditions. One of the significant limitations of this study is the use of primary CML PBMNCs for studying miRNA, as there is inherent heterogeneity in the data due to patient-specific variability. Previous studies in CML on miRNA regulating CML stem cells using cell lines have provided repeatable and comparable results. While Cell lines are cost-effective and provide repeatable and similar results, primary samples are more representative of the clinical features of the disease but are more variable and less reproducible.

Figure 4.2.5: Gene ontology and pathway enrichment analysis of predicted target genes of miR-30b-5p. A) GO biological processes B) Interactive network view of significantly enriched target genes. C) Reactome 2021 D) Interactive network view.

We investigated the role of hypoxia-related miRNAs in the survival and maintenance of CML cells. Notably, miR-30b-5p exhibited significant upregulation specifically under hypoxic conditions in CML cells. Previous studies have identified miR-30b as a prognostic marker and reported to influence the proliferation and migration of solid tumors. Additionally, the knockout of miR-30b led to reduced proliferation and colony-forming capacity of CML cells cultured in hypoxic condition. Further, Target prediction showed that dyregulated miRNA was associated with TGF- β signaling, which is already reported to be associated CML progression. One of the major limitations is the use of primary CML patient samples due to their inherent variability. Despite these limitations, this research illuminates the role of hypoxia-related miRNAs, particularly miR-30b-5p, in the survival of CML cells, thereby offering insights into potential avenues for therapeutic intervention.

4.3 Generation and characterization of immortalized CML MSC line and assessing its role in the maintenance of CML LSCs

Despite the impressive efficacy of TKIs in inducing remission and controlling the disease, a subset of leukemic cells known as Leukemia Stem Cells (LSCs) has emerged as a ⁷⁹major obstacle to achieving treatment-free remission (TFR) (Corbin et al., 2011b; Hamilton et al., 2006; Holyoake et al., 1999; Perl and ⁴Carroll, 2011).⁸⁰ The bone marrow mesenchymal stromal cells (BMMSCs) play a crucial role in leukemogenesis and the protection of leukemic stem cells (LSC) from TKIs (Guarnerio et al., 2018; Zhang et al., 2012).⁴ Recent² evidence suggests that the persistence of LSCs might be attributed⁸¹ to the sheltered environment provided by the BM niche. ²The BM hematopoietic microenvironment is replete with growth factors and cytokines that potentially offer survival signals to the residual CML cells (Hou et al., 2020; Nair et al., 2010; Shahrabi et al., 2014).⁴ ²Williams et al. have reported that cytokines present in the bone marrow microenvironment (BMM) can stimulate leukemic cell growth and contribute to imatinib resistance in the mouse model of BCR/ABL, Arf-null lymphoblastic leukemia⁸² (Williams et al., 2007).⁴ Zhang² et al. demonstrated that MSCs derived from imatinib-resistant CML patients exhibited protective effects against cell death induced by IM or NI in K562 and BV173 cells compared to MSCs from IM responders. ²This protection was attributed to elevated IL-7 secretion from the MSCs (Zhang et al., 2019).⁴

Furthermore, MSCs derived from CML patients displayed an enhanced capacity to support normal and CML BM CD34+CD38- cells (Dolinska et al., 2023). Despite these findings, there is limited knowledge on how the MSCs are altered due to their interaction with CML LSCs; further research is needed. It is challenging to conduct extensive research to study the alterations in CML patient-derived BMMSCs. ***Primary MSCs possess several drawbacks, including cell senescence and the loss of multipotent differentiation capacity. Immortalizing primary MSCs offers several potential benefits and applications to overcome these limitations.*** Immortalized MSCs enable the generation of a consistent and abundant cell supply, provide a source for long-acting therapeutic cells, avoid cell senescence, and maintain differentiation capacity. In the present study, we characterize MSCs derived from CML patients at diagnosis, post-treatment, and from normal healthy volunteer bone marrow to assess alterations in their trilineage differentiation potential. Since primary CML MSCs have finite growth, and HS-5 (healthy donor-derived MSCs) cannot replicate the CML microenvironment, we generated and characterized immortalized MSCs from a CML patient (iCML MSCs), comparing them with primary CML MSCs. The iCML MSCs can be valuable for investigating the mechanisms underlying MSC-mediated protection in CML.

4.3.1. Comparison of BM MSCs from CML patients vs. Normal BM

To understand the changes incurred by the stromal cells during CML, mesenchymal stem cells were isolated from the bone marrow of CML-CP patients at diagnosis, post-treatment, and normal bone marrow cells. The CML and NBM-derived MSCs were spindle-shaped and reached confluency approximately in 2-3 weeks (Figure 4.3.1A). When the immunophenotype profile between NBM and CML patients was compared, all were positive for stromal

markers CD73 and CD90 and negative for hemopoietic lineage CD45 and CD34 (Figure 4.3.1B).

303 MSCs are multipotent progenitors that can differentiate into osteocytes, adipocytes, and chondrocytes (Somoza et al., 2014). The MSCs' ability to differentiate into various lineages is crucial in maintaining the structure and composition of the BM niche (Woods and Guezguez, 2021). To assess any alteration in the differentiation capacity of MSCs derived from CML patients at diagnosis, post-treatment, and NBM, we differentiated the MSCs into adipocytes, osteocytes, and chondrocytes by supplementing specific differentiation media. Both CML and NBM-derived MSCs could be differentiated into all three lineages.

Figure 4.3.1: Propagation and characterization of CML and healthy donor-derived MSCs. A) Schematic representation of the experimental outline of CML MSC propagation B) Immunophenotypic characteristics of CML vs. Healthy MSCs. MSCs were positive for CD73 and CD90 and negative for hematopoietic lineage markers CD45 and CD34.

Although there was no significant difference in the adipogenic (Figure 4.3.2A-B) or osteogenic differentiation potential (Figure 4.3.2C) between CML MSCs vs. NBM MSCs, chondrogenic differentiation potential was enhanced in CML MSCs compared to NBM MSCs (Figure 4.3.2D). There was no difference in the trilineage differentiation potential between the MSCs derived from treatment naïve and post-treatment BM. Previous studies have reported that leukemic-stromal interaction alters the trilineage differentiation potential of MSC in leukemic patients (Dolinska et al., 2023; Zhang et al., 2019). The altered chondrogenic differentiation potential of MSC derived from CML patients might have been caused by leukemic cell-stromal cell interaction. These changes may

collectively contribute to the functional alterations of CML MSCs, including their hematopoiesis-supportive function and differentiation (Zhang et al., 2012). Further studies are needed to investigate the role of altered chondrogenic differentiation in CML MSCs and its role in the LSCs maintenance.

Figure 4.3.2: Trilineage differentiation potential of CML and Normal BM MSC:

Representative image of **A)** adipogenic differentiation (after oil Red O stain) **B)** Quantification of in vitro adipogenic differentiation potential of NBM and CML MSC **C)** Osteogenic differentiation (by Alizarin Red stain and immunohistochemistry of collagen X) **D)** Chondrogenic differentiation potential (by Toluidine blue, Alcian Blue, Picosirus red, Safranin O and Collagen II protein expression).

4.3.2. Generation of immortalized CML MSCs (iCML MSC):

The healthy donor-derived MSC line (HS-5) has been extensively used as a model to investigate the interplay between the leukemic and stromal cells. However, it has been reported that HS-5 cells lack the ability to differentiate into chondrocytes (Roeklein and Torok-Storb, 1995). CML MSCs showed enhanced chondrogenic differentiation potential, and primary CML MSCs have a limited lifespan and tend to undergo senescence, losing their functional abilities after a few passages (typically 2-3). To address these limitations, we developed an immortalized CML MSC line and show that the iCML MSC line can be cultured up to 25 passages, allowing for long-term investigations and screening of molecules that can target CML LSCs and their interactions with the BMM.

For immortalizing MSCs, primary CML MSCs were transduced with HEE lentivirus carrying Human Kusabira Orange (huKO+) fluorescent protein, and

the medium was supplemented with doxycycline (dox) to induce the expression of HEE virus (**Figure 4.3.3A**). After² four days of dox induction, huKO+bright cells were single-cell-sorted and then characterized further. After² isolating 62 single cells expressing the huKO+ fluorescent protein, we carefully examined their characteristics (**Figure 4.3.3B**). Among² these clones, we chose one that displayed spindle-shaped morphology and positive expression of CD73 and CD90, recognized as MSC markers (**Figure 4.3.3D**). Karyotyping² analysis to assess further the genetic stability of the immortalized CML MSC clone showed no additional chromosomal abnormalities after the immortalization process (**Figure 4.3.3C**).

Immortalized CML MSCs (iCML MSCs) retained the capacity for trilineage differentiation, closely resembling the differentiation potential observed in primary CML MSCs (**Figure 4.3.4A-C**); however, the HS-5 could not be⁹⁵ differentiated⁹⁵ into chondrocytes (**Figure 4.3.4C**). Further², the expression of *PPARG*, *RUNX2*, and *ACAN* genes was evaluated⁹⁶ as markers after adipogenic, osteogenic, and chondrogenic differentiation, respectively. Compared to⁹⁷ undifferentiated cells⁹⁷, we observed increased *PPARG* and *RUNX2* expression in adipo and osteo-differentiated⁹⁸ cells (**Figure 4.3.5A-B**). As² expected, *ACAN* expression was higher in CML MSCs than HS-5, confirming the enhanced chondrogenic differentiation potential observed in CML MSC (**Figure 4.3.5C**). The² iCML MSCs retained the chondrogenic differentiation potential as the primary CML MSCs, suggesting⁹⁹ that the iCML MSC clone can be an appropriate model for studying the interaction between BMM and CML cells.

4.3.3 iCML MSCs reduce imatinib-induced apoptosis of primary chronic myeloid leukemia CD34+ cells.

We then studied the functional role of iCML MSCs in regulating the growth of CML CD34+ cells and their protective effect against TKI. MSCs² interact with the

leukemia¹⁰¹ cells through their secreted factor/direct cell contact to regulate¹⁰² the growth¹⁰³ of leukemic cells (Nair et al., 2010)⁴ and chemoprotect the leukemic cells from therapeutic insult (Zhang et al., 2019).⁴ To confirm iCML 'MSCs'⁵⁸ role in mediating the protection of CML LSCs, we cocultured CML CD34+ cells with iCML MSC and then treated them with IM as described in **Figure 4.3.6A**.² CML CD34+ cells cocultured with iCML MSCs showed a significant^{104,105} enhanced cell viability compared to the CML-CD34+ cultured alone (132% vs 100%, p=0.0199).² CML-CD34+ cells cocultured with iCML MSC and maintained in hypoxia showed a significant increase in the percentage of viable cells compared to the control (137% vs. 100%, p=0.0252).² We also observed that CML-CD34+ cells cultured with MSCs and then treated with imatinib (IM 20nM) showed reduced cell death in comparison to the control (85% vs 53%, p<0.0001) (**Figure 4.3.6B**).² These results suggest that iCML MSC retained its functional property and protected the CML CD34+ cells from IM treatment, similar to primary CML MSCs.

We then tested if the secretory factors released from the iCML MSC line support the growth of leukemic cells. Conditioning media¹⁰⁶ was collected, and the leukemic cells were cultured¹⁰⁷ with and without the conditioning medium. The proliferation and percentage of CD34+ cells were measured¹⁰⁸ sequentially. The growth of leukemic cells increased gradually by 15 times when cultured in the presence of conditioning media (**Figure 4.3.6C-D**).² Also, the percentage of CD34+CD38- cells gradually increased from 16% to 56%.² In contrast, the leukemic cells cultured in basal media showed a subtle increase in proliferation but did not show an increase in CD34+ percentage (**Figure 4.3.6C-D**).² These results suggest that the secretory factor released from the iCML MSC may selectively support the growth and proliferation of CD34+ leukemic cells.

Figure 4.3.3: Generation and characterization of immortalized CML MSCs. **A)** Experimental outline **B)** Representative image of MSC transduced with HEE lentivirus expressing huKO+ after dox induction **C)** Karyotypic analysis of primary CML MSC before and after immortalizing **D)** Immunophenotypic characterization of immortalized CML MSCs expressing MSCs marker and negative for hematopoietic lineage markers.

Figure 4.3.4: iCML MSC retained adipogenic differentiation potential

Representative image of **A)** Oil Red O stain and absorbance at OD540 showing adipogenic differentiation **B)** osteogenic differentiation by Alizarin Red stain, Von ¹⁰⁹kossa stain and immunohistochemistry of collagen-X **C)** Chondrogenic differentiation by toluidine blue, Alcian blue, Picosirus red, and Collagen II in p-CML, iCML MSCs, and HS-5 after adipogenic differentiation. ^{2,110}pCML – Primary CML MSCs, iCML MSC – immortalized CML MSCs, and HS- 5 – Healthy donor-derived immortalized MSCs.

Figure 4.3.5: iCML MSC retained the osteogenic differentiation potential

Quantitative RT-PCR results showing **A)** PPARG, **B)** RUNX2, and **C)** ACAN expression in p-CML, iCML MSCs, and HS-5 after adipogenic, osteogenic and chondrogenic differentiation respectively. ^{2,111}pCML – Primary CML MSCs, iCML MSC – immortalized CML MSCs, and HS- 5 – Healthy donor-derived immortalized MSCs.

4.3.4. ²Coculture of CML CD34+ with iCML MSCs showed dysregulation of genes associated with Calcium metabolism (S100A8, S100A9)

Our RNA seq data revealed that genes associated with chemotaxis, cell migration, and adhesion ¹¹²are differentially expressed in CML CD34+ compared with healthy donor CD34+. ²To identify the genes related to bone marrow microenvironment-mediated protection in CML LSC, we cocultured CML CD34+ cells with iCML MSC ¹¹³and tested the expression of differentially expressed genes using NanoString technology.

Figure 4.3.6: iCML MSC protected the CML CD34+ cells from TKI treatment and selectively increased the growth of CD34+ cells in ex vivo. ²**A)** Experimental outline of iCML MSC coculture with CML CD34+ **B)** Violin plot showing the percentage of live cells with and without IM treatment **C)** Growth rate of leukemic cells cultured with and without conditioning medium **D)** Representative flow plots showing the increase in the percentage of CD34+ cells cultured with conditioning medium. ²**CM:** Conditioning medium

As reported earlier, we observed upregulation of CDH2 in CML CD34+ cocultured with CML MSCs (Zhang et al., 2012). ⁴Furthermore, ²genes associated with calcium metabolism ¹¹⁴were also increased in CML CD34+ cells cocultured with iCML MSCs (**Figure 4.3.7A-C**). ²Investigating the functional role of these genes may provide valuable insights into identifying novel therapeutic targets for eradicating CML CD34+ cells.

Figure 4.3.7: Genes associated with calcium metabolism upregulated in CML CD34+ cocultured with iCML MSCs. **A)** Heatmap showing the 21 gene panel differentially expressed in CML-CD34+ cells **B)** Experimental outline of coculture system **C)** Significant downregulation of S100A8, S100A9 in CML-CD34+ cocultured with MSCs.

4.3.5. ²Cytokine profiling of iCML MSCs

MSCs secrete various cytokines, growth factors, and chemokines, contributing to immune modulation, tissue remodeling, and cellular homeostasis during regeneration. Since the conditioned media isolated from CML MSC lines have been shown to protect and selectively improve the growth of leukemic cells, we conducted cytokine profiling to assess the cytokines released by these MSCs that support the growth of leukemic cells. Cytokine profiling revealed that 13 cytokines were abundantly secreted by the CML MSC lines. As previously reported, IL-6 was abundant in the CML MSCs lines. Additionally, IGFBP6, known to support the growth of CML cells, as reported earlier, was also found to be secreted by CML MSCs (Cambria et al., 2023). In addition, cytokines associated with cell adhesion, such as TIMP1 and TIMP-2, were also seen (Ries et al., 1999). The cytokines abundantly secreted by CML MSCs were found to be significantly involved in immune regulation, supporting the hypothesis that CML MSCs create an immune modulatory microenvironment that promotes growth and provides protection against TKIs.

Figure 4.3.8 Cytokine profiling in iCML MSC. A-E) Representative pictures of the cytokine antibody array F) Relative expression of secreted factors detected using the cytokine array.

Like normal hematopoietic progenitors, leukemia cells physically interact with the BM niche and deliver signals capable of re-programming MSCs and endothelial cells (ECs), consequently altering the hematopoietic niche (Zehentmeier and Pereira, 2019). The present study shows that CML MSCs have morphology and immunophenotypes similar to NBM MSCs. As reported earlier, CML MSCs at diagnosis and post-treatment showed no trilineage differentiation potential (Estrada-González et al., 2014) but exhibited

enhanced chondrogenic differentiation compared to NBM MSCs. This² altered differentiation potential might be caused¹²⁴ by leukemic-stromal interaction. In² normal hematopoiesis, chondrocytes play an essential role in the secretion of inflammatory cytokines, and the increased chondrogenic differentiation potential in CML MSCs might replicate an inflammatory microenvironment in CML patients (Nievergall et al., 2016).⁴

The healthy donor-derived MSC line (HS-5) is extensively utilized¹²⁵ as a model for exploring the interaction between leukemia¹²⁶ and stromal cells. It² has a limitation of the reported lack of chondrogenic differentiation ability. Due to² the increased chondrogenic differentiation potential observed in CML MSCs,
the use of HS-5 might not be optimal for investigating the BMM-mediated
protection in CML leukemic stem cells (LSCs)(Roecklein and Torok-Storb, 1995)⁴
. Meanwhile,¹²⁷² Primary CML MSCs, despite their relevance, suffer from a limited lifespan and a tendency to undergo senescence after a few passages (typically 2-3), leading to a loss of functional abilities. We² developed an immortalized CML MSC line to overcome this challenge. Immortalization² enables indefinite culturing, facilitating long-term investigations and hence the ability to be used¹²⁸ for screening molecules capable of targeting CML LSCs and their interactions with the bone marrow microenvironment. iCML² MSCs showed no change in chromosomal aberration compared to the primary CML MSCs.

Further, trilineage differentiation revealed that it had retained the trilineage differentiation potential as the primary CML MSCs. Functional² analysis revealed that the iCML MSCs retained¹²⁹ the capacity for trilineage differentiation, closely resembling the differentiation potential observed in primary CML MSCs. This² characteristic makes the iCML MSC clone an appropriate model for studying the interaction between bone marrow microenvironment (BMM) and CML cells. iCML² MSCs protected CML CD34+

cells from IM treatment, confirming that iCML MSCs support the CML CD34+ cells from chemotherapy. ²The ²secretary factor released from the iCML MSC may selectively support the growth and proliferation of CD34+ cells. ²Genes ¹³⁰associated with calcium metabolism ¹³⁰were increased in CML CD34+ cells cocultured with iCML MSCs. Investigating the functional role of these genes may provide valuable insights into identifying novel therapeutic targets for eradicating CML CD34+ cells. ²In ¹³¹solid ¹³¹tumors such as Gliomas, immortalized MSCs and their plasma membranes can be used as natural drug carriers for targeting gliomas and providing a safe, adequate, quality-controlled, and continuous source for ¹³²the treatment of gliomas ⁴(Zhang et al., 2022). ²MSCs from elderly patients are immortalized by transducing them with SV40LT and hTERT. ²These ¹³³iMSCs have a high osteogenic potential and are used in *in vitro* models to study bone tissue engineering ⁴(Piñeiro-Ramil et al., 2021). ²Human Adipose Tissue Mesenchymal Stem/Stromal Cells 1 (HATMSC1) cell line phenotype ¹³⁴was found to be consistent, and the cytokine production appeared to be higher compared to the primary MSCs ⁴(Paprocka et al., 2021). ²iCML MSC showed enhanced chondrogenic potential because of clonal selection, and retaining the trilineage differentiation potential may be beneficial in studying leukemia-stromal cell interaction.

Further cytokine profiling of CML MSCs revealed that cytokines associated with immune response and inflammation are enhanced compared with HS-5, supporting the finding that CML MSCs might provide an inflammatory ³⁰⁷microenvironment to support the growth of CML CD34+. ²Further studies are warranted to confirm the results.

In summary, the immortalized CML line cell maintains the main characteristics observed in primary CML MSCs, thus making this a valuable model for studying

leukemic-stromal interaction and functional studies of candidate genes involved in mediating TKI resistance in CML LSCs.

4.4 Generation and characterization of iPSCs from primary CML cells for modeling CML LSCs.

The persistence of leukemic stem cells (LSCs) remains a primary factor contributing to disease recurrence in CML (Corbin et al., 2011b). Owing to the rarity of LSCs, obtaining a sufficient number of cells for high throughput screening to understand TKI resistance mechanisms and screening drugs to eliminate them poses a challenge (Corbin et al., 2011b; Holyoake et al., 1999).

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Induced pluripotent stem cells (iPSCs) derived from patients with genetic diseases or cells with genetic alterations can be banked and differentiated into large quantities of disease-relevant cells, making them perfect for studying disease development and drug screening (Takahashi et al., 2007b).² In the context of CML, iPSCs generated from patients help researchers understand how oncogenes and patient-specific chromosomal abnormalities influence leukemia-like¹³⁶ phenotypes (Papapetrou, 2019b).² CML-iPSCs² derived from the same patient at different stages of the disease and from patients with distinct responses to TKI therapy offer valuable resources for investigating the molecular and epigenetic mechanisms underlying CML progression and drug resistance.² This² approach aids in identifying novel therapeutic targets and potential drug candidates for CML treatment (Corbin et al., 2011b; Miyauchi et al., 2018).⁴ Additionally,² iPSCs can be effectively genetically manipulated using gene editing methods to study specific genes involved in disease pathogenesis (Imeri et al., 2023).⁴

Earlier studies have successfully generated CML-iPSCs from peripheral blood mononuclear cells and CD34+ hematopoietic stem and progenitor cells isolated from CML patients (Hosoi et al., 2014; Hu et al., 2011; Kumano et al., 2012; Miyauchi et al., 2018; Telliam et al., 2016).⁴ These² CML-iPSCs exhibit resistance to IM, similar to CML-LSCs, making them cellular models¹³⁷ for investigating the mechanisms responsible for IM resistance (Bedel et al., 2013; Charaf et al., 2017; Kumano et al., 2012).⁴ Moreover,² hematopoietic progenitors derived from CML-iPSCs demonstrated some of the pathophysiologic characteristics of the disease (Bedel et al., 2013; Hosoi et al., 2014; Hu et al., 2011; Kumano et al., 2012; Miyauchi et al., 2018).⁴ However,² it remains unclear how BCR::ABL1 fusion protein expression and TKI treatment affect the efficiency and quality of iPSC generation from CML cells.

This study presents the successful generation of iPSCs from CD34+ cells of CML patients with varying responses to TKI therapy. The purpose was to model the disease and explore the impact of BCR::ABL1 oncoprotein expression on the maintenance of CML-iPSCs. Unlike previous studies, we discovered that the expression and activity of BCR::ABL1 influences the survival and maintenance of CML-CD34-iPSCs. It was crucial to suppress the TKI activity of the BCR::ABL1 fusion protein through TKI supplementation to ensure the viability of CML-iPSCs. The CML-iPSCs produced using our protocol could be further differentiated into BCR::ABL1-positive hematopoietic progenitors. These patient-specific CML-iPSCs and their differentiated hematopoietic cells hold great potential for many applications, including high throughput CRISPR-Cas9 screening, which can help identify novel pathways involved in CML pathogenesis and drug resistance.

4.4.1 Generation of iPSCs from normal and CML CD34+ cells:

CD34+ cells from two CML patients are known to constitute the leukemia stem/progenitor cells associated with disease recurrence. Our goal was to reprogram these cells from the patients who achieved complete cytogenetic response and one who achieved suboptimal response after TKI therapy,. The patients' demographics are shown in the Table 4.4.1. In addition, we reprogrammed CD34+ cells from a healthy volunteer as a control.

Table 4.4.1: Patient demographics.

CD34+ cells were purified from the patients and the healthy donor and cultured in a CD34+ expansion medium. The percentage of CD34+ cells remained consistent while the number of cells increased by 15 to 20-fold (Mean: 20.2 ± 3.34) (**Figure 4.4.1A**). Actively proliferating cells from day 3 of the culture were nucleofected with episomal plasmids (Miyauchi et al., 2018) (Miyauchi et al., 2018). After 2 days of nucleofection, the cells were transferred to Matrigel

coated¹⁴⁶ plate. Subsequently², the half-culture media was replaced¹⁴⁷ with reprogramming¹²³ media on alternating days. Between² day 14 and day 18, iPSC colonies with typical morphology were generated from normal CD34+ cells (as shown in **Figure 4.4.1B**), and two clones¹⁴⁸ were picked¹⁴⁹ between day 20 and day 24 (**Figure 4.4.1B**). Normal^{2,150} iPSC clones retained typical iPSC morphology and expressed high¹⁵¹ levels of pluripotency markers without differentiation for 12 passages (**Figures 4.4.1C and 1D**). In² contrast, CML-CD34+ cells from TKI-responsive patients formed several⁵⁸ "dome-shaped"⁵⁸ colonies surrounded by adherent cells formed by the spontaneous differentiation of iPSCs (**Figures 4.4.1B and C**). Eleven^{2, 58} "dome-shaped"⁵⁸ colonies were isolated from 3 patients, and all these clones expressed TRA-1-60 in the initial passages (**Figure 4.4.1D**). Such² dome-shaped colonies were described¹⁵² (Bedel et al., 2013; Kumano et al., 2012)⁴, however¹⁵³, such a morphology of CML-iPSCs has not been described by other studies. After² 3-4 passages, the⁵⁸ "dome-shaped"⁵⁸ colonies peeled off from the bottom of the plates and floated in the medium, leaving the differentiated cells attached to the plate.

The⁵⁸ "dome-shaped"⁵⁸ CML-CD34-iPSC colonies expressed the same types of *BCR::ABL1* fusion transcripts (e13a2 and e14a2) present in the peripheral blood mononuclear cells used for reprogramming¹²³ (**Figure 4.4.2A and B**). Karyotyping² confirmed¹⁵⁴ that all iPSC clones had t(9;22) presence (**Figure 4.4.2C**). Furthermore², there were no mutations¹⁵⁵ detected in the *BCR::ABL1* kinase domain among any of the clones.

4.4.2 IM supplementation aids in the maintenance, stability, and pluripotency of CML iPSCs

Previous studies have shown that TKI treatment enhances pluripotency marker expression in *BCR::ABL1*+ iPSC clones (Charaf et al., 2017)⁴. However², it was unclear¹⁵⁷ in these studies whether TKI supplementation enhances¹⁵⁶ the stability of

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CML-CD34-iPSCs. ²We hypothesized that the atypical morphology and high rate of spontaneous differentiation of CML-CD34-iPSC colonies were due to the constitutive expression of the BCR::ABL1 fusion protein and its tyrosine kinase (TK) activity. ²To address this, we supplemented the medium with 10μM IM to inhibit the TK activity of the BCR::ABL1 fusion protein. ²Within two days of IM supplementation, we observed that several attached colonies with typical iPSC morphology derived from the dome-shaped colonies (**Figure 4.4.3A**) and there was an increase in the TRA-1-60+ cell population (**Figures 4.4.3B and C**), with all clones showing positive pluripotency marker expression (**Figure 4.4.3D**). ²These clones could also differentiate into three germ ¹⁵⁸layers, ectoderm, endoderm, and mesoderm (**Figure 4.4.4**). ²However, when IM ¹⁵⁹was withdrawn, the IM-supplemented CML-CD34-iPSC colonies reverted to their original ⁵⁸"dome-shaped" ⁵⁸morphology within 2-4 days (**Figure 4.4.5A**), with a decrease in cell viability (**Figure 4.4.5B**) and TRA-1-60 expression (**Figure 4.4.5C**). ²Conversely, when control iPSCs ¹⁶⁰were treated with IM, there was a reduction in the colony number, indicating that IM supplementation ¹⁶¹specifically enhances ¹⁶²the maintenance of CML-CD34-iPSCs (**Figure 4.4.6**).

Figure 4.4.1: ¹²³Reprogramming of Normal and CML CD34+ cells. ²(A) Experimental outline for ¹²³reprogramming of CML-CD34+ and normal-CD34+ cells. ²The purified CD34+ cells were expanded in culture, nucleofected with episomal plasmids, transferred to Matrigel-coated plates containing the ¹²³reprogramming medium, and isolated colonies and passaged. ²HEM: Hematopoietic expansion media comprising SFEM II ¹⁶³containing CD34+ expansion supplement and UM729. (B) Representative images of the emerging iPSC colonies generated from CML-CD34+ and normal-CD34+ cells. ²CML-CD34-iPSCs ⁵⁸had "dome-shaped" ⁵⁸colonies ¹⁶⁴and normal-CD34-iPSCs had typical flat morphology. (C) CML-CD34-iPSC and

normal-CD34-iPSC colonies. Red arrows indicate the non-iPSC cells spontaneously differentiated from CML-CD34-iPSCs. Scale bar: 50µM. (D) TRA-1-60 immunofluorescence of normal-CD34-iPSC and "dome-shaped" CML-CD34-iPSC colonies. Scale bar: 250µM. The green color indicates TRA-1-60 Yellow arrows indicate TRA-1-60+ "dome-shaped" CML-CD34-iPSC colonies and red arrows indicate differentiated cells.

Figure 4.4.3: **Characterization of CML-CD34-iPSC clones.** (A) Agarose gel image of BCR::ABL1 transcripts in CML-CD34-iPSC clones. (B) Quantification of BCR::ABL1 transcripts present in the CML-CD34-iPSC clones from 4 patients (normalized to ABL1 expression) by ddPCR. Red bars indicate the BCR::ABL1 transcripts % in the peripheral blood samples collected at presentation, and the black bars indicate those in the CML-CD34-iPSC clones (cultured with 10µM IM supplementation). (C) Karyotyping analysis of iPSC lines showing t(9;22) (q34;q11) translocation in the CML-CD34-iPSC lines except one clone showing normal karyotype. An iPSC line generated from normal-CD34+ cells showing normal karyotype is also shown. #Ph+G13, G17 represents CML-CD34-iPSC clones derived from CML patients, TKI- Tyrosine kinase inhibitor, SOP- Suboptimal response, R-Responder, IM-Imatinib Mesylate, Ph- represent clone showing normal karyotype.

The CRK-like proto-oncogene (CRKL) is a downstream target of BCR::ABL1 and can be phosphorylated by the fusion protein (Hamilton et al., 2006; Nichols et al., 1994). In order to determine the status of BCR::ABL1 kinase activity, the expression of p-CRKL was analyzed in CML-CD34-iPSCs before and after IM treatment (Figure 4.4.5D). The results showed that the expression of p-CRKL decreased in CML-CD34-iPSCs after IM treatment. This suggests that the

suppression of BCR::ABL1 mediated tyrosine phosphorylation resulted in the stable maintenance of CML-CD34-iPSCs.

Figure 4.4.3 : IM aids in the maintenance of CML-CD34-iPSCs. (A)

Representative images of the "dome-shaped" CML-CD34-iPSC colonies exhibit significant differentiation generated in the absence of IM and with typical iPSC morphology after IM supplementation for 24 hours. Scale bar: 20µM. (B) Flow cytometry analysis of TRA-1-60 expression in CML-CD34-iPSCs cultured in the presence and absence of IM. CML-CD34-iPSCs cultured in the presence of IM showed a significantly reduced level of differentiation compared to the colonies maintained in the absence of IM. (C) Transformation of "dome-shaped" colonies to typical iPSCs colonies after IM treatment. "Dome-shaped" colonies transform to TRA-1-60+ iPSC colonies with flat morphology. Red arrows: differentiated cells, yellow arrows: "dome-shaped" TRA-1-60+ colonies, and blue arrows: typical iPSC colonies formed beneath the "dome-shaped" colonies. Scale bar: 250µM. (D) Representative immunofluorescence of pluripotency markers in CML-CD34-iPSC and normal-CD34-iPSC clones stained with anti-SSEA-4, anti-NANOG, anti-TRA-1-60, anti-OCT4A, anti-TRA-1-81 and anti-SOX2 antibodies. Scale bar: 100µM

Figure 4.4.4 : Trilineage differentiation of CML-iPSC lines (G13, G15, G17 and G18) and a normal iPSC line. The cells differentiated to ectoderm, endoderm and mesoderm, exhibited the expression of Brachyury, SOX17 and PAX6, as measured by immunofluorescence analysis. DAPI was used for staining the nuclei. Scale bar: 250µM.

4.4.3 The effect of IM in reprogramming of CML-CD34+ cells

We assessed the impact of IM supplementation on the reprogramming of CML-CD34+ cells, as we observed that the reduction of BCR::ABL1 kinase activity

improves the stability and pluripotency of CML-iPSCs. CML CD34+ cells from two IM-responsive patients were nucleofected and cultured in the reprogramming medium supplemented with 10µM IM (**Figure 4.4.7A**). Reprogramming of CML-CD34+ cells without IM supplementation resulted in the formation of "dome-shaped" colonies. After IM supplementation, these colonies transformed into typical iPSC morphology, expressing the TRA-1-60 pluripotent marker (**Figure 4.4.7B**). However, IM supplementation throughout reprogramming it failed to yield iPSC colonies with either the typical morphology or those with "dome-shaped" morphology. Our findings indicate that while IM facilitates the stability of CML-iPSCs by inhibiting BCR::ABL1 kinase activity, it hinders the reprogramming of CML-CD34+ cells. Overall, our results demonstrate that IM supplementation transforms the "dome-shaped" colonies formed during the reprogramming of CML-CD34+ cells into iPSCs with flat morphology. Additionally, IM improves the pluripotency of CML-CD34-iPSCs by inhibiting BCR::ABL1-mediated TK activity.

4.4.4. Reprogramming of CD34+ cells from an IM refractory CML patient

The HPCs (Hemopoietic progenitor cells) derived from the CML iPSCs generated from therapy-resistant patients offer a useful tool to explore the underlying mechanisms of resistance. Therefore, CD34+ cells obtained from IM-refractory patients were subjected to reprogramming, with and without IM supplementation (**Figure 4.4.7A**). In contrast, we

Figure 4.4.5: Withdrawal of IM affects cell proliferation, morphology, and pluripotency of CML-CD34-iPSCs. (A) The morphology and flow cytometry analysis of CML-CD34-iPSCs after IM withdrawal for 48 and 96 hours. The IM-supplemented CML-CD34-iPSCs formed "dome-shaped" colonies after IM withdrawal resulting in reduced TRA-1-60 expression. **(B)** Number of CML-CD34-

iPSCs after 48h of IM withdrawal. CML-CD34-iPSCs showed reduced cell viability after IM withdrawal (C) Immunofluorescence analysis showing TRA-1-60 expression in the CML-CD34-iPSC clones cultured with (IM+) and without IM (IM-). (D) Immunoblot analysis of phospho-CRKL expression in CML-iPSC clones cultured with and without IM. Relative Phospho-CRKL protein expression was estimated using Image J software.

Figure 4.4.6: Alkaline phosphatase staining of a healthy donor CD34-iPSC line treated with different concentrations of IM showing a reduction in the colony number after IM treatment.

Figure 4.4.7: Supplementation of IM did not improve the reprogramming of CML-CD34-iPSCs. (A) Experimental outline to assess the effect of IM in reprogramming CML-CD34-iPSCs. (B) Immunofluorescence showing TRA-1-60 expression in CML-CD34-iPSCs after IM supplementation during reprogramming. (C) Alkaline Phosphatase staining of CML iPSC generated from an IM-refractory CML patient's CD34+ in the absence (a) and presence of IM (b). Scale bar: 50µM

observed iPSC colonies emerged earlier in IM-refractory patient's compared to the responder (~3 weeks Vs. ~4 weeks). However, IM refractory patient-generated more colonies with continuous IM supplementation than without IM (Figure 4.4.7C). We isolated a total of 9 colonies, with 8 of them displaying a distinct "dome-shaped" appearance (as shown in Figure 4.4.1C), while the remaining colonies exhibited the typical iPSC morphology (Figure 4.4.8A). Upon

conducting karyotyping analysis on the "dome-shaped" colonies derived from the IM refractory patient, we observed the presence of t(9;22). Conversely, the clone with the typical iPSC morphology showed a normal karyotype (Figure 4.4.8A and 4.4.8B). These findings strongly indicate that the "dome-shaped" morphology observed in CML-CD34-iPSCs results from BCR::ABL1 expression in these cells.

Figure 4.4.8: *Reprogramming of IM refractory CML-CD34+ cells (A)*

Representative images of one Ph-CML-CD34-iPSC colony with typical morphology generated from an IM refractory patient. (B) Karyotyping analysis of IM refractory CML-CD34-iPSC clone with typical iPSC morphology showing normal karyotype. IM- Imatinib mesylate, G18- CML patient sample id. Scale bar: 50µM.

4.4.5. Second and third generation TKIs also aid in the maintenance of CML iPSCs.

We investigated the effects of second generation TKIs, DA and NIL, and third generation TKI ponatinib on the maintenance of CML-CD34-induced pluripotent stem cells (CML-CD34-iPSCs). We replaced IM with different concentrations of DA, NIL, and ponatinib and observed their effects on CML-CD34-iPSCs. However, when used at lower concentrations, these TKIs formed "dome-shaped" colonies (Figure 4.4.9). Our findings collectively indicate that suppressing BCR::ABL1 TK activity enhances the pluripotency of CML-iPSCs.

Figure 4.4.9: *Effect of Second and third generation TKI in maintaining CML-CD34-iPSCs. Images of iPSCs after treating the IM-supplemented CML-CD34-iPSCs with different concentrations of Dasatinib (0.25, 0.5, 1 and 2nM), Nilotinib (5, 10, 20 and 40nM) and Ponatinib (0.5, 0.75, 1 and 2 nM). Scale bar: 50µM*

4.4.6. Hematopoietic differentiation of CML iPSCs

Previous research has shown that CML-iPSCs can be successfully transformed²⁰⁹ into hematopoietic progenitor cells (HPCs),²⁰⁸ despite the presence of BCR::ABL1 oncoprotein and its TK activity (Bedel et al., 2013; Kumano et al., 2012; Miyauchi et al., 2018).⁴ However,² the efficiency of hematopoietic differentiation of CML-iPSCs has been reported to be lower than that of normal iPSCs (Hu et al., 2011; Miyauchi et al., 2018).⁴ Additionally,² the HPCs obtained from CML-iPSCs have only partially regained TKI sensitivity (Hosoi et al., 2014)⁴ (Hosoi et al., 2014).⁴ In light of our findings that IM supplementation

Figure 4.4.10: Effect of IM on hematopoietic differentiation of single cell expanded CML-CD34-iPSCs. (A) Experimental outline of hematopoietic differentiation from single-cell sorted CML-CD34-iPSCs. **(B)** Representative work outline of different experiment groups (E1 to E5) with IM supplementation during different stages of hematopoietic differentiation.²⁰⁹ **(C)** Representative flow cytometry results showing the percentage of CD34+ and CD45+ generated under different conditions of hematopoietic differentiation.²¹⁰ **(D)** Total cells and CD34+CD45+ cells generated during hematopoietic differentiation.²¹¹ is necessary to maintain CML-CD34-iPSCs, we studied whether IM supplementation is also necessary²¹² for the differentiation of CML-iPSCs into HPCs.²¹³

To answer our question, we carried out single cell sorting²¹⁴ to obtain iPSC colonies that had an equal number of cells (Figure 4.4.10A).²¹⁵ These colonies were subjected to hematopoietic differentiation and then IM was supplemented during different stages (Figure 4.4.10B).²¹⁶ We differentiated CML-CD34-iPSCs using two different concentrations of IM (1μM and 2μM) as well as without any IM supplementation.²¹⁷ We were able to obtain suspension cells consisting of HPCs both with and without IM. Interestingly,²¹⁸ CML-CD34-iPSCs

differentiated without IM before and after mesoderm induction (E1) showed a 5-fold increase in the yield of CD34+CD45+ HPCs and the total number of suspension cells compared to those differentiated in the presence of IM throughout the entire differentiation (E3 and E5) (**Figure 4.4.10D**). Flow² cytometry analysis of hematopoietic markers revealed that approximately 41% of the suspension cells were CD34+CD45+ HPCs (**Figure 4.4.10C**). By² utilizing single-cell sorting of iPSCs, we generated a sufficient number of HPCs for a drug screening experiment.

Based on the previous results, iPSCs were seeded as small aggregates and then subjected to differentiation with and without IM supplementation after mesoderm induction (**Figure 4.4.11A**). Flow² cytometry analysis showed that around 50% of the suspension cells were CD34+CD45+ HSPCs (**Figure 4.4.11B**). We² found that CML-CD34-iPSCs that were differentiated²²³ without IM resulted in around²²⁴ 30 times more HPCs than those with IM supplementation. The² decline in HPC differentiation with IM can be attributed²²⁵ to the sensitivity of CML-iPSC-HPCs to IM (**Figure 4.4.11C**). Hematopoietic² colony formation revealed that the HPCs generated without IM had more myeloid progenitors compared to²²⁶ those from a normal²²⁷ iPSC clone (**Figure 4.4.11D**). We² validated these findings by differentiating CML-CD34-iPSCs from two more patients (**Figure 4.4.11E**), which also resulted²²⁸ in more CD34+CD45+ cells without IM (**Figure 4.4.11F,G**).²²⁹ Consistently,² differentiation without IM resulted in more HPCs compared to²³⁰ the other experimental groups.

Figure 4.4.11: IM affects the HSPCs differentiation of CML-CD34-iPSCs. (A) Schematic representation of hematopoietic differentiation of CML-iPSCs to analyze the effect of IM after mesoderm differentiation **(B)** Representative flow cytometry results of the percentages of CD34+CD45+ cells generated from

healthy donor and CML-CD34-iPSCs. (D) Hematopoietic colony forming capacity of HSPCs derived from CML-CD34-iPSCs and normal CD34-iPSCs. (E) Experimental outline ²³¹HSPC differentiation with and without IM at different stages of differentiation. (F) ²Representative flow cytometry results of the percentages of CD34+CD45+ cells generated from healthy ²³²donor and CML-CD34-iPSCs generated from two ²³³different patients. (G) ²Bar graphs showing the relative cell numbers and CD34+CD45+ cells generated by CML-iPSC colonies (G17 and G18) generated from two ²³⁴different patients during differentiation under different conditions. ²IM^{-/-} represents CML-CD34-iPSCs was subjected to HSPC differentiation without IM; IM^{+/-} represents IM ²³⁵was withdrawn after mesoderm induction. ²IM^{+/+} represents IM ²³⁶was supplemented throughout HSPCs differentiation.

A crucial goal in treating CML is to eliminate CML-LSCs, as doing so can help prevent the disease recurrence, prolong survival, enhance the quality of life, and enable curative treatments(Corbin et al., 2011b; Etienne et al., 2017; Mahon and Etienne, 2014). ⁴However, ²due to the scarcity of these cells, studying the molecular mechanisms contributing to their survival has been challenging. ²A potential solution to this obstacle is generating iPSCs from CML CD34+ cells. ²These iPSCs can be expanded and differentiated into hematopoietic cells, providing a continuous and abundant supply for in-depth proteome, epigenome, transcriptome, and drug screening studies.

Previous studies have successfully generated CML iPSCs from PBMNCs(Hu et al., 2011)⁴and CD34+ cells (Bedel et al., 2013; Kumano et al., 2012; Miyauchi et al., 2018; Telliam et al., 2016)⁴ using various vectors such as lentiviral (Bedel et al., 2013),⁴ retroviral (Hosoi et al., 2014; Kumano et al., 2012),⁴ Sendai (Telliam et al., 2016)⁴ or episomal vectors (Hu et al., 2011; Miyauchi et al., 2018).⁴ However, ²

these studies ²³⁷ did not address the atypical morphology and low ¹² reprogramming efficiency observed in CML iPSCs. ² Additionally, the impact of BCR::ABL1 expression on the morphology and stabilization of CML-iPSC colonies was not investigated (Imeri et al., 2023).⁴

In our observations, the ¹²³ reprogramming efficiency of CML CD34+ cells was approximately 50 times lower than that of normal CD34+ cells, as previously reported (Hu et al., 2011; Miyauchi et al., 2018).⁴ This ² lower efficiency could be ²³⁸ attributed to the presence of the *BCR::ABL1* oncogene, which might interfere with the ¹²³ reprogramming process of CML CD34+ cells (Ito et al., 2022).⁴ ² Furthermore, CML CD34+ cells formed colonies later than normal CD34+ cells and exhibited atypical colony morphology (Bedel et al., 2013).⁴ ² Other researchers, such as Bedel et al., reported that CML iPSCs displayed a distinct morphology similar to cell aggregates found in mouse embryonic stem cells (Bedel et al., 2013; Kumano et al., 2012).⁴

The instability of CML-CD34-iPSCs may be due to the presence of the *BCR::ABL1* oncogene, which might disrupt the action of ¹²³ reprogramming factors required to generate stable iPSC colonies. ² Previous research by Ito et al. indicated that the use of molecularly targeted drugs like IM, capable of suppressing the activity of the BCR-ABL oncogene, could enhance the transcriptional regulation of ¹²³ reprogramming factors and boost pluripotency marker expression (Ito et al., 2022).⁴ This ² study also demonstrated that IM supplementation increased pluripotency marker expression in BCR::ABL+ K562 cells, further confirming that oncogene suppression might enhance cancer ⁵⁸ cells' reprogramming.¹²³ ² Our study corroborated these findings, revealing that IM supplementation was essential for generating stable CML iPSCs without spontaneous differentiation. ² The presence of IM improved the stability and maintenance of all isolated CML-CD34-iPSC colonies, enabling them to be

²³⁹cultured for over 20 passages without significant differentiation. CML-CD34-iPSCs² cultured with IM showed increased proliferation, while IM withdrawal led to reduced proliferation and TRA-1-60 expression. These² results align with previous findings indicating faster growth of CML-iPSCs in the presence of TKI (Bedel et al., 2013; Charaf et al., 2017).⁴

Interestingly, the phosphorylation of CRKL was decreased in IM-treated primary CD34+ HPCs from CML patients (Suknuntha et al., 2015)⁴ and CML iPSCs (Figure 4.4.3D), suggesting that BCR-ABL TK activity in hematopoietic cells and CML iPSCs may involve similar pathways. The² discovery that IM can stabilize the pluripotency of CML-iPSCs unveils a novel mechanism of pluripotency maintenance. Both² CML-iPSCs and LSCs express the BCR-ABL fusion protein but are resistant to IM.²⁴⁰ Therefore,² CML-iPSCs generated using our approach, along with the HPCs derived from these iPSCs, offer a valuable platform for CRISPR-Cas9 screening to investigate the mechanisms of TKI resistance in these cells and identify potential drugs to effectively eliminate CML-LSCs.²⁴¹

Summary, conclusions, and future directions

³¹² Introduction²⁴² of targeted therapy with tyrosine kinase inhibitor (TKI) imatinib in chronic myeloid leukemia (CML) has revolutionized the management of this disease, resulting in patients on TKI therapy having life expectancy similar to healthy populations. However,² one major challenge to TKI therapy's⁵⁸ success in CML is the persistence of leukemic stem cells (LSCs) that TKIs do not eliminate. In² this doctoral work, we sought to understand the mechanisms by which CML LSCs escape TKI therapy and explored ways to modulate identified targets in eliminating these LSCs, as outlined in Figure--

We took three approaches: First,²⁴³ transcriptomic profiling in primary CML CD34+ cells and healthy donor CD34+ cells to look for differentially expressed

gene signatures. ²Subsequently, we performed molecular inhibition of one of the topmost upregulated targets in CML CD34+ cells. ²We assessed whether this would reduce the stemness in-vitro and leukemogenic potential in-vivo in a cell ²⁴⁴line derived xenograft mouse model. ²Further, as LSCs are a rare population of cells, we used iPSC-based disease ²⁴⁵modeling of CML and found an exciting observation that to form stable iPSCs, BCR::ABL has to ²⁴⁶be inhibited.

Second, we attempted to unravel the contribution of dysregulated miRNAs in primary CML cells cultured under hypoxia, postulating that hypoxia-associated miRNAs play a role in the survival and maintenance of CML LSCs.

Third, to explore the role of ²⁴⁷bone marrow MSC niche in protecting CML LSCs, we successfully generated and characterized an immortalized CML MSC cell line and assessed its role in maintaining CML LSCs.

The salient findings from each objective are listed below:

Objective 1: Transcriptomic profiling of CML CD34+ cells to study the differentially expressed genes between CML LSCs and normal hematopoietic stem cells.

v Transcriptomics profiling revealed 1563 genes upregulated and 1400 genes downregulated in the CML CD34+ cells.

v Pathway enrichment analysis showed genes associated with the cell cycle, Fanconi ²⁴⁸anemia pathway, and metabolism were significantly altered in CML CD34+ cells. ²These changes pointed towards enhanced oxidative phosphorylation, indicating potential metabolic vulnerabilities that could be ²⁴⁹exploited for therapeutic intervention. ²in CML CD34+ cells.

v Genes associated with serine proteases, including cathepsin G (CTSG), cathepsin L (CTSL), neutrophil elastase (ELANE), and ribonucleases (RNASE) were upregulated in CML CD34+ cells. ²Subsequent validation ³⁸in an entirely new cohort of (n=59) primary ²⁵⁰cml cd34+ cells corroborated the upregulated genes.

v Molecular modulation of CTSG markedly reduced colony-forming potential *in vitro*, underscoring its probable role in promoting LSC stemness and clonogenicity. ² These observations ²⁵¹ were further corroborated in an *in vivo* xenotransplantation model, wherein CTSG knockdown showed a significant increase in overall survival, suggesting its role in leukemogenesis.

Objective 2: The role of hypoxia-associated miRNAs in CML LSC survival

v Apart from CML cell lines, we established hypoxic culture conditions in primary CML cells, which is quite challenging. ² We further confirmed that ²⁵² the cells were maintained in hypoxic conditions by showing reduced proliferation, entry into a slow-cycling state, and elevated expression of HIF-1 α and its downstream targets.

v MiRNA profiling of CML primary cells cultured under hypoxia showed dysregulation of 20 miRNAs. ² Among the differentially expressed miRNAs, miR-210, a well-known hypoxia-regulated miRNA, was significantly upregulated.

v miR-30b-5p were explicitly upregulated in CML cells under hypoxic conditions, whereas unaltered in healthy donor PBMNCs. ² The role of miR-30b is ²⁵³ exclusively studied in solid ²⁵⁴ tumors, but its role in CML LSC is unknown. ² MiR-30b-5p ²⁵³ knockout reduced proliferation and colony-forming capacity in K562 cells under hypoxic conditions, suggesting that mir30b5p might play a role in the maintenance of CML cells.

v Target prediction revealed genes associated with TGF- β as a potential target of miR-30b-5b. ² TGF- β ²⁵⁵ signaling is ²⁵⁶ reported to be associated with CML LSCs; further studies are needed to confirm the findings.

Objective 3: ²⁵⁷ Generation and characterization of immortalized CML MSC lines and ²⁵⁷ assessing their roles in maintaining LSCs.

v CML-MSCs and normal MSCs were confirmed immunophenotypically for MSC markers. ² Although both normal and CML MSCs retained the trilineage

differentiation potential (Adipogenic, Osteogenic, and Chondrogenic), CML MSCs exhibited enhanced chondrogenic differentiation compared to NBM MSCs.

v The iCML MSCs expressed all the MSC markers ²⁵⁸with no chromosomal aberration and retained the trilineage differentiation potential as the primary CML-MSCs.

v iCML MSCs protected the CML cells from Imatinib-induced apoptosis and selectively increased the growth of CD34+ leukemic cells in ex vivo conditions. iCML ²MSC could be ideal for studying cell-cell interaction.

v CML MSCs secreted cytokines associated with immune response and inflammation, suggesting the possibility of an immune microenvironment in CML BMM. These ²results need to be validated further.

The iCML MSC is a valuable model for studying leukemic-stromal interaction and functional studies of candidate genes mediating TKI resistance in CML LSCs.

Objective 4: Generation and characterization of iPSCs from primary CML cells for modeling CML LSCs. ²⁵⁹

314 | As LSCs are a rare population of cells, we generated and characterized induced Pluripotent Stem Cells (iPSCs) from CML CD34+ cells. These ²iPSCs can be expanded and transformed into hematopoietic cells, providing a steady supply for in-depth proteome, epigenome, transcriptome, and drug screening studies.

315 | v The reprogramming ¹²³efficiency of CML CD34+ cells was significantly lower than that of normal CD34+ cells, likely due to the presence of the BCR::ABL1 oncogene, which may interfere with the reprogramming ¹²³process. CML ²CD34+ cells also exhibited delayed colony formation and atypical morphology.

v CML-iPSCs are highly unstable, and supplementation with imatinib mesylate (IM) aided in the maintenance of ²⁶⁰CML-iPSCs. Results suggest that suppression

of BCR::ABL1 fusion protein is essential for the maintenance of CML-iPSCs in the pluripotent state.

v Hematopoietic differentiation of CML-iPSCs in the absence of imatinib resulted in an increased generation of hematopoietic cells. Colony forming assay showed bias towards myeloid lineage compared with the CML-iPSCs differentiated with imatinib.

v The CML-iPSCs generated could be differentiated into BCR::ABL1 positive hematopoietic progenitors.

The CML-iPSCs and the resulting HSPC are excellent tools for conducting high-throughput screening assays such as CRISPR-Cas9 or small molecule compound library.

Conclusions

Global transcriptomic analysis of CML vs. normal CD34+ cells provided valuable insights into the molecular intricacies of CML, highlighting altered pathways, cell surface markers, and the potential role of CTSG in LSC survival.

These findings offer a foundation for future research to develop targeted therapeutic strategies to disrupt these survival mechanisms and improve treatment outcomes for CML patients.

MiRNA profiling in primary CM cells in hypoxic condition showed upregulation of miR-30b-5p. miR-30b-5p expression in hypoxic conditions was restricted only to CML cells without alteration in healthy donor PBMNCs. Further, miR-30b-5p knock out in K562 cell line markedly reduced colony forming capacity and proliferation in hypoxic conditions. In-silico analysis revealed miR-30b-5p may potentially alter the TGF- β signalling in hypoxic conditions miR-30b-5p could be potential biomarker for primary CML cells.

Limitations of the present study:

The transcriptomic study ²⁷⁷was performed in CD34+ fraction, ²⁷⁸which could be a mixture of progenitors and stem cells in both healthy donor and CML cells. ²The gene signatures ²⁷⁹obatined need ³⁸validation in CML LSCs (CD34+CD38-) fraction to identify bonafide CML LSCs signatures.

One of the ²⁸⁰major limitations in the miRNA study is the use of primary CML patient bulk cells for miRNA profiling; due to their inherent variability in the primary samples would have masked the prominent ²⁸¹dsregulated miRNA. ²⁸²Ideal ²⁸³scenerio would have been to use ²⁸⁴cell line model for discovery and ³⁸validation in primary CML cells.

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[P1] [P2] [P3] [P4] [P5] [P6] [P7] [P8]

Top of Form

Bottom of Form

[P1] ²⁸⁵Hypoxia associated miRNA

[P2] Add DNA repair pathway in transcriptome cartoon

[P3] Only characterization of iCML can remove vs HS-5

[P4] iPSC cartoon add 1m ²⁸⁶supplemtaion and change from ²⁸⁷dome shaped to flat colonies for CML

[P5] The arrow for ^{288,289}ipsc objective should come from CML CD34+ cells; ²⁹⁰rightnow ²⁹¹it is starting from ²⁹²CTSG box

[P6] iCML MSC is functionally similar to primary CML MSC but can be grown in culture for ²⁹³longer periods;

unique cytokine signatures in CML MSC can serve as potential druggable targets to overcome BM MSC microenvironment in CML

[P7] Mir30b5p is a potential target that helps maintain CML stem cells in quiescent state?^{294 295}

[P8] You should highlight that validation³⁸ of target genes was done in n number of CD34+ cells

1.	leukemia → leukaemia	Mixed dialects of English	Correctness
2.	<i>. Hypoxia-inducible; . It; . In; . Hypoxia; . Several; . For; . Notably; . Low; . Emerging; . Although; . The; . Previous; . These; . Both; . Cell; . We; . Similar; . Briefly; . Principal; . This; . A; . Among; . CML; . E; . To; . Also; . qPCR results of A)miR-30b-5p B) miR-363-5p C)miR-660-5p in C...</i>	Text inconsistencies	Correctness
3.	activates and stabilises	Wordy sentences	Clarity
4.	<i>(Simsek et al., 2010); (Licausi et al., 2011); (Forristal et al., 2015; Gao et al., 2015; Zou et al., 2013); (Ng et al., 2014; Singh et al., 2022); (Khan et al., 2021; Licausi et al., 2011); (Lin et al., 2020; Salati et al., 2017; Wang et al., 2015); (Ruiz et al., 2021); (Hubbi and Semenza, 2015); ...</i>	Citation style options	Correctness
5.	tumour-promoting	Mixed dialects of English	Correctness
6.	leukemia → leukaemia	Mixed dialects of English	Correctness
7.	greater → more significant, more tremendous	Word choice	Engagement
8.	been shown	Passive voice misuse	Clarity
9.	been shown	Passive voice misuse	Clarity
10.	, while	Punctuation in compound/complex sentences	Correctness
11.	induces → causes	Word choice	Engagement
12.	tumor → tumour	Mixed dialects of English	Correctness
13.	tumors → tumours	Mixed dialects of English	Correctness
14.	role → position	Word choice	Engagement

15.	hypothesising	Wordy sentences	Clarity
16.	tumors → tumours	Mixed dialects of English	Correctness
17.	were tested	Passive voice misuse	Clarity
18.	2 → two	Improper formatting	Correctness
19.	fold → 2-fold	Misspelled words	Correctness
20.	were taken	Passive voice misuse	Clarity
21.	can be used	Passive voice misuse	Clarity
22.	. The	Improper formatting	Correctness
23.	molecules,	Punctuation in compound/complex sentences	Correctness
24.	the use of	Wordy sentences	Clarity
25.	. This	Improper formatting	Correctness
26.	This	Intricate text	Clarity
27.	as compared to → than	Wordy sentences	Clarity
28.	are designed	Passive voice misuse	Clarity
29.	, which showed	Incorrect phrasing	Correctness
30.	, as	Punctuation in compound/complex sentences	Correctness
31.	were identified	Passive voice misuse	Clarity
32.	2 → two	Improper formatting	Correctness
33.	were used	Passive voice misuse	Clarity
34.	an overlapping	Determiner use (a/an/the/this, etc.)	Correctness

35.	<i>This</i>	Intricate text	Clarity
36.	5 → five	Improper formatting	Correctness
37.	<i>CML cell lines cultured in normoxia and hypoxia were assessed</i>	Passive voice misuse	Clarity
38.	<i>Validation; validation</i>	Text inconsistencies	Correctness
39.	<i>was found</i>	Passive voice misuse	Clarity
40.	found to be	Wordy sentences	Clarity
41.	<i>be significantly upregulated</i>	Passive voice misuse	Clarity
42.	<i>been extensively evaluated</i>	Passive voice misuse	Clarity
43.	tumors → tumours	Mixed dialects of English	Correctness
44.	tumor → tumour	Mixed dialects of English	Correctness
45.	esophageal → oesophagal	Mixed dialects of English	Correctness
46.	<i>was evaluated</i>	Passive voice misuse	Clarity
47.	<i>were extracted</i>	Passive voice misuse	Clarity
48.	<i>Increased GLUT1 expression was observed in MEG01, KY01, MOLM6, KCL22, K562, and EM-2 cell lines confirming the induction of hypoxia.</i>	Unclear sentences	Clarity
49.	<i>was evaluated</i>	Passive voice misuse	Clarity
50.	<i>To generate Cas9 expressing K562 cell line</i>	Misplaced words or phrases	Correctness
51.	<i>were first transduced</i>	Passive voice misuse	Clarity
52.	<i>lenti-Cas9</i>	Unknown words	Correctness

53.	<i>were subjected</i>	Passive voice misuse	Clarity
54.	<i>was transduced</i>	Passive voice misuse	Clarity
55.	<i>transduction efficiency was found</i>	Passive voice misuse	Clarity
56.	proliferation → increase	Word choice	Engagement
57.	altering → changing, limiting	Word choice	Engagement
58.	3'untranslated; "; "; MSCs'; ' patients'; "; " ; patient's; cells'; therapy's	Text inconsistencies	Correctness
59.	A total of seventy	Wordy sentences	Clarity
60.	<i>were predicted</i>	Passive voice misuse	Clarity
61.	tumor → tumour	Mixed dialects of English	Correctness
62.	<i>been studied</i>	Passive voice misuse	Clarity
63.	signaling → signalling	Mixed dialects of English	Correctness
64.	tumor → tumour	Mixed dialects of English	Correctness
65.	signaling → signalling	Mixed dialects of English	Correctness
66.	results → effects	Word choice	Engagement
67.	, specifically	Punctuation in compound/complex sentences	Correctness
68.	have been reported	Incomplete sentences	Correctness
69.	it to	Pronoun use	Correctness
70.	tumors → tumours	Mixed dialects of English	Correctness
71.	proliferation → expansion, increase	Word choice	Engagement
72.	condition → conditions	Incorrect noun number	Correctness

73.	<i>Additionally, the knockout of miR-30b led to reduced proliferation and colony-forming capacity of CML cells cultured in hypoxic condition.</i>	Incomplete sentences	Delivery
74.	dyregulated → dysregulated, deregulated	Misspelled words	Correctness
75.	signaling → signalling	Mixed dialects of English	Correctness
76.	<i>is already reported</i>	Passive voice misuse	Clarity
77.	with CML	Wrong or missing prepositions	Correctness
78.	major → significant	Word choice	Engagement
79.	major → significant	Word choice	Engagement
80.	. The	Improper formatting	Correctness
81.	<i>be attributed</i>	Passive voice misuse	Clarity
82.	leukemia → leukaemia	Mixed dialects of English	Correctness
83.	normal → regular	Word choice	Engagement
84.	on → of	Wrong or missing prepositions	Correctness
85.	<i>are altered</i>	Passive voice misuse	Clarity
86.	<i>is needed</i>	Passive voice misuse	Clarity
87.	<i>the immunophenotype profile between NBM and CML patients was compared</i>	Passive voice misuse	Clarity
88.	<i>be differentiated</i>	Passive voice misuse	Clarity
89.	altered → modified, limited	Word choice	Engagement
90.	<i>been caused</i>	Passive voice misuse	Clarity

91.	<i>been reported</i>	Passive voice misuse	Clarity
92.	lack the ability to → <i>cannot</i>	Wordy sentences	Clarity
93.	show → <i>showed</i>	Incorrect verb forms	Correctness
94.	<i>were transduced</i>	Passive voice misuse	Clarity
95.	<i>be differentiated</i>	Passive voice misuse	Clarity
96.	<i>was evaluated</i>	Passive voice misuse	Clarity
97.	<i>Compared to undifferentiated cells, we observed increased PPARG and RUNX2 expression in adipo and osteo-differentiated cells (Figure 4.3.5A-B).</i>	Unclear sentences	Clarity
98.	<i>osteo-differentiated</i>	Unknown words	Correctness
99.	, suggesting → <i>. This suggests</i>	Hard-to-read text	Clarity
100.	leukemia → <i>leukaemia</i>	Mixed dialects of English	Correctness
101.	leukemia → <i>leukaemia</i>	Mixed dialects of English	Correctness
102.	regulate → <i>control, restrain</i>	Word choice	Engagement
103.	growth → <i>development</i>	Word choice	Engagement
104.	a significant → <i>significantly</i>	Wordy sentences	Clarity
105.	significant → <i>significantly</i>	Misuse of modifiers	Correctness
106.	<i>Conditioning media was collected</i>	Passive voice misuse	Clarity
107.	<i>the leukemic cells were cultured</i>	Passive voice misuse	Clarity
108.	<i>The proliferation and percentage of CD34+ cells were measured</i>	Passive voice misuse	Clarity

109.	<i>kossa</i>	Unknown words	Correctness
110.	pCML → ECML	Misspelled words	Correctness
111.	pCML → ECML	Misspelled words	Correctness
112.	<i>are differentially expressed</i>	Passive voice misuse	Clarity
113.	and tested → . We tested	Hard-to-read text	Clarity
114.	<i>were also increased</i>	Passive voice misuse	Clarity
115.	remodeling → remodelling	Mixed dialects of English	Correctness
116.	<i>been shown</i>	Passive voice misuse	Clarity
117.	growth → development	Word choice	Engagement
118.	<i>Cytokine profiling revealed that 13 cytokines were abundantly secreted by the CML MSC lines.</i>	Passive voice misuse	Clarity
119.	reported → noted, said	Word choice	Engagement
120.	<i>were found</i>	Passive voice misuse	Clarity
121.	found to be	Wordy sentences	Clarity
122.	leukemia → leukaemia	Mixed dialects of English	Correctness
123.	<i>re-programming; reprogram; reprogrammed; reprogramming; Reprogramming</i>	Text inconsistencies	Correctness
124.	<i>be caused</i>	Passive voice misuse	Clarity
125.	<i>is extensively utilized</i>	Passive voice misuse	Clarity
126.	leukemia → leukaemia	Mixed dialects of English	Correctness
127.	<i>Due to the increased chondrogenic differentiation potential observed in</i>	Incomplete sentences	Delivery

CML MSCs, the use of HS-5 might not be optimal for investigating the BMM-mediated protection in CML leukemic stem cells (LSCs)(Roecklein and Torok-Storb, 1995).

128.	<i>be used</i>	Passive voice misuse	Clarity
129.	retained → kept, possessed, included	Word choice	Engagement
130.	<i>were increased</i>	Passive voice misuse	Clarity
131.	tumors → tumours	Mixed dialects of English	Correctness
132.	the treatment of → treating	Wordy sentences	Clarity
133.	iMSCs → MCs	Misspelled words	Correctness
134.	<i>was found</i>	Passive voice misuse	Clarity
135.	modeling → modelling	Mixed dialects of English	Correctness
136.	leukemia-like → leukaemia-like	Mixed dialects of English	Correctness
137.	models for → models for, model for	Misspelled words	Correctness
138.	<i>be further differentiated</i>	Passive voice misuse	Clarity
139.	normal → ordinary, regular	Word choice	Engagement
140.	leukemia → leukaemia	Mixed dialects of English	Correctness
141.	, → ,, .	Comma misuse within clauses	Correctness
142.	showin → shown	Misspelled words	Correctness
143.	the Table → in Table	Incorrect phrasing	Correctness
144.	2 → two	Improper formatting	Correctness

145.	<i>were transferred</i>	Passive voice misuse	Clarity
146.	Matrigel-coated → Matrigel-coated	Misspelled words	Correctness
147.	<i>the half-culture media was replaced</i>	Passive voice misuse	Clarity
148.	two clones → two clones	Improper formatting	Correctness
149.	<i>were picked</i>	Passive voice misuse	Clarity
150.	Normal → Regular	Word choice	Engagement
151.	levels of	Wordy sentences	Clarity
152.	<i>were described</i>	Passive voice misuse	Clarity
153.	, however → . However, ; however	Punctuation in compound/complex sentences	Correctness
154.	that all	Wordy sentences	Clarity
155.	no mutations were	Wordy sentences	Clarity
156.	enhances → improves	Word choice	Engagement
157.		Tone suggestions	Delivery
158.	layers, → layers:	Incorrect punctuation	Correctness
159.	<i>was withdrawn</i>	Passive voice misuse	Clarity
160.	<i>were treated</i>	Passive voice misuse	Clarity
161.	specifically	Wordy sentences	Clarity
162.	enhances explicitly	Word choice	Engagement
163.	containing → controlling	Word choice	Engagement
164.	, and	Punctuation in compound/complex sentences	Correctness

165.	. Yellow	Incorrect punctuation	Correctness
166.	, and	Punctuation in compound/complex sentences	Correctness
167.	Quantification of → Quantifying	Wordy sentences	Clarity
168.	is also shown	Passive voice misuse	Clarity
169.	be phosphorylated	Passive voice misuse	Clarity
170.	In order to → To	Wordy sentences	Clarity
171.	was analyzed	Passive voice misuse	Clarity
172.	This	Intricate text	Clarity
173.	BCR::ABL1-mediated	Misspelled words	Correctness
174.	4.4.3 :	Improper formatting	Correctness
175.	maintanence → maintenance	Misspelled words	Correctness
176.	in the absence of → without	Wordy sentences	Clarity
177.	presence → fact	Word choice	Engagement
178.	colonies → settlements, territories	Word choice	Engagement
179.	4.4.4 :	Improper formatting	Correctness
180.	: Trilineage	Improper formatting	Correctness
181.	normal → regular, standard	Word choice	Engagement
182.	mesoderm,	Punctuation in compound/complex sentences	Correctness
183.	was used	Passive voice misuse	Clarity

184.	Seale → scale	Incomplete sentences	Delivery
185.	of	Wrong or missing prepositions	Correctness
186.	the formation of	Wordy sentences	Clarity
187.	it	Pronoun use	Correctness
188.	it	Wordy sentences	Clarity
189.	useful → valuable, helpful	Word choice	Engagement
190.	resistance mechanisms	Wordy sentences	Clarity
191.	were subjected	Passive voice misuse	Clarity
192.	reprogramming,	Punctuation in compound/complex sentences	Correctness
193.	, resulting	Punctuation in compound/complex sentences	Correctness
194.	4.4.6:Alkaline → 4.4.6: Alkaline	Misspelled words	Correctness
195.	observed → Observed	Improper formatting	Correctness
196.	patient's → patients, patient	Incorrect noun number	Correctness
197.	patients generated	Confused words	Correctness
198.	colonies → settlements	Word choice	Engagement
199.	colonies → settlements, territories	Word choice	Engagement
200.	4.4.5.Second → 4.4.5 Second	Misspelled words	Correctness
201.	third-generation	Misspelled words	Correctness
202.	second-generation	Misspelled words	Correctness
203.	third-generation	Misspelled words	Correctness

204.	the maintenance of → maintaining	Wordy sentences	Clarity
205.	<i>We replaced IM with different concentrations of DA, NIL, and ponatinib and observed their effects on CML-CD34-iPSCs.</i>	Unclear sentences	Clarity
206.	third-generation	Misspelled words	Correctness
207.	<i>be successfully transformed</i>	Passive voice misuse	Clarity
208.	),	Punctuation in compound/complex sentences	Correctness
209.	different → various	Word choice	Engagement
210.	Representative → Expected	Word choice	Engagement
211.	showing → show	Incorrect verb forms	Correctness
212.	different → other	Word choice	Engagement
213.	generated → induced	Word choice	Engagement
214.	is → Is	Improper formatting	Correctness
215.	necessary → required, essential	Word choice	Engagement
216.	to differentiate	Wordy sentences	Clarity
217.	single-cell → single-cell	Misspelled words	Correctness
218.	<i>To answer our question, we carried out single cell sorting to obtain iPSC colonies that had an equal number of cells (Figure 4.4.10A).</i>	Unclear sentences	Clarity
219.	<i>were subjected</i>	Passive voice misuse	Clarity
220.	, and	Punctuation in compound/complex sentences	Correctness

221.	as well as → and	Wordy sentences	Clarity
222.	were able to obtain → obtained	Wordy sentences	Clarity
223.	were differentiated	Passive voice misuse	Clarity
224.	around → about, approximately	Word choice	Engagement
225.	be attributed	Passive voice misuse	Clarity
226.	compared to → than	Wordy sentences	Clarity
227.	a normal → an average, a standard, a typical	Word choice	Engagement
228.	which also resulted → resulting	Wordy sentences	Clarity
229.	, G	Improper formatting	Correctness
230.	compared to → than	Wordy sentences	Clarity
231.	of HSPC	Wrong or missing prepositions	Correctness
232.	donor → donors	Incorrect noun number	Correctness
233.	different	Wordy sentences	Clarity
234.	different	Wordy sentences	Clarity
235.	was withdrawn	Passive voice misuse	Clarity
236.	was supplemented	Passive voice misuse	Clarity
237.		Tone suggestions	Delivery
238.	be attributed	Passive voice misuse	Clarity
239.	be cultured	Passive voice misuse	Clarity
240.	are resistant to → resist	Wordy sentences	Clarity

241.	to eliminate CML-LSCs effectively	Inappropriate colloquialisms	Delivery
242.	The introduction	Determiner use (a/an/the/this, etc.)	Correctness
243.	We took three approaches: First, transcriptomic profiling in primary CML CD34+ cells and healthy donor CD34+ cells to look for differentially expressed gene signatures.	Unclear sentences	Clarity
244.	line-derived → line-derived	Misspelled words	Correctness
245.	modeling → modelling	Mixed dialects of English	Correctness
246.	be inhibited	Passive voice misuse	Clarity
247.	the bone	Determiner use (a/an/the/this, etc.)	Correctness
248.	anemia → anaemia	Mixed dialects of English	Correctness
249.	could be exploited	Passive voice misuse	Clarity
250.	eml → CML	Misspelled words	Correctness
251.	were further corroborated	Passive voice misuse	Clarity
252.	the cells were maintained	Passive voice misuse	Clarity
253.	is exclusively studied	Passive voice misuse	Clarity
254.	tumors → tumours	Mixed dialects of English	Correctness
255.	signaling → signalling	Mixed dialects of English	Correctness
256.	is reported	Passive voice misuse	Clarity
257.	Objective 3: Generation and characterization of immortalized CML MSC lines and assessing their roles in maintaining LSCs.	Unclear sentences	Clarity

258.	with no → without	Wordy sentences	Clarity
259.	modeling → modelling	Mixed dialects of English	Correctness
260.	the maintenance of → maintaining	Wordy sentences	Clarity
261.	the maintenance of → maintaining	Wordy sentences	Clarity
262.	<i>Global transcriptomic analysis of CML vs. normal CD34+ cells provided valuable insights into the molecular intricacies of CML, highlighting altered pathways, cell surface markers, and the potential role of CTSG in LSC survival.</i>	Intricate text	Clarity
263.	priamry → primary	Misspelled words	Correctness
264.	eondition → conditions	Incorrect noun number	Correctness
265.	upregualtion → upregulation, up-regulation	Misspelled words	Correctness
266.	eondtions → conditions	Misspelled words	Correctness
267.	knock-out → knockout	Confused words	Correctness
268.	the K562	Determiner use (a/an/the/this, etc.)	Correctness
269.	eolony forming → colony-forming	Misspelled words	Correctness
270.	eondtions → conditions	Misspelled words	Correctness
271.	In-sillice → In-silicon, In-silica	Misspelled words	Correctness
272.	analyis → analysis	Misspelled words	Correctness
273.	may → might	Faulty tense sequence	Correctness
274.	potentailly → potentially	Misspelled words	Correctness

275.	eondtions → conditions	Misspelled words	Correctness
276.	a potential	Determiner use (a/an/the/this, etc.)	Correctness
277.	was performed	Passive voice misuse	Clarity
278.	which could be	Wordy sentences	Clarity
279.	obatined → obtained	Misspelled words	Correctness
280.	major → significant	Word choice	Engagement
281.	deregulated → dysregulated, deregulated, regulated	Misspelled words	Correctness
282.	The ideal	Determiner use (a/an/the/this, etc.)	Correctness
283.	seenerio → scenario	Misspelled words	Correctness
284.	a cell	Determiner use (a/an/the/this, etc.)	Correctness
285.	Hypoxia-associated	Misspelled words	Correctness
286.	supplemtaion → supplementation	Misspelled words	Correctness
287.	dome shaped → dome-shaped	Misspelled words	Correctness
288.	the ipsc	Determiner use (a/an/the/this, etc.)	Correctness
289.	ipsc	Unknown words	Correctness
290.	rightnow → right	Misspelled words	Correctness
291.	, it	Punctuation in compound/complex sentences	Correctness
292.	the CTSG	Determiner use (a/an/the/this, etc.)	Correctness

		etc.)	
293.	longer → more extended	Word choice	Engagement
294.	a quiescent	Determiner use (a/an/the/this, etc.)	Correctness
295.	state? → state.	Closing punctuation	Correctness
296.	<i>Hypoxia-inducible factor 1α (HIF-1α) is a crucial transcription factor</i>	Flavonoids targeting HIF-1: implications on cancer metabolism	Originality
297.	<i>cellular processes, including cell proliferation, survival, and stemness</i>	The PRMT5-LSD1 axis confers Slug dual transcriptional activities and promotes breast cancer progression Journal of Experimental & Clinical Cancer Research Full Text https://jeccr.biomedcentral.com/articles/10.1186/s13046-022-02400-7	Originality
298.	<i>unexplored. The current study aimed to determine the role of</i>	Ablation of apoptosis-stimulating of p53 protein 1 protects mice from acute hepatic injury and dysfunction via NF-κB pathway in CCl4-induced hepatotoxicity	Originality
299.	<i>Cell cycle analysis revealed an accumulation of cells in the</i>	The HDAC Inhibitor LBH589 Enhances the Antimyeloma Effects of the IGF-1RTK Inhibitor Picropodophyllin	Originality
300.	<i>The bone marrow mesenchymal stromal cells (BMMSCs) play a crucial role in</i>	A review of aberrant DNA methylation in bone marrow mesenchymal stromal cells: a new possible access of acute myeloid leukemia	Originality
301.	<i>Despite these findings, there is limited knowledge on how</i>	Parental Participation in the Education of Female Students with Learning Difficulties: The Views of Saudi Elementary Teachers and Parents.	Originality

302.	<i>To understand the changes incurred by the stromal</i>	K562 chronic myeloid leukemia cells modify osteogenic differentiation and gene expression of bone marrow stromal cells	Originality
303.	<i>MSCs are multipotent progenitors that can differentiate into</i>	Current and Future Directions of Stem Cell Therapy for Bladder Dysfunction Stem Cell Reviews and Reports https://link.springer.com/article/10.1007/s12015-019-09922-2	Originality
304.	<i>the percentage of viable cells compared to the control</i>	Aconitum Heterophyllum Phytoniosomes Cytotoxic Activity Ligands https://www.ijpsonline.com/articles/emin-vitroem-and-emin-silicoem-cytotoxic-activity-of-emaconitum-heterophyllumem-phytoniosomes-and-its-ethanolic-root-extract-a-com-4861.html?view=mobile	Originality
305.	<i>showed reduced cell death in comparison to the</i>	The <i>FMR1</i> gene and fragile X-associated tremor/ataxia syndrome	Originality
306.	<i>and their plasma membranes can be used as natural drug carriers for targeting gliomas and providing a safe, adequate, quality-controlled, and continuous source for the treatment of gliomas</i>	Immortalized Mesenchymal Stem Cells: A Safe Cell Source for Cellular or Cell Membrane-Based Treatment of Glioma	Originality
307.	<i>Further studies are warranted to confirm the results.</i>	Use of octreotide for the prevention of pancreatic fistula after elective pancreatic surgery: A systematic review and meta-analysis — United Arab Emirates University https://research.uaeu.ac.ae/en/publications/use-of-octreotide-for-the-prevention-of-pancreatic-fistula-after-	Originality

308.	<i>Induced pluripotent stem cells (iPSCs) derived from patients with</i>	iPSC-derived neurons profiling reveals GABAergic circuit disruption and acetylated α -tubulin defect which improves after iHDAC6 treatment in Rett syndrome	Originality
309.	<i>due to the constitutive expression of the BCR:</i>	Activation of protein kinase A (PKA) by 8-Cl-cAMP as a novel approach for antileukaemic therapy	Originality
310.	<i>useful tool to explore the underlying mechanisms of</i>	Discovery of novel HSP90 inhibitors that induced apoptosis and impaired autophagic flux in A549 lung cancer cells	Originality
311.	<i>C) Representative flow cytometry results showing the percentage of</i>	A functional genetic toolbox for human tissue-derived organoids eLife https://elifesciences.org/articles/67886	Originality
312.	<i>Introduction of targeted therapy with tyrosine kinase inhibitor</i>	Hyper-CVAD Compared With BFM-like Chemotherapy for the Treatment of Adult Acute Lymphoblastic Leukemia. A Retrospective Single-Center Analysis	Originality
313.	<i>As LSCs are a rare population of cells,</i>	Abstract 3153: Imatinib mesylate treatment improves reprogramming efficiency, and morphology of chronic myeloid leukemia derived induced pluripotent stem cells	Originality
314.	<i>we generated and characterized induced Pluripotent Stem Cells (iPSCs) from</i>	Generation of one control and four iPSCs clones from patients with Emery-Dreifuss muscular dystrophy type 1	Originality
315.	<i>cells, likely due to the presence of the</i>	Identification and distribution of the adrenocortical homolog, chromaffin tissue, and corpuscles of Stannius in <i>Amia calva</i> L	Originality

316.	<i>P1] [P2] [P3] [P4] [P5] [P6] [P7] [P8</i>	staticCFunction - Kotlin Programming Language https://kotlinlang.org/api/latest/jvm/stdlib/kotlinx.cinterop/static-c-function.html	Originality
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Plasma imatinib levels and *ABCB1* polymorphism influences early molecular response and failure-free survival in newly diagnosed chronic phase CML patients

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Achieving early molecular response (EMR) has been shown to be associated with better event free survival in patients with chronic phase chronic myeloid leukemia (CP-CML) on Imatinib therapy. We prospectively evaluated the factors influencing the 2-year failure free survival (FFS) and EMR to imatinib therapy in these patients including day29 plasma Imatinib levels, genetic variants and the gene expression of target genes in imatinib transport and biotransformation. Patients with low and intermediate Sokal score had better 2-year FFS compared to those with high Sokal Score ($p = 0.02$). Patients carrying *ABCB1*-C1236T variants had high day29 plasma imatinib levels ($P = 0.005$), increased EMR at 3 months ($P = 0.044$) and a better 2 year FFS ($P = 0.003$) when compared to those with wild type genotype. This translates to patients with lower *ABCB1* mRNA expression having a significantly higher intracellular imatinib levels ($P = 0.029$). Higher day29 plasma imatinib levels was found to be strongly associated with patients achieving EMR at 3 months ($P = 0.022$), MMR at 12 months ($P = 0.041$) which essentially resulted in better 2-year FFS ($p = 0.05$). Also, patients who achieved EMR at 3 months, 6 months and MMR at 12 months had better FFS when compared to those who did not. This study suggests the incorporation of these variables in to the imatinib dosing algorithm as predictive biomarkers of response to Imatinib therapy.

Imatinib mesylate, a tyrosine kinase inhibitor (TKI), has revolutionized the treatment of chronic myeloid leukemia (CML) changing it from a life-threatening disease to a condition that can be controlled in the vast majority of cases by oral medication. For those patients who develop resistance or intolerance to imatinib, choices of second or third generation TKIs are available. However, responses are the best when such changes in therapy are done prior to disease progression to an accelerated phase or blast crisis. This decision process on continuing therapy or changing TKI is compounded by the high cost of these second generation drugs in a predominantly self-paying system as exists in our country. Identifying the sub-optimal responders and early switch of therapy is essential since the achievement of major molecular response (MMR) or deep molecular response (DMR) within 3–6 months post TKI therapy has been shown to significantly improve progression free survival^{1–4}.

Several factors have been reported to contribute to sub-optimal response or resistance to imatinib^{5,6}. Among the *BCR-ABL1* dependent mechanisms, development of mutations in the kinase domain of *BCR-ABL1*^{7–9} plays a major role. Several imatinib independent mechanisms of resistance have also been reported including overexpression of efflux transporters^{10–12}, decreased expression of influx transporters^{13–15}, decreased plasma levels^{16,17}, binding to plasma proteins¹⁸, and genetic polymorphisms in the enzymes and transporters involved in imatinib transport or biotransformation^{19,20}. With the exception of one study reporting an intronic deletion polymorphism

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in the *BIM* (*BCL2Like11*) gene to be associated with imatinib resistance^{21–23}, there are no other prospective studies in patients with CML of Asian origin where all the parameters have been systematically evaluated. This is important due to the ethnic differences in genetic polymorphisms, which in turn could influence the differences in systemic exposure to anticancer drugs^{24,25}.

Although there are several studies^{15,17,21,22,26} exploring the factors influencing MMR and DMR in the international scale, there is no comprehensive analysis of the factors influencing EMR and MMR which in turn influence failure free survival (FFS). Our aim was to prospectively document response to imatinib as first line therapy using serial molecular monitoring to document EMR and MMR to evaluate the factors influencing response to imatinib therapy in newly diagnosed patients with CML-CP.

Patients and methods

Patients. All newly diagnosed, imatinib naïve adult CP- CML patients visiting Department of Haematology, Christian Medical College, Vellore were enrolled in the study after obtaining written informed consent. Ethical approval for this study has been approved by Institutional review board (IRB Min no: IRB (EC)-4–11–06–2008 dated June 19, 2008) at Christian medical college, Vellore -632,004, Tamilnadu, India. All methods in the study were performed in accordance with relevant guidelines and regulations.

Morphology/RT-PCR/Cytogenetics/FISH analysis. All patients at diagnosis were analyzed for the presence of *BCR-ABL1* fusion gene by Fluorescence in-situ hybridization (FISH). Interphase FISH analysis was performed using fixed cell suspensions obtained by direct or unstimulated overnight cultures of peripheral blood or bone marrow as reported previously²⁷. Although karyotyping is not routinely performed for CML patients in our centre, it was done in those patients who gave consent for bone marrow aspiration. Peripheral blood was collected at diagnosis and RNA was extracted using Trizol method. cDNA was synthesized from 2 µg RNA using random hexamers and reverse transcriptase enzyme (High capacity c-DNA synthesis kit, Thermo Scientific) followed by RT-PCR to identify the *BCR-ABL1* fusion transcript [e13a2/e14a2 or e1a2).

Plasma and intracellular levels of Imatinib. Blood samples were collected on day29 post imatinib therapy in EDTA anticoagulated tubes and plasma was separated and stored immediately. Trough plasma imatinib and desmethyl imatinib (imatinib metabolite) concentration was assessed using HPLC (High Performance Liquid Chromatography) Ultra-violet detection method as reported previously²⁸ with minor modifications. Dasatinib was used as an internal standard.

Peripheral blood mononuclear cells (PBMNCs) ($15 \times 10^6/\text{mL}$) from CML patients at diagnosis was incubated at 37 °C for 24 h and treated with 5 µM imatinib for 5 h. The cells were washed with phosphate buffered saline (PBS) and the cell pellets were stored at – 80 °C until analysis. Just before processing, the samples were thawed, the cells were lysed by sonication for 10 min on ice. Intracellular levels of Imatinib were analyzed by the same method as that of plasma imatinib incorporating minor changes from a previously published protocol²⁸.

BCR-ABL1 molecular monitoring. Peripheral blood was collected at diagnosis, 3, 6, 9, 12 and 18 months' post imatinib therapy to check the *BCR-ABL1* transcript level. Additional samples were analyzed depending on clinical needs. The *BCR-ABL1* transcript levels were quantified using real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) method as reported previously²⁹.

BCR-ABL1 kinase domain mutation detection. Mutations in the *BCR-ABL1* kinase domain were evaluated in patients with suboptimal response as reported previously³⁰. The sequences were aligned using SeqScape (Applied Biosystems) and the type of the mutation was predicted using Mutation Taster (<https://www.mutationtaster.org/>).

RNA expression of influx and efflux transporters. Total RNA was extracted using Trizol method and 1–2 µg RNA was used for c-DNA synthesis (High capacity cDNA Reverse Transcription Kit, Applied Biosystems). RNA expression of various Imatinib efflux and influx transporters were analyzed using TaqMan based assays [assay IDs: *ABCB1* (HS01067802-m1), *ABCG2* (HS01053787-m1), *SLC22A1* (HS00427554-m1), *ABCA3* (HS00184543-m1), *ABCA5* (HS00363322-m1), *ABCA6* (HS00365329-m1), *ABCB5* (HS02889060-m1), *ABCB6* (HS00180568-m1), *ABCB7* (HS00188776-m1), *ABCB8* (HS00894817-m1), *ABCB10* (HS00429240-m1), *ABCB11* (HS00184824-m1), *ABCC1* (HS00219905-m1), *ABCC3* (HS009784173-m1), *ABCC4* (HS00988717-m1), *ABCC11* (HS01090768-m1)] and the expression was normalized to the housekeeping gene *GAPDH* (4352934E).

RNA expression of influx and efflux transporters in CD34⁺ fraction. CD34⁺ cells were enriched from the CML patient sample and healthy donors using Easysep magnetic enrichment kit (<https://www.stemcell.com/easysep-human-cd34-positive-selection-kit-ii.html>). RNA extraction, c-DNA synthesis and expression of influx (*hOCT1*) and efflux (*ABCB1* & *ABCG2*) transporter analysis was also done as explained previously.

Polymorphisms in imatinib transporters and drug metabolizing enzymes. Genomic DNA was extracted from peripheral blood using standard phenol–chloroform method and 50–100 ng DNA was used for each PCR. All coding exons with flanking introns were screened for *SLC22A1* and known single nucleotide polymorphisms (SNP) in Imatinib efflux transporters *ABCB1* (exon 26), *ABCG2* (promoter, exon 2 and exon 5), *OCTN1/SLC22A4* (exon 9) were screened by PCR followed by sequencing and variants were analyzed by SeqS-

cape software. Three SNPs in Cyp3A4/3A5 were analyzed by restriction fragment length polymorphism (RFLP). The primer sequences and the source of the methods are listed in Supplementary Table 1.

***BIM* deletion polymorphism.** Genomic DNA was used to assess the *BIM* deletion polymorphism by PCR as previously reported²¹. The PCR conditions used were: 95 °C for 10 min and 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 10 min. Emerald Master mix (TaKaRa BIO Inc., Shiga, Japan) was used for this PCR. The amplified products when subjected to 3% agarose gel electrophoresis showed the band size of 216 bp for wild-type and 173 bp if there was deletion.

***GSTT1*, *GSTM1* deletions, *GSTA1**B and *GSTP1**B polymorphisms.** *GSTT1* and *GSTM1* deletion, *GSTA1**B and *GSTP1**B ([Ile105Val] rs1965) polymorphisms were screened by methods as reported previously³¹. Primers used for PCR reaction and the conditions are listed in supplementary Table 1.

Assessment of response and definition of outcome. Response criteria including complete hematological response (CHR) and molecular response were defined according to the European Leukaemia Net (ELN) guidelines³². Hematologic response was defined as normalized peripheral blood cell counts (WBC < 10 X10⁹/L and platelet count < 450 X10⁹/L) without evidence of peripheral blasts, promyelocytes, or myelocytes, and without evidence of extramedullary disease including disappearance of palpable splenomegaly lasting for at least 4 weeks. Molecular response was classified based on *BCR-ABL1* to control gene transcript ratios, expressed on the International Scale (IS) as reported previously from our lab²⁹. EMR was defined as *BCR-ABL1* to control gene ratio of ≤ 0.1% in the IS at 3 and ≤ 1% at 6 months; MMR was defined as *BCR-ABL1* to control gene transcript ratio of ≤ 0.1% in the IS, 12 months after imatinib therapy. In patients without an MMR at 12 months, mutation analysis of the *BCR-ABL1* fusion transcript was performed by direct sequencing.

Patients were classified as intolerant to imatinib therapy if the patient meets one or more of the criteria as reported previously³³. Patients who progressed to accelerated phase or blast crisis were classified as non-responders at all-time points. FFS was assessed by defining an event as: loss of hematological or cytogenetic response, progression to advanced phase or blast crisis, stem-cell transplant, death from any cause, change in imatinib dose or switch to a second generation TKI due to suboptimal response, as reported previously³⁴. Overall survival (OS) was calculated from the initiation of imatinib therapy until the date of death from any cause or the date of last follow-up.

Statistical analysis. All statistical analyses were performed using SPSS (IBM SPSS statistics version 21.0, Armonk, NY) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA; www.graphpad.com) software; *p* value < 0.05 was used for significance testing. Genotype distribution was tested for Hardy–Weinberg equilibrium (HWE). Comparison of RNA expression levels of candidate genes was done using one-way ANOVA and student's *t*-tests. For analyzing PFS, patients who stopped imatinib or switched treatment during follow-up were censored at the time of stopping or switching.

Results

Patient demographics. The demographics of the CP-CML patients enrolled in this study are listed in Table 1. There were 108 males and 52 females with a median age of 36 (range 18–65) years. One hundred and ten patients had low or intermediate Sokal score while 50 patients had high Sokal Score. Each patient started with an oral dose of 400 mg of imatinib daily. The median follow-up of the patients post therapy was 83 months (range: 12 to 120 months). Of the 146 patients in whom karyotyping was done, 128 had only t(9;22) and 18 had the t(9;22) with additional chromosomal abnormalities.

Response to imatinib therapy. One hundred and fifty of the 160 patients (94%) achieved complete hematological response at 3 months. The proportion of patients who achieved EMR at 3, 6 months and MMR at 12 months' post imatinib treatment is listed in Table 2. Neither the *BCR-ABL1* transcript type nor Sokal score showed significant association with achieving EMR/MMR. The rate of FFS at 24 months' post imatinib therapy in these patients was 78 ± 3.3%. The events included switch of TKI (*n* = 18), escalation (*n* = 33) of imatinib dose due to in-ability to afford or access 2nd/3rd generation TKI, palliative therapy due to severe toxicity (*n* = 7), imatinib dose reduced due to intolerance (*n* = 5), stem cell transplantation (*n* = 1) and progressive disease (*n* = 3) requiring chemotherapy or death. The decision to switch TKI or change the dose of TKI was entirely upto the discretion of the treating doctor as per the department policy and ELN guidelines.

Spectrum of *BCR-ABL1* kinase domain mutation in imatinib non-responders. In patients who failed to achieve milestone responses to imatinib therapy, presence of mutation in the *BCR-ABL1* kinase domain was tested. Of the 80 patients tested, 22 patients had mutations in the *BCR-ABL1* kinase domain. The spectrum of *BCR-ABL1* kinase domain mutation identified in these patients is listed in Supplementary Table 2. T315I and G250E were the common mutations identified.

Genotype/Alele frequencies of genetic variants. The allele frequencies of the 29 genetic variants in 12 genes [drug transporter genes *SLC22A1*, *ABCB1* & *ABCG2* and drug metabolizing enzyme genes *GST*, *CYP3A4/A5*] screened in this patient cohort is listed (Supplementary Table 3). The genotypes of all the genetic variants were in Hardy–Weinberg equilibrium. The *SLC22A1* variants were also screened in normal healthy volunteers (*n* = 100), as there was no Indian data at the time we started this study (Supplementary Table 4). The

Age	Median (Range) : 36 years (18–65)
Sex	Males: 108; Females: 52
Sokal Score	Low (< 0.8) N = 49
	Intermediate (0.8–1.2) N = 61
	High (> 1.2) N = 50
Median follow-up (range)	83 (12–120 months)
Karyotype at diagnosis	Only 9;22 n = 128
	t(9;22)with additional chromosome abnormalities n = 18
	Not available N = 14
BCR-ABL1 Fusion transcript	
e13a2 type	51
e14a2 type	109
Drug	
Glivec	140
Veenat	20

Table 1. Patient Demographics (n = 160 CP-CML).

	3 months		6 months		12 months	
Molecular response*	< 10% (66/115; 57%)		< 1% (66/135; 49%)		< 0.1% (61/145; 42%)	
	e13a2	e14a2	e13a2	e14a2	e13a2	e14a2
	17	49	18	48	36	25
Transcript type#	> 10% (49/115; 43%)		> 1% (69/135; 51%)		> 0.1% (84/145; 58%)	
	e13a2	e14a2	e13a2	e14a2	e13a2	e14a2
	17	32	24	45	61	23

Table 2. Incidence of early molecular response (EMR) at 3 & 6 months and major molecular response (MMR) status at 12 months in CML-CP patients on imatinib therapy. *Denominators represent the number of patients for whom basal data was available at scheduled time point & those who were still on imatinib at that time. #Transcript type not associated with molecular response at 3, 6 and 12 months.

allele frequencies of *SLC22A1* variants were comparable between patients and normal controls. The *SLC22A1* exon5 variant (Arg287Gly) was in complete linkage disequilibrium with an exon 6 variant (Thr340Met). An 8 bp ins polymorphism in intron7 was in complete linkage disequilibrium with an exon7 coding variant (Met-408Val; rs628031) which results in a splice variant. When we tried to amplify the full length of *SLC22A1*cDNA in patients with the intron 7 ins polymorphism in the mutant state, there was a truncated transcript but no full length *SLC22A1* transcript while the heterozygotes showed both truncated and full length transcripts; the samples with wild type genotype showed only the full length transcript upon RT-PCR.

RNA expression of imatinib influx transporter *hOCT1* and ABC transporters. Expression of Imatinib influx (*hOCT1/SLC22A1*) and efflux transporters (*ABCB1*, *ABCG2*, *SLC22A1*, *ABCA3*, *ABCA5*, *ABCA6*, *ABCB5*, *ABCB6*, *ABCB7*, *ABCB8*, *ABCB10*, *ABCB11*, *ABCC1*, *ABCC3*, *ABCC4* and *ABCC11*) showed wide interpatient variability (Supplementary Table 5).

Plasma and intracellular imatinib and desmethyl imatinib levels. In this cohort, 87.5% of patients (n = 140) were on Glivec and 12.5% patients (n = 20) were on Veenat, a generic imatinib formulation from India. The trough plasma imatinib and desmethyl imatinib levels on day29 was available only in 67 patients. This is due to reasons such as patients not visiting the clinic by the end of one month, taking imatinib at night so trough level sampling was not possible or have withheld imatinib due to intolerance. There was no significant difference in trough plasma imatinib levels between patients receiving Glivec vs. generic imatinib. The median plasma imatinib and desmethyl imatinib concentrations on day29, were 1050 ng/mL (106–5035 ng/mL) and 191 ng/mL (31–2161 ng/mL) respectively. The median intracellular imatinib level after ex-vivo incubation of primary CML cells (n = 64) with imatinib was 1225 ng/mL (range 181–12848 ng/mL).

Plasma imatinib levels influence EMR and MMR to imatinib therapy. The median plasma imatinib level on day29 was significantly higher in those who achieved EMR at 3 months compared to those who did not (1280 ng/ml vs 887 ng/ml; p = 0.022 Fig. 1a). The median plasma imatinib levels on day29 was significantly higher in those who achieved MMR at 12 months compared to those who did not (1207 ng/ml vs 1022 ng/ml; p = 0.0417 respectively; Fig. 1b).

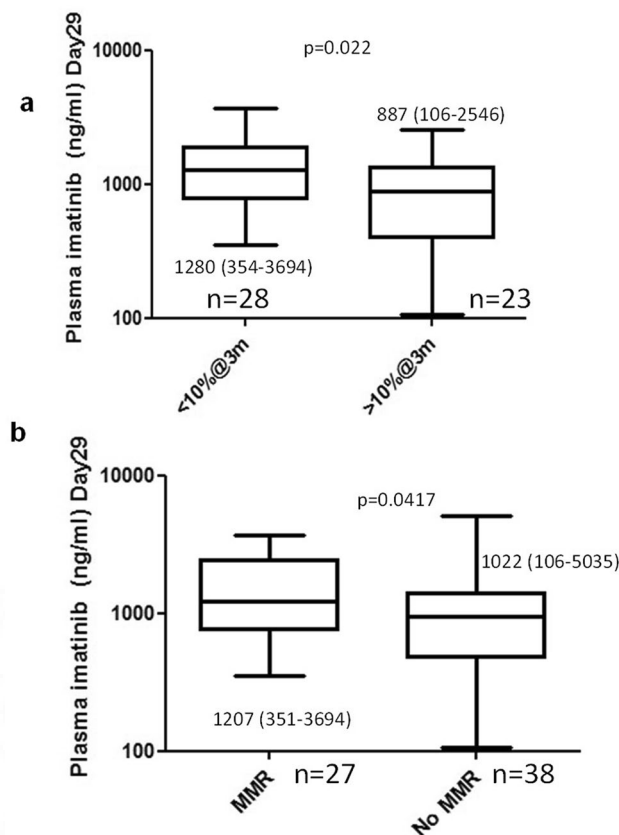


Figure 1. Plasma imatinib levels predict early molecular response at 3 & 6 months and MMR at 12 months post imatinib therapy. **(a)** Plasma imatinib levels (median, range) on day29 with early molecular response at 3 months and **(b)** in patients with and without MMR to imatinib at 12 months. Statistical significance was calculated using Mann–Whitney U test.

Plasma Imatinib levels are influenced by genetic variants in *ABCB1*. When the role of genetic variants in the target imatinib metabolism/ transport genes on trough plasma imatinib levels was evaluated, patients with variant *MDR1/ABCB1*-C1236T had significantly high day29 plasma imatinib concentration ($p = 0.005$) compared to those with wild type genotype (Fig. 2a). None of the other variants were significantly associated with plasma imatinib levels.

RNA expression of ABC transporter genes significantly influence intracellular but not plasma imatinib levels. The expression of influx and efflux transporters was compared with plasma and intracellular imatinib levels. Patients who had *ABCB1* expression above median showed significantly lower intracellular imatinib levels ($p = 0.0293$) (Fig. 2b). Patients who had *ABCA3* and *ABCC4* expression above median had lower intracellular imatinib levels (*ABCA3* $p = 0.0747$; *ABCC4* $p = 0.0536$) (Fig. 2c, d).

RNA expression of imatinib transporter genes in the CD34⁺ fraction and response to therapy. The expression of Imatinib efflux (*ABCB1*, *ABCG2*) and influx (*SLC22A1*) transporters in CD34⁺ as well as bulk CML cells was compared. There was a significantly increased expression of efflux transporters *ABCG2* ($p = 0.001$) and *ABCB1* ($p = 0.007$) and decreased expression of *SLC22A1* ($p < 0.001$) in CD34⁺ fraction compared to the total cellular RNA (Fig. 3a). Decreased expression of *hOCT-1* mRNA was observed in CML CD34⁺ cells compared to CD34⁺ cells derived from normal healthy donors (Fig. 3b). There was no significant association between RNA expression of these transporters in the CD34⁺ cells with EMR and MMR.

Genetic polymorphisms in imatinib transporter and drug metabolizing genes influence EMR and MMR to imatinib therapy. We further analyzed the influence of genetic polymorphisms in target genes on the incidence of EMR at 3 & 6 months and MMR at 12 months. The incidence of EMR at 3 months was significantly higher in patients with *MDR1*-C1236T variant genotype ($p = 0.044$) (Table 3) compared to those with wild type genotype. The incidence of EMR at 6 months was higher in patients with *MDR1*-C3435T variant genotype ($p = 0.058$) (Table 3) compared to those with wild type genotype. Also, the incidence of EMR at 6 months was higher in patients with *GSTM1* wild type genotype compared to those with null genotype ($p = 0.071$).

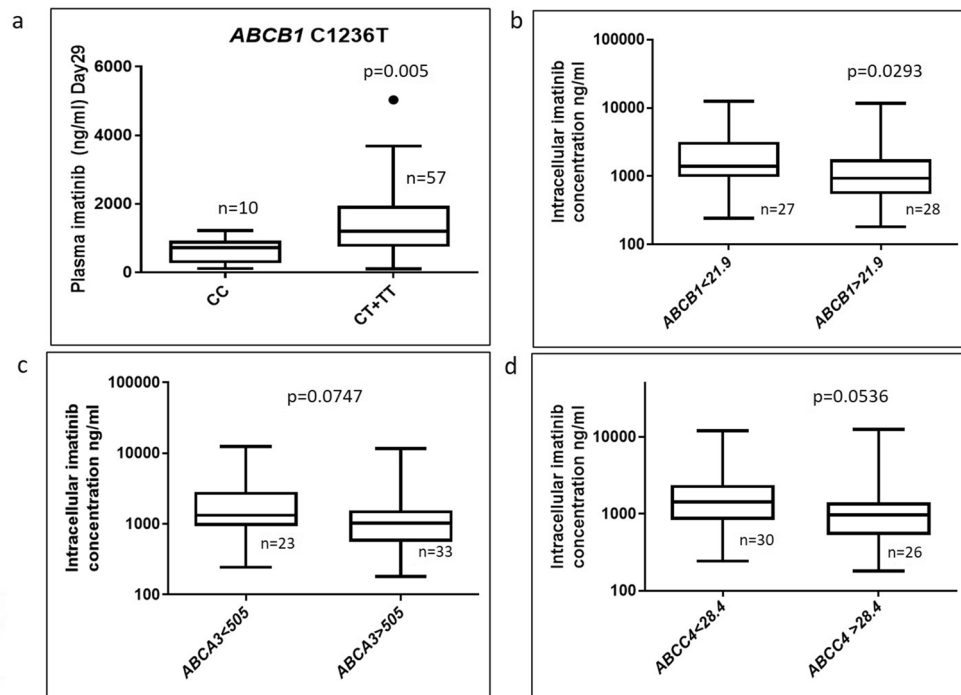


Figure 2. *ABCB1* polymorphism influences plasma imatinib levels and RNA Expression of ABC transporters influence intracellular Imatinib levels (b) Plasma imatinib levels on day29 post imatinib therapy in patients with *ABCB1/MDR1* C1236T genotypes (wt (CC) n = 10; Het + Mut (CT + TT) n = 57). (b) Intracellular imatinib levels in patients with above or below median expression of *ABCB1* (median expression-21.9), (c) *ABCA3* (median expression-505 below median n = 23 vs above median n = 33) and (d) *ABCC4* (median expression-28.4; below median n = 30 vs above median n = 26) RNA. The RNA expression of each gene was normalised to GAPDH and relative expression to CML001 using 2^{-ddCT} method. Statistical significance was calculated using Mann–Whitney U test.

Factors influencing 2-year FFS post imatinib therapy. We further analyzed the influence of all the basic demographic (including age, sex, Sokal score, *BCR-ABL1* transcript type) as well as variables including imatinib plasma levels, RNA expression and genetic polymorphisms of imatinib influx/efflux transporters on 2-year FFS post imatinib therapy. Patients with low (<0.8) and intermediate (0.8–1.2) Sokal score showed better 2-year FFS compared to those with high Sokal score (>1.2) (40/110 vs. 28/50 patients with low/intermediate vs. high Sokal score respectively had failure to imatinib therapy; $p=0.02$). None of the other basic demographic factors was significantly associated with FFS or EMR. Patients who had *MDR1* variant genotype showed significantly better FFS compared to the wild type genotype (*MDR1*- 1236; $p=0.005$, *MDR1*- 2677; $p=0.004$ and *MDR1*- 3435; $p=0.004$) (Fig. 4a–c). Also, patients with higher median Imatinib levels (>1757 ng/mL) on day29 had significantly better FFS ($p=0.057$) (Fig. 4d). Patients with *ABCA6* expression below median showed significantly better FFS compared to those with above median levels ($p=0.007$) (Fig. 4e). There was a trend to significantly lower rate of FFS in patients with *ABCC4* expression above median compared to below median ($p=0.066$) (Fig. 4f). Patients who achieved EMR at 3 and 6 months had significantly better rate of FFS compared to those who did not achieve EMR (Supplementary Fig. 1a–b). Patients who achieved MMR at 12 months also showed significantly better rate of FFS compared to those who did not achieve MMR ($p=0.002$) (Supplementary Fig. 1c). High Sokal score (Hazard ratio: 1.53; p value: 0.05), *MDR1* C2677T genotype (Hazard ratio: 0.401; p value: 0.014) and not achieving EMR at 3 months post imatinib therapy (Hazard ratio: 3.875; p value: 0.0001) were significant risk factors for 2-year FFS in multivariate analysis (Tables 4, 5). Plasma imatinib levels were available only in 51 patients for whom molecular response was also available and hence could not be included as a variable in multivariate analysis.

Discussion

Targeted therapy with imatinib is still the first line treatment of choice in newly diagnosed patients with CML in chronic phase (CML-CP). However, a proportion of patients do not achieve milestone molecular responses with imatinib and are classified as sub-optimal responders. Identifying these patients followed by early switch of therapy will help improve FFS. Although several studies have evaluated factors influencing attainment of MMR after imatinib therapy, to the best of our knowledge, there is no prospective study evaluating various factors influencing EMR and FFS after imatinib therapy, especially in a uniform cohort of CP-CML patients. We conducted a prospective single centre observational study in CP-CML patients with serial molecular monitoring and comprehensive pharmacogenetic analysis to evaluate factors influencing EMR to imatinib and FFS. Response to imatinib as seen by achieving milestone molecular response post imatinib therapy was similar to previous

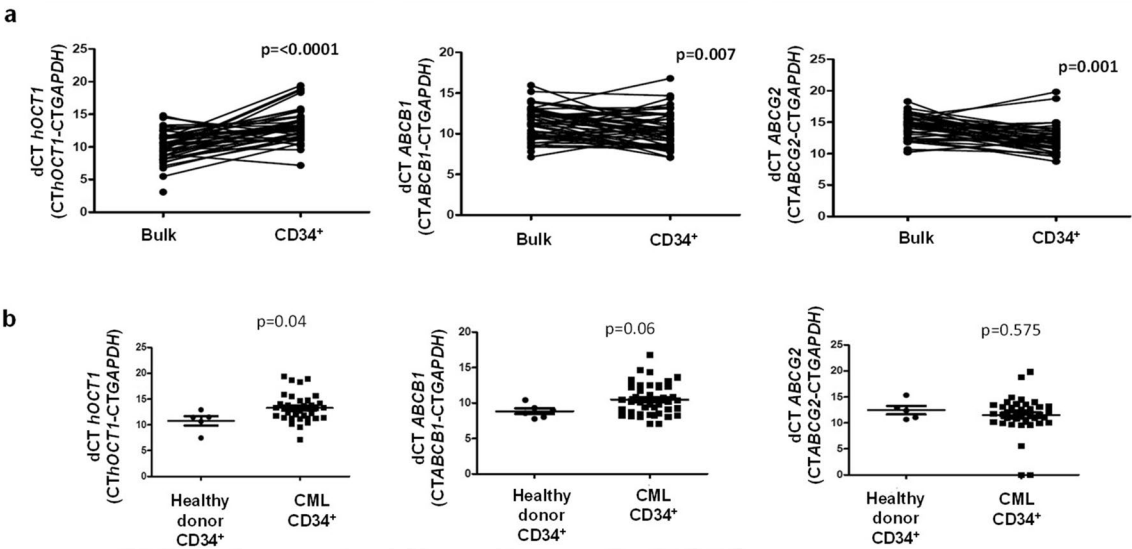


Figure 3. Expression of imatinib influx and efflux transporters in bulk Vs CD34⁺ primary CML cells. **(a)** Expression of *hOCT1*, *ABCB1/MDR1* and *ABCG2* in CML bulk cells compared with expression of transporter expression in CML CD34⁺ (Leukemic stem cells) LSCs cells. Statistical significance was calculated using paired-T test. **(b)** Expression of *hOCT1*, *ABCB1/MDR1* and *ABCG2* in CD34⁺ cells from normal healthy donors vs. primary CML CD34⁺ cells where higher the dCt lower the expression and vice versa. Statistical significance was calculated using Mann–Whitney U test.

	Genotype	<i>BCR-ABL1</i> < 10% n (%)	<i>BCR-ABL1</i> > 10% n (%)	<i>p</i> value
<i>MDR1</i> 1236 genotype & EMR at 3 months (n = 114)	Wt	7 (35)	13 (65)	0.045*
	Het/Mut	58 (61.7)	36 (38.2)	
	Genotype	<i>BCR-ABL1</i> < 1% n (%)	<i>BCR-ABL1</i> > 1% n (%)	<i>p</i> value
<i>MDR1</i> 3435 genotype & EMR at 6 months (n = 134)	Wt	6 (28.5)	15 (71.5)	0.058
	Het/Mut	59 (52.2)	54 (47.8)	
<i>GSTM1</i> genotype & EMR at 6 months (n = 134)	Positive	47 (55.3)	38 (44.7)	0.071
	Negative	18 (38.3)	29 (61.7)	

Table 3. *ABCB1/MDR1* C1236T, C3435T and *GSTM1* genotype influences early molecular response at 3 and 6 months. Statistical significance was calculated using Fisher exact test.

reports^{35,36}. Mutations in the *BCR-ABL1* kinase domain explained only 28% of the patients with sub-optimal response, which is also similar to previous reports^{37–39}. None of the basic demographic parameters including age, sex, Sokal score and *BCR-ABL1* transcript type showed significant association with achieving EMR/MMR post imatinib therapy in the present study. Similar to previous reports from India, the median age of diagnosis in of CML in our cohort is lower compared to Western patients (36 yrs vs. 47yrs)⁴⁰, majority of the patients younger than 40 years. This is probably the reason for age not showing significant association with response to imatinib therapy. While the distribution of low, intermediate and high Sokal scores was similar to Western data in CP-CML patients as reviewed by Ganesan and Kumar⁴⁰, patients with high Sokal score showed significantly poor 2-year FFS in this study, similar to previous reports^{41,42}.

Several studies have reported that genetic variants in the influx and efflux transporters of imatinib namely *hOCT1*, *ABCG2* and *MDR1* to be associated with imatinib levels^{16,20,43,44}. In the present study, similar to previous reports, patients with variant *MDR1* genotype showed significant association with plasma imatinib levels. The variants of *MDR1* C1236T, C3435T and G2326T were shown to result in decreased *MDR1* expression^{45–47} which in turn results in higher plasma imatinib levels and hence better EMR/MMR/FFS. Increased RNA expression of ABC transporters *ABCB1*, *ABCA3* and *ABCC4* were significantly associated with lower intracellular imatinib levels in our study. It has been reported previously that increased expression of *ABCB1*⁴⁸, *ABCA3*⁴⁹ and *ABCC4*⁵⁰ to be associated with imatinib resistance. Unlike previous reports, none of the genetic variants in *hOCT1* were significantly associated with plasma imatinib levels or molecular response in the present study.

Higher trough plasma Imatinib levels have been shown to be associated with significantly better molecular response (Table 4), including the present study. The median plasma imatinib levels in patients with good response vs. suboptimal response in our study is similar to previous reports^{16,17,26,51,52}. However, from the available data from India, although various measures of response including clinical, cytogenetic or MMR were considered,

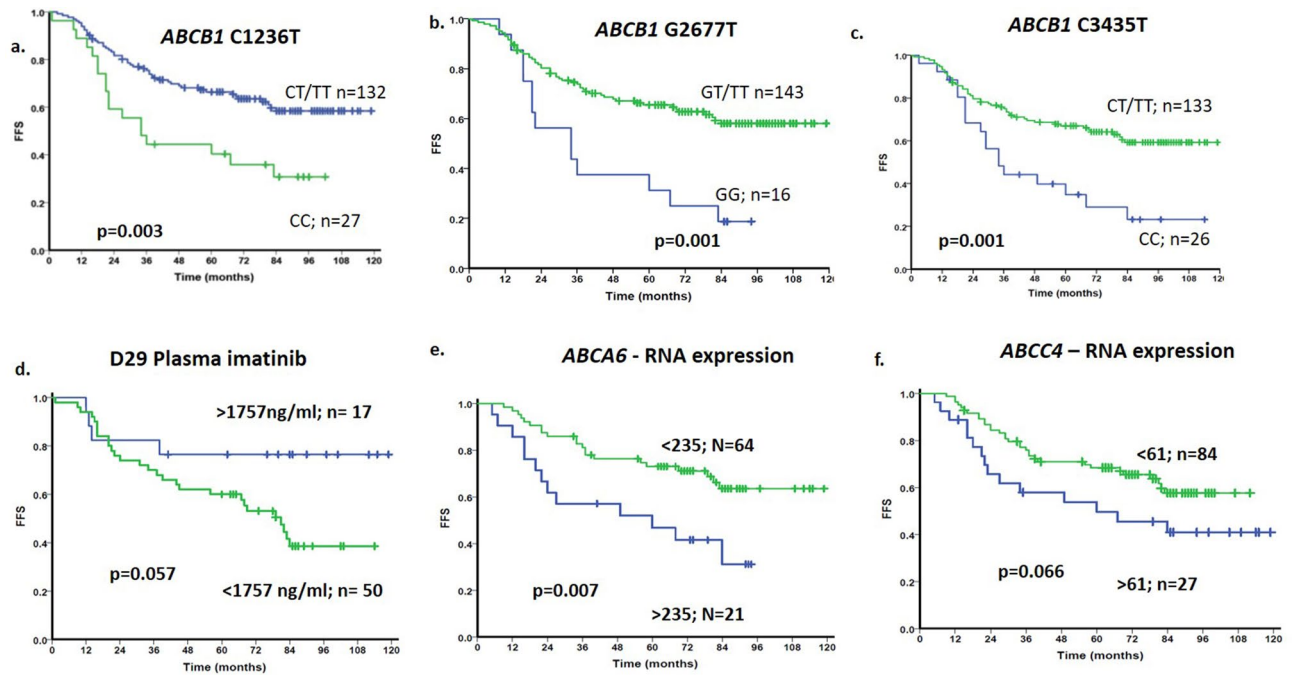


Figure 4. *MDR1* polymorphisms, plasma imatinib levels, and RNA expression of efflux transporters influence FFS after imatinib therapy in patients with CP-CML. (a)–(c) Influence of *MDR1* genotypes C1236T, G2677T & C3435T on FFS after imatinib therapy. (d) Influence of plasma imatinib levels on day29 on FFS. (e)–(f) Influence of *ABCA6* and *ABCC4* expression on FFS after imatinib therapy. RNA expression was normalised to *GAPDH* as the housekeeping gene and expressed relative to the expression in CML001 using $2^{-\Delta\Delta CT}$ method.

Variables	p value	Hazard ratio
Sokal score	0.001*	1.835 (1.272–2.647)
EMR at 3 months	0.000*	4.092 (2.269–7.381)
EMR at 6 months	0.000*	6.291 (3.281–12.061)
MMR at 12 months	0.000*	10.627 (4.552–24.809)
Desmethyl imatinib day-29	0.052	0.997(0.993–1.000)
plasma imatinib day-29	0.010*	0.999 (0.999–1.000)
ABCA6	0.009*	0.399 (0.199–0.799)
ABCC1	0.055	1 (1.000–1.011)
ABCC4	0.070	0.567 (0.307–1.048)
<i>MDR1</i> G2677T	0.001*	0.374 (0.204–0.686)
<i>MDR1</i> C3435T	0.002*	0.416 (0.242–0.715)
Sokal score	0.05*	1.513 (1.001–2.287)
<i>ABCB1</i> (<i>MDR1</i>) C2677T variant genotype	0.014*	0.401 (0.193–0.833)
Not achieving EMR at 3 months	0.0001*	3.875 (2.132–7.046)

Table 4. Univariate and Multivariate analysis for 2-year failure-free survival.

the imatinib levels in good responders seems to be higher than the previous reports from outside India^{43,44,53,54}, and the present study. The two Indian studies that showed association between molecular response and plasma Imatinib levels have assessed either hematological response^{43,53} or considered *BCR-ABL1* ratio below 1% as molecular response. The median trough plasma Imatinib levels on day29 can be a used as biomarker for assessment of response to imatinib therapy to rule out issues related to poor compliance as well as identifying poor or ultra-rapid metabolizers who could benefit from adjustment of imatinib doses.

RNA expression of influx and efflux transporters of imatinib have been shown to influence response to imatinib therapy. Several studies have reported increased expression or functional activity of *hOCT1* to be associated with better response to imatinib^{13,14,55}. However, the RNA expression of these transporters did not show any association with EMR or MMR in the present study. Interestingly, when the expression of these transporters in CML CD34⁺ vs. bulk cells was compared, there was significantly increased *ABCB1* and *ABCG2* expression and decreased *hOCT1* in the CD34⁺ cells compared to CML bulk cells. This is in line with the fact that imatinib

Reference	N	Response	Responder		Non responders		p value
			N	Mean C0 ng/mL	N	Mean C0 ng/mL	
Larson et al., 2008 ¹⁶	351	CCyR	297	1099 ± 554	54	812 ± 409	0
Takahashi et al., 2010 ⁵¹	254	CCyR	218	1057 ± 585	36	835 ± 524	0.033
		MMR	166	1107 ± 594	88	873 ± 528	0.002
Picard et al., 2007 ¹⁷	68	CCyR	56	1123 ± 617	12	694 ± 556	0.03
		MMR	34	1452 ± 649	34	869 ± 427	0.001
Ishikawa et al., 2010 ²⁶	60	MMR	38	1093	22	853	0.002
				562–2150		361–1205	
Forrest et al., 2009 ⁵²	78	CCyR	53	1010 ± 469	24	1175 ± 656	0.29
		MMR	51	1067 ± 473	27	1063 ± 643	0.74
Singh et al., 2009 ⁵³	40	Clinical response	20	2340 ± 520	20	690 ± 150	0.002
Malhotra et al., 2014 ⁴³	131	Molecular response (< 1%)	104	2110 ± 1180	27	1310 ± 720	0.001
Natarajan et al. 2019 ⁵⁴	173	MMR	71	2333 ± 1112	102	1643 ± 1384	P < 0.001
Arora et al., 2013 ⁴⁴	46	CCyR	NA	2157 ± 1287	NA	1884 ± 809	P > 0.05
Present study	51	EMR@3 months	28	1532 ± 939	23	971 ± 714	0.022*
	65	MMR@12 months	28	1566 ± 991	37	1160 ± 921	0.042*

Table 5. Association of steady state plasma imatinib levels with response- comparison with previous studies.

does not eliminate CML stem cells^{56,57} probably due to this dysregulated expression of the transporters in the CML CD34⁺ cells.

Polymorphisms in imatinib influx/ efflux transporters have been reported to influence response to imatinib therapy and progression free survival^{15,19,58–60}. Similar to these reports, *MDR1* and *ABCG2* variants were associated with better EMR/MMR in the present study. Higher plasma imatinib levels^{16,60}, attainment of EMR (at 3 or 6 months)^{61–63} & MMR at 12 months^{64,65} have been reported to result in better FFS in patients with CML on imatinib therapy. In the present study, *MDR1* variants, day29 plasma imatinib level of > 1757 ng/mL, lower *ABCA6*, *ABCC4* RNA expression as well as achieving EMR at 3, 6 months and MMR at 12 months were associated with significantly better FFS. While increased *ABCC4* RNA expression has been reported previously in suboptimal response to imatinib^{15,17,21,22,26}, the role on increased *ABCA6* on imatinib response is not clear.

Our study suggests that factors such as steady state plasma Imatinib levels, *MDR1* polymorphisms and ABC transporter expression influence EMR/MMR to imatinib therapy, which in turn influence FFS in patients with CP-CML. The possibility to tailor dose of imatinib considering these factors in order to improve molecular response to imatinib and better FFS, remains to be tested.

Received: 18 May 2020; Accepted: 2 November 2020

Published online: 26 November 2020

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Acknowledgements

This study is supported by Department of Biotechnology India-Programme support Grant: BT/01/COE/08/03; Centre of Excellence grant from Department of Biotechnology India: BT/COE/34/SP13432/2015 and Indian Council of Medical Research Centre for Advanced Research Grant 70/14/14-CAR to Dr. Poonkuzhali Balasubramanian. RVS, VM and PB are supported by Wellcome DBT India Alliance (IA/S/17/1/503118, IA/CPHS/18/1/503930 and IA/S/15/1/501842) respectively. UK is supported by an early career fellowship program of Wellcome DBT India Alliance (IA/CPHE/17/1/503351), Government of India. SK is supported by University Grants Commission, ESB by DST Inspire fellowship and SV by Indian Council of Medical Research, Government of India. We sincerely acknowledge the encouragement and support provided by Dr. Mammen Chandy, Professor and Former Head, Department of Haematology, CMC Vellore, currently Director, Tata Medical Centre, Kolkata in the initial stages of this study. The help provided by Ms. Preetha Markose & Dr. Ajay Abraham in the initial stages of this study, Mr. Christopher Benjamin, Mr. Selvakumar, Ms. Kalaiselvi in CML patient recruitment for the study and by Dr. Eunice S. Edison in managing the DNA sequencing core facility are gratefully acknowledged.

Author contributions

B.M.R., E.S.B., S.G., S.A., S.K., S.V., E.M. and P.B. performed experiments, analysed the results. P.B. designed the research. B.M.R. and P.B. wrote the manuscript. K.M.L. contributed to statistical analyses. N.B.J., V.M.S., S.R.V., A.A., U.K., A.J.D., F.N.A., A.K., B.G., A.S., V.M. – enrolled patients, managed them and provided clinical data. P.B. and V.M. reviewed the manuscripts and approved the final version.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41598-020-77140-9>.

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RECEIVED 15 March 2023

ACCEPTED 18 May 2023

PUBLISHED 31 May 2023

CITATION

Rajamani BM, Illangeswaran RSS,
Benjamin ESB, Balakrishnan B,
Jebanesan DZP, Das S, Pai AA,
Vidhyadharan RT, Mohan A,
Karathedath S, Abraham A, Mathews V,
Velayudhan SR and Balasubramanian P
(2023), Modulating retinoid-X-receptor
alpha (RXRA) expression sensitizes
chronic myeloid leukemia cells to
imatinib *in vitro* and reduces disease
burden *in vivo*.
Front. Pharmacol. 14:1187066.
doi: 10.3389/fphar.2023.1187066

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Pai, Vidhyadharan, Mohan, Karathedath,
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Modulating retinoid-X-receptor alpha (RXRA) expression sensitizes chronic myeloid leukemia cells to imatinib *in vitro* and reduces disease burden *in vivo*

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Introduction: The ligand-activated transcription factors, nuclear hormone receptors (NHRs), remain unexplored in hematological malignancies except for retinoic acid receptor alpha (RARA).

Methods: Here we profiled the expression of various NHRs and their coregulators in Chronic myeloid leukemia (CML) cell lines and identified a significant differential expression pattern between inherently imatinib mesylate (IM)-sensitive and resistant cell lines.

Results: Retinoid-X-receptor alpha (RXRA) was downregulated in CML cell lines inherently resistant to IM and in primary CML CD34⁺ cells. Pre-treatment with clinically relevant RXRA ligands improved sensitivity to IM *in-vitro* in both CML cell lines and primary CML cells. This combination effectively reduced the viability and colony-forming capacity of CML CD34⁺ cells *in-vitro*. *In-vivo*, this combination reduced leukemic burden and prolonged survival. Overexpression (OE) of RXRA inhibited proliferation and improved sensitivity to IM *in-vitro*. *In-vivo*, RXRA OE cells showed reduced engraftment of cells in the bone marrow, improved sensitivity to IM, and prolonged survival. Both RXRA OE and ligand treatment markedly reduced BCR::ABL1 downstream kinase activation, activating apoptotic cascades and improving sensitivity to IM. Importantly, RXRA OE also led to the disruption of the oxidative capacity of these cells.

Conclusion: Combining IM with clinically available RXRA ligands could form an alternative treatment strategy in CML patients with suboptimal response to IM.

KEYWORDS

chronic myeloid leukemia (CML), imatinib, RXRA, RXRA ligands, resistance

1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by a reciprocal chromosomal translocation t(9;22) that generates the BCR::ABL1 fusion protein, a constitutively active tyrosine kinase essential for the pathogenesis of the disease. Molecular targeted therapy with tyrosine kinase inhibitors (TKI) such as imatinib mesylate (IM) has dramatically improved the outcome of this disease. However, a proportion of patients fail to achieve or retain molecular response with TKIs leading to disease progression.

The mechanism of TKI resistance in CML has been extensively studied and broadly classified as BCR::ABL1 dependent and independent. BCR::ABL1-dependent mechanisms include mutations in the BCR::ABL1 kinase domain and over expression of BCR::ABL1 or gene expression. BCR::ABL1 independent resistance mechanisms include the persistence of leukemic stem cells, activation of multiple signaling pathways, and epigenetic events (Milojkovic and Apperley, 2009; Dohse et al., 2010; Patel et al., 2017; Loscocco et al., 2019). Mutations in the BCR::ABL1 kinase domain are seen in ~30% of CML patients with suboptimal responses to imatinib. Some patients fail multiple TKIs without having documented kinase domain mutations. Effectively targeting these TKI-insensitive cells by combination therapies that interfere with various signaling pathways has been attempted previously (Prost et al., 2015; Irvine et al., 2016; Wagle et al., 2016; Agarwal et al., 2017; Finch et al., 2017). Although combining repurposed clinical agents such as venetoclax (Ko et al., 2014; Carter et al., 2016), pioglitazone (Prost et al., 2015), and tigecycline (Kuntz et al., 2017) with TKIs are shown to eliminate leukemic stem cells (LSCs) selectively, these drugs are not translated into clinics for CML treatment. Targeting nuclear hormone receptors (NHRs) with specific ligands has improved disease outcomes and survival in solid tumors (Culig, 2014; Wong et al., 2014; de Bono et al., 2018; Rathkopf et al., 2018; Smith et al., 2018; Munster et al., 2019). However, except for the role of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (APL) (Grignani et al., 1998; Lengfelder et al., 2005; Zheng et al., 2005; Park et al., 2008), no studies show the role of NHRs in modulating imatinib resistance in CML cells.

NHRs are a family of ligand-dependent transcription factors frequently dysregulated in various cancer types, rendering this family of proteins potential therapeutic targets for cancers (Petrie et al., 2007; Feldman et al., 2014; Quintero Barceinas et al., 2015). NHR ligands bind to the ligand binding domain of the receptor, which dissociates the co-repressors from the receptor and facilitates the binding of the co-activator for receptor activation (Sonoda et al., 2008). NHR ligand treatments improved disease outcomes with limited toxicity in various solid tumors. The most widely explored NHRs are estrogen (ER) and androgen receptor (AR), and their ligands are used as monotherapy or combination therapy for breast and prostate cancer. Long-term treatment of ER ligand tamoxifen in patients with breast cancer reduced disease mortality and death compared to no tamoxifen therapy (Group EBCTCG EBCTC, 2011; Davies et al., 2013). AR ligands (Flutamide, Bicalutamide, Enzalutamide, and Apalutamide) increased metastasis-free survival in patients with metastatic prostate cancer (Culig, 2014; Wong et al., 2014; de Bono et al., 2018; Rathkopf et al., 2018; Smith

et al., 2018). Glucocorticoid receptor (GR) ligand, Relacorilant in combination with Nab-paclitaxel improved outcomes with limited toxicity in patients with adenocarcinoma and ovarian cancer (Munster et al., 2019). Retinoid-X-receptor alpha (RXRA) ligand bexarotene in combination with chemotherapy was more effective associated with better survival, and improved response in refractory cutaneous T-cell lymphoma (CTCL) (Duvic et al., 2001a; Duvic et al., 2001b; Pileri et al., 2013), metastatic breast cancer (Esteve et al., 2003; Yen et al., 2004; Yen and Lamph, 2005) and non-small cell lung carcinoma (NSCLC) (Khuri et al., 2001; Edelman et al., 2005). The role of NHRs in myeloid malignancies is relatively less explored except for the use of RARA (retinoic acid receptor alpha) ligand all-trans retinoic acid (ATRA) as a differentiation agent in APL. Preliminary results from our laboratory suggest that NHRs such as PPARG and RXRA were downregulated in chemoresistant acute myeloid leukemia (AML) cell lines (Karathedath et al., 2013), suggesting that NHRs can be modulated to improve chemosensitivity.

There are limited reports on the use of RXRA ligands in hematological malignancies. Retinoid-X-receptors (RXR) are members of the NHR superfamily (Evans and Mangelsdorf, 2014). 9-cis retinoic acid, 9 cis-13,14-Dihydroretinoic acid, and long-chain fatty acids are natural ligands for RXR (Arnold et al., 2012; Jones et al., 2015; Rühl et al., 2015; Niu et al., 2017). In mammalian cells, three genes code for three RXR isoforms- RXRA, RXRB, and RXRG (Nohara et al., 2009). RXRs either homodimerize or heterodimerize with other NHRs for transcriptional activation. The heterodimeric partners of RXRs include retinoic-acid receptor (RAR), vitamin-D-receptor (VDR), peroxisome proliferator-activated receptors (PPAR), liver-X-receptor (LXR), pregnane-X-receptor (PXR), and the constitutive androstereone receptor (CAR) (Bugge et al., 1992; Kliewer et al., 1992; Willson and Kliewer, 2002; Calkin and Tontonoz, 2012). RARA and RXRA expression is downregulated in AML cells compared to myelodysplastic syndrome (Welch et al., 2014). Several studies have shown the efficacy of bexarotene as a pan-RXR ligand in AML. Bexarotene treatment induced differentiation and apoptosis in AML blast cells *in-vitro* and in AML cell lines (Altucci et al., 2005; Sanchez et al., 2014). AML patients treated with bexarotene showed differentiation *in-vivo* (Tsai et al., 2008). Bexarotene, combined with decitabine, was well tolerated and showed a modest response in older relapsed AML patients (Welch et al., 2014). The dual activation of RXRA-RARA heterodimers by the specific ligands, ATRA and bexarotene effectively targeted MLL-AF9 myelomonocytic leukemic cells *in-vitro* and improved the survival *in-vivo* in mice models (Di Martino et al., 2021a). DT448/9PP mutated RXRA receptor overexpression enhanced transcriptional activity leading to differentiation and loss of colony-forming ability in KMT2A-MLLT3 transformed AML cells (Di Martino et al., 2021b). Bexarotene treatment increased the protein expression of RXR isoforms (RXRA, RXRB & RXRG) in SH-SY5Y cells (Dheer et al., 2018). Bexarotene treatment increased the protein expression of RXR and PPARG in brain cells (Zuo et al., 2019). RXRA ligand treatment increased the mRNA expression of other NHRs, such as PPARG, LXRA, & LXR (Di Martino et al., 2021a), in MLL-AF9 AML mice *in-vivo*.

Few studies have addressed the role of NHRs in modulating TKI sensitivity in CML. PPARG agonist pioglitazone was reported to target CML cells effectively by down-regulating transcription factors such as

STAT5, CITED2, and HIF2a, which are overexpressed in CML stem cells (Prost et al., 2015). PPARG ligand-thiazolidinedione has been reported to synergize with TKIs and target CML stem cells (Prost et al., 2015; Glodkowska-Mrowka et al., 2016). CML patients treated with pioglitazone combined with imatinib achieved a deeper molecular response of MR4.5 at 12 months (Rousset et al., 2017). However, the role of other NHRs and the effect of their ligands in overcoming inherent TKI resistance in CML is still not completely understood.

We investigated the differential expression profile of NHRs in CML cell lines that are inherently imatinib sensitive vs. resistant. We also assessed the effect of RXRA ligand treatment combined with imatinib on improving TKI sensitivity in CML cell lines, primary cells *in-vitro*, and in a CML cell line-derived xenograft mice model.

2 Materials and methods

2.1 Chemicals and reagents

Imatinib Mesylate (SML1027), methyl thiazolyldiphenyl-tetrazolium bromide (MTT), 9-cis retinoic acid (9cRA), Bexarotene (Bexa), acitretin (ACI), SR11237, all trans retinoic acid (ATRA), testosterone, 17 β -estradiol (17 β E), pioglitazone (PIO), rosiglitazone (rosi), Triiodothyronine (T3) and GSK4716 were from Sigma-Aldrich (St Louis, MO, United States). Rabbit monoclonal p-CRKL (3,181), p-STAT5 (9,351), p-AKT (4,060), caspase-3 (9,661), PARP (5,625), and RXRA (3,085) were from Cell Signaling Technologies (Danvers, MA, United States). β -actin (A5441) was from Sigma-Aldrich (St. Louis, MO), and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000), goat anti-mouse IgG (1:10,000) from Cell Signaling Technologies (Danvers, MA, United States). Imatinib Mesylate used for mice model experiments is a gift from NATCO, India.

2.2 Cell lines and primary cells

CML cell lines JURL-MK1, KCL22, Lama84, KYO1, EM2, and KU812, were a kind gift from Dr. Markus Muschen, University of California San Diego (UCSD), with approval from DSMZ, Germany. Cell lines were cultured in RPMI medium with 10% FBS and antibiotics (100 U/ml-penicillin and 100 μ g/mL-streptomycin) in a humidified atmosphere with 5% CO₂ at 37°C.

Bone marrow or peripheral blood samples were collected after obtaining written informed consent from imatinib naïve adult CP-CML patients at diagnosis before the initiation of therapy. This study was approved by the Institutional Review Board of Christian Medical College, Vellore (IRB Min No. 8704). Primary CML cells from blood or bone marrow were processed and enriched for CML CD34⁺ cells, as reported previously (Rajamani et al., 2020).

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from CML cell lines (JURL-MK1, KU812, KYO1, EM2, Lama84, and KCL22) and treated with or without NHR ligands using Trizol reagent. RNA (1 μ g) was converted to cDNA using random hexamer and reverse transcriptase enzyme (High-capacity cDNA synthesis kit, Thermo Scientific).

2.4 NHR profiling

To identify the basal expression of NHRs and coregulators in CML cell lines, we used an NHR RT² PCR array (SA Biosciences, Germany) (<https://geneglobe.qiagen.com/product-groups/rt2-profiler-pcr-arrays>). The expression of each NHR was normalized using five housekeeping genes (*ACTB*, *GAPDH*, *B2M*, *HGPRT* & *RPLP0*), as recommended by the manufacturers. The data was analyzed using SA biosciences web-based analysis software (www.sabiosciences.com/pcr/arrayanalysis.php).

2.5 q-RT-PCR analysis of NHR expression

The expression of *AHR* (TaqMan assay ID: Hs00169233_m1), *AR* (Hs00171172_m1), *ESR1* (Hs01046816_m1), *ESRRG* (Hs00976243_m1), *PPARG* (Hs01115513_m1), *RXRA* (Hs0107640_m1), *RXRB* (Hs 00232774_m1) and *THRA* (Hs00268470_m1) were evaluated by qRT-PCR. Imatinib influx transporter *SLC22A1/hOCT1* (Hs00427554-m1) expression was measured using TaqMan assay after treating the primary CML cells with NHR ligands. *GAPDH* was used as the housekeeping gene for these targets. Fold change in the expression of other NHRs such as *RARA*, *PPARG*, and *VDR* was measured by RQ-PCR using SYBR green chemistry (SYBR premix Ex TaqII (Tli RNaseH Plus), Takara, Bio, Shiga, Japan), and *ACTB* was used as the housekeeping gene to calculate the relative expression ($\Delta\Delta C_t$) of these targets.

2.6 In-vitro-cytotoxicity assay

CML cell lines JURL-MK1, KCL22, Lama84, KYO1, EM2, and KU812 [1×10^5], were treated in a 96-well plate with increasing concentrations of imatinib (0–500 nM) for 48 h. Cell viability was measured at standard wavelength 570 nm and reference wavelength 630 nm using EL800 microplate reader with KC junior software (Biotek Winooski, VT, United States).

CML cell lines (KCL22 & Lama84) were pretreated with NHR agonists (agonists and their concentrations as listed in Supplementary Table S1) for 24 h, followed by treatment with increasing concentration of imatinib (0 nM–500 nM). All the experiments were performed in triplicates. The inhibitory concentration of 50% (IC-50) was calculated using Adapt software.

As suggested by previous reports (Austin et al., 2015; Wang et al., 2017), bulk primary CML cells [3×10^5] were pretreated with NHR agonists for 24 h, followed by treatment with increasing concentration (0–100 μ M) of imatinib for 48 hrs.

2.7 RNA expression by nanostring

RNA was extracted from CML CD34⁺ cells ($n = 39$) and healthy donor CD34⁺ cells ($n = 9$) using total RNA purification plus kit (Norgen Biotek Corp, Canada). Expression of *RXRA* was assessed using nCounter, and data counts were normalized with housekeeping genes–*ACTB*, *GAPDH* & *GUS* using nSolver software (NanoString, Technologies, Seattle, WA).

2.8 Colony forming unit assay

Primary CML (bulk & CD34⁺) or healthy donors (peripheral blood mononuclear cells—PBMNCs & CD34⁺) cells (2×10^5) were treated with and without RXRA ligands 9-cis retinoic acid, acitretin, or bexarotene in combination with imatinib for 24 h; 1×10^4 cells were seeded in methylcellulose medium (MethoCult™ H3334 classic, StemCell Technologies, Vancouver, Canada). Colonies were visualized using a phase-contrast microscope ($\times 10$ objective) and enumerated after 14 days.

2.9 Apoptosis assay

Primary CML CD34⁺ cells (2×10^5) were treated with RXR ligands 9-cis retinoic acid, acitretin, or bexarotene for 24 h, followed by imatinib for 48 h. Cells were washed with 1XPBS and stained with Annexin-V conjugated with allophycocyanin (APC) and 7-Aminoactinomycin D (7AAD) (BD-bioscience, San Diego, CA). BD-Accuri C6 (BD Biosciences, Franklin Lakes, NJ) with BD Accuri C6 software (Version 1.0.264.21) was used for flow cytometry analysis.

2.10 Western blot analysis for BCR-ABL downstream signaling pathway

CML cell lines (KCL22, Lama84) or primary CML cells were treated with RXRA ligands (9-cis retinoic acid, acitretin, or bexarotene) for 24 h, followed by imatinib for 48 h. The whole-cell lysate was prepared using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease-phosphatase inhibitor cocktail (Roche, IN, United States) and 2 mM phenyl methyl sulfonyl fluoride (Sigma Aldrich). Total protein concentration was measured using the Bradford assay. 50 µg of protein was resolved in a 10% SDS PAGE. Anti-p-CRKL, anti-p-AKT, anti-p-STAT5, anti-caspase3, and anti-PARP antibodies were used, and β -actin was used as the loading control.

2.11 Overexpression of RXRA in CML cell line

The RXRA plasmid containing the full-length cDNA, pLX304 (clone ID- HsCD00437134), was purchased from the DNASU plasmid repository (Arizona, United States). Lentivirus particles were prepared by transfecting 293T cells with the RXRA cDNA plasmid and packaging plasmids. Lentiviruses were collected after 48, 60, and 72 h, pooled together, filtered, and concentrated using a lenti-X concentrator (Takara, Bio, Shiga, Japan), and stored at -80°C until use. Lama84 cells were transduced with lentiviruses in the presence of polybrene, followed by spinfection. RXRA OE virus transduced Lama84 cells were selected by blasticidin (1 mg/mL).

2.12 CML cell line-derived xenograft (CDX) mice model

293T cells were transfected with plasmid lentiX-Luc2 containing a luciferase reporter for lentivirus production. KCL22 cells were transduced with lentivirus containing the luciferase reporter. Luciferase transduced KCL22 (1×10^6 cells) were then transplanted into sub-lethally irradiated (2.5Gy) 8–10 weeks old NSG mice (Jackson Laboratory, United States) via tail-vein. Disease development was monitored by measuring luciferase expression by injecting D-luciferin substrate and imaging using an IVIS Spectrum *in-vivo* imaging system (PerkinElmer, United States). One week after transplantation, the mice were treated orally with either imatinib mesylate (100 mg/kg dissolved in water) (NATCO-Pharma Limited, India) or acitretin (10 mg/kg dissolved in corn oil) or in combination for 21 days. Engraftment of KCL22 cells in the mice was assessed by measuring luciferase expression every week for 6–7 weeks. All animal experiments were carried out per protocols approved by the Institutional Animal Care and Use Committee (IAEC No. 5/2019).

RXRA/empty vector transduced Lama84 (2×10^6) cells were transplanted into sub-lethally irradiated (2.5Gy) 8–10 weeks old NSG mice via tail-vein. The leukemic burden in peripheral blood was assessed using flow cytometry by measuring human CD45 expression (BD-bioscience, CA). The percentage of survival of mice was evaluated by Kaplan-Meier survival analysis.

2.13 Extracellular flux analysis using Seahorse XFe24 analyzer

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse-XFe24 Analyzer (Agilent Technologies). Briefly, Lama84 EV and RXRA OE 4×10^5 cells per well (4 replicates) were seeded in a 24-well XF24 plate coated with retronectin (Takara Bio, Shiga, Japan). Thirty minutes before analysis, the medium was replaced with Seahorse XF media (Agilent Technologies, Santa Clara, CA, United States), and the plate was incubated at 37°C . Three sequential measurements of OCR and ECAR were taken at basal, proton leak (oligomycin), maximal respiration (FCCP), and OXPHOS inhibition (rotenone and Antimycin-A) to assess bioenergetics.

2.14 Statistical analysis

Paired *t*-test, non-parametric *t*-test, and ANOVA with Kruskal-Wallis's correction were used where appropriate. All statistical analysis were done using GraphPad Prism-v5 software, and a *p*-value < 0.05 was considered statistically significant.

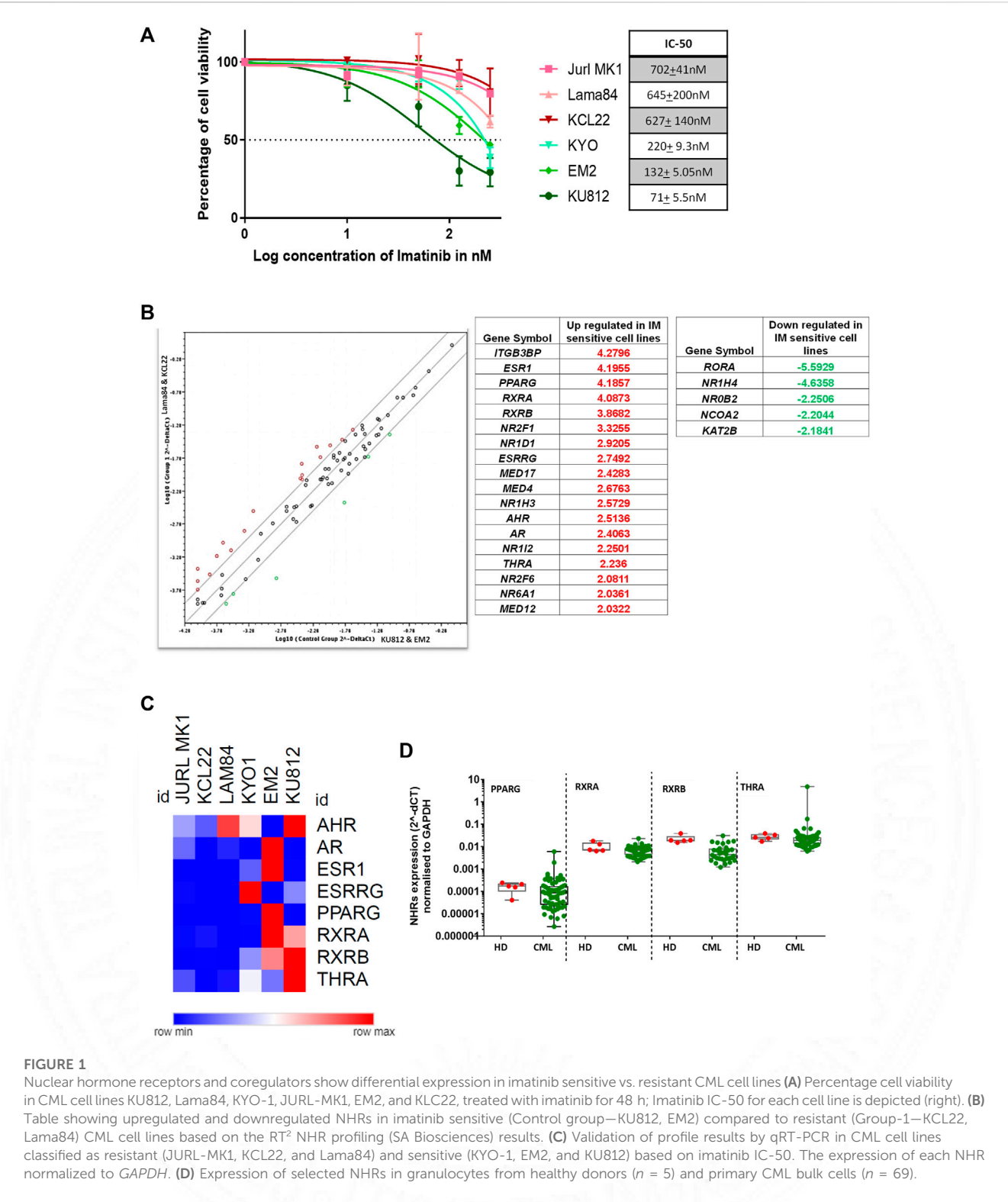


FIGURE 1 Nuclear hormone receptors and coregulators show differential expression in imatinib sensitive vs. resistant CML cell lines (A) Percentage cell viability in CML cell lines KU812, Lama84, KYO-1, JURL-MK1, EM2, and KLC22, treated with imatinib for 48 h; Imatinib IC-50 for each cell line is depicted (right). (B) Table showing upregulated and downregulated NHRs in imatinib sensitive (Control group—KU812, EM2) compared to resistant (Group-1—KCL22, Lama84) CML cell lines based on the RT² NHR profiling (SA Biosciences) results. (C) Validation of profile results by qRT-PCR in CML cell lines classified as resistant (JURL-MK1, KCL22, and Lama84) and sensitive (KYO-1, EM2, and KU812) based on imatinib IC-50. The expression of each NHR normalized to *GAPDH*. (D) Expression of selected NHRs in granulocytes from healthy donors ($n = 5$) and primary CML bulk cells ($n = 69$).

3 Results

3.1 Differential expression profile of NHRs in imatinib sensitive vs. resistant CML cell lines

We performed an *in-vitro* cytotoxicity assay to identify CML cell lines that are sensitive or resistant to IM. KU812, EM2, and

KYO1 cell lines were identified as sensitive, and JURL-MK-1, KCL22, and Lama84 cell lines as resistant to IM. The kill curves and IC-50 of the cell lines tested are listed (Figure 1A). To identify whether the NHR expression differs between the IM sensitive (EM2, KU812) and resistant (KCL22 and Lama84) cell lines, we performed NHR profiling using SA Biosciences PCR-based array. There was differential expression (keeping a cut-off $\leq$ or >2 folds) of 9 NHRs

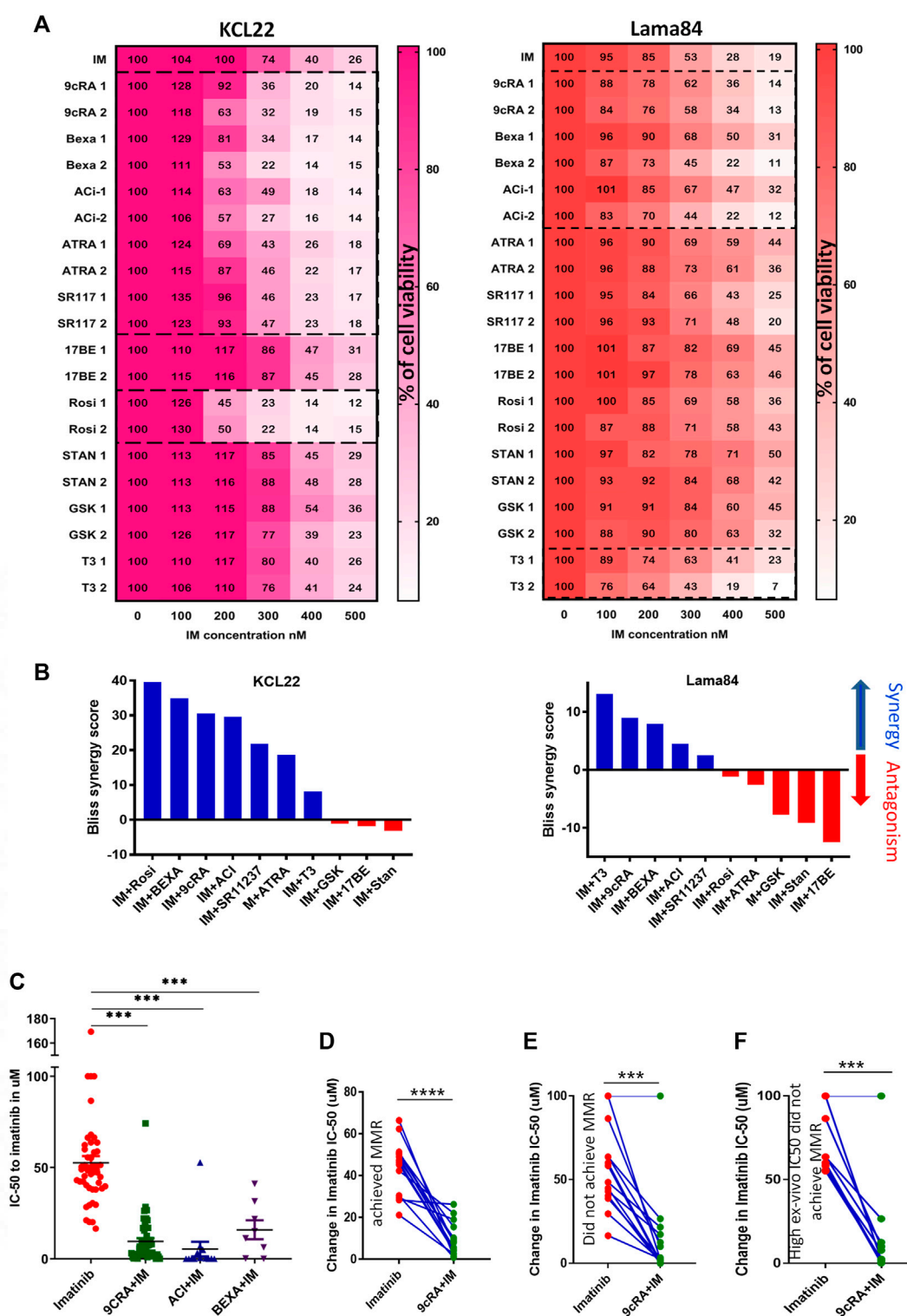


FIGURE 2

RXRA ligand treatment significantly reduced IC-50 to IM in CML cell lines and primary CML cells (A) Representative heat maps showing the cell viability measured at 48 h in CML cell lines KCL22 and Lama84 treated with ligands specific to downregulated NHRs in combination with imatinib. The X-axis shows the increasing concentrations of imatinib (100–500 nM), and the y-axis shows different NHR ligands (two different concentrations). (B) Bliss synergy score were analysed in cell lines treated with NHR ligands in combination with imatinib using synergy finder. The higher the synergy scores, the higher the synergy, and the lower the synergy scores, the antagonism. (C) The difference in IC-50 between imatinib alone vs. in combination with ligand [9-cis-retinoic acid ($n = 42$), acitretin ($n = 13$), and bexarotene ($n = 8$)] was compared using paired t -test and the p -value calculated by Tukey's multiple comparison test. The change in imatinib IC-50 with and without 9-cRA in primary CML cells treated with 9-cRA from patients who achieved MMR at 12 months (D), those who did not achieve MMR at 12 months (E), and those who did not achieve MMR with high IC-50 to imatinib (F). p -value was calculated by paired t -test. Significance values: *** $p < 0.001$.

and 13 coregulators between IM-sensitive and resistant cell lines (Figure 1B). Compared to resistant cell lines, 8 NHRs (*ESR1*, *PPARG*, *RXRA*, *RXRβ*, *ESRRG*, *AHR*, *AR*, *THRA*) and ten coregulators (*ITGB3BP*, *NR2F1*, *NR1D1*, *MED17*, *MED4*, *NR1H3*, *NR1I2*, *NR2F6*, *NR6A1*, *MED12*) were upregulated and *RORA* & 4 coregulators (*NR1H4*, *NR0B2*, *NCOA2* & *KAT2B*) were downregulated in imatinib-sensitive cell lines. A Representative Heatmap illustrates the expression profile of NHRs and coregulators (Supplementary Figure S1). Further validation by q-RT-PCR revealed NHRs such as *RXRA*, *RXRβ*, and *THRA* to be upregulated in the IM-sensitive cell lines compared to IM-resistant cell lines. In contrast, all the other NHRs (*AR*, *ESR1*, *ESRRG*, *PPARG*) were upregulated in at least one of the IM-sensitive cell lines (Figure 1C). To check if the expression pattern of NHRs was unique to CML, we compared the expression of selected NHRs between healthy donor (HD) granulocytes and primary CML cells. There was no significant difference in the expression pattern (Figure 1D).

3.2 RXRA ligand treatment significantly reduced IC-50 to imatinib in CML cell lines and primary CML cells

We next attempted to see if treatment with ligands specific to the downregulated NHRs in the inherently IM-resistant cell lines (KCL22, Lama84) could improve IM sensitivity. The concentration of the ligands used for this treatment is listed (Supplementary Table S1). Treatment with RXRA ligands (9cRA, bexarotene, acitretin, ATRA, SR11237) and PPARG ligand rosiglitazone decreased cell viability in combination with IM compared to IM alone in the KCL22 cells (Figure 2A). In the Lama84 cells, RXRA ligands bexarotene, acitretin, 9cRA, and THRA ligand T3 decreased cell viability (Figure 2A). The combination index (CI) was calculated using the bliss synergy score mathematical model to test the synergy between the combination of ligand and IM. KCL22 cells treated with rosiglitazone, bexarotene, 9cRA, acitretin, SR11237, ATRA, and T3 showed high synergy with IM, whereas GSK4716, 17-β estradiol, and stanozolol showed antagonism with IM (Figure 2B). Ligands such as T3, 9cRA, bexarotene, acitretin, and SR11237 showed synergy, and rosiglitazone, ATRA, GSK4716, stanozolol and 17-β estradiol showed antagonism with IM in Lama84 cell line (Figure 2B).

Based on these results, the ligands that sensitized either cell line to IM were tested in the primary CML cells. Treatment with RXRA ligands (9cRA, acitretin, and bexarotene) improved IM IC-50 *in-vitro* in primary bulk cells obtained from imatinib naive CML patients (Figure 2C). Other NHR ligands such as ATRA, 17-β-estradiol, pioglitazone, and T3 did not improve IC-50 to IM (Supplementary Figures S2A–E). Based on the patients' molecular response status at 12 months, the primary CML samples were grouped as attained major molecular response (MMR) or no MMR. 9cRA significantly decreased IM IC-50 *in-vitro* in samples from patients who achieved in all samples irrespective of the molecular response status (Figures 2D, E; Supplementary Figure S3). Primary CML cells from patients who did not achieve MMR and with high IC50 to IM (above

median IC-50-53uM) also were sensitized to IM with 9cRA combination treatment (Figure 2F).

3.3 RXRA ligand treatment decreased cell viability and colony-forming unit in primary CML CD34⁺ cells

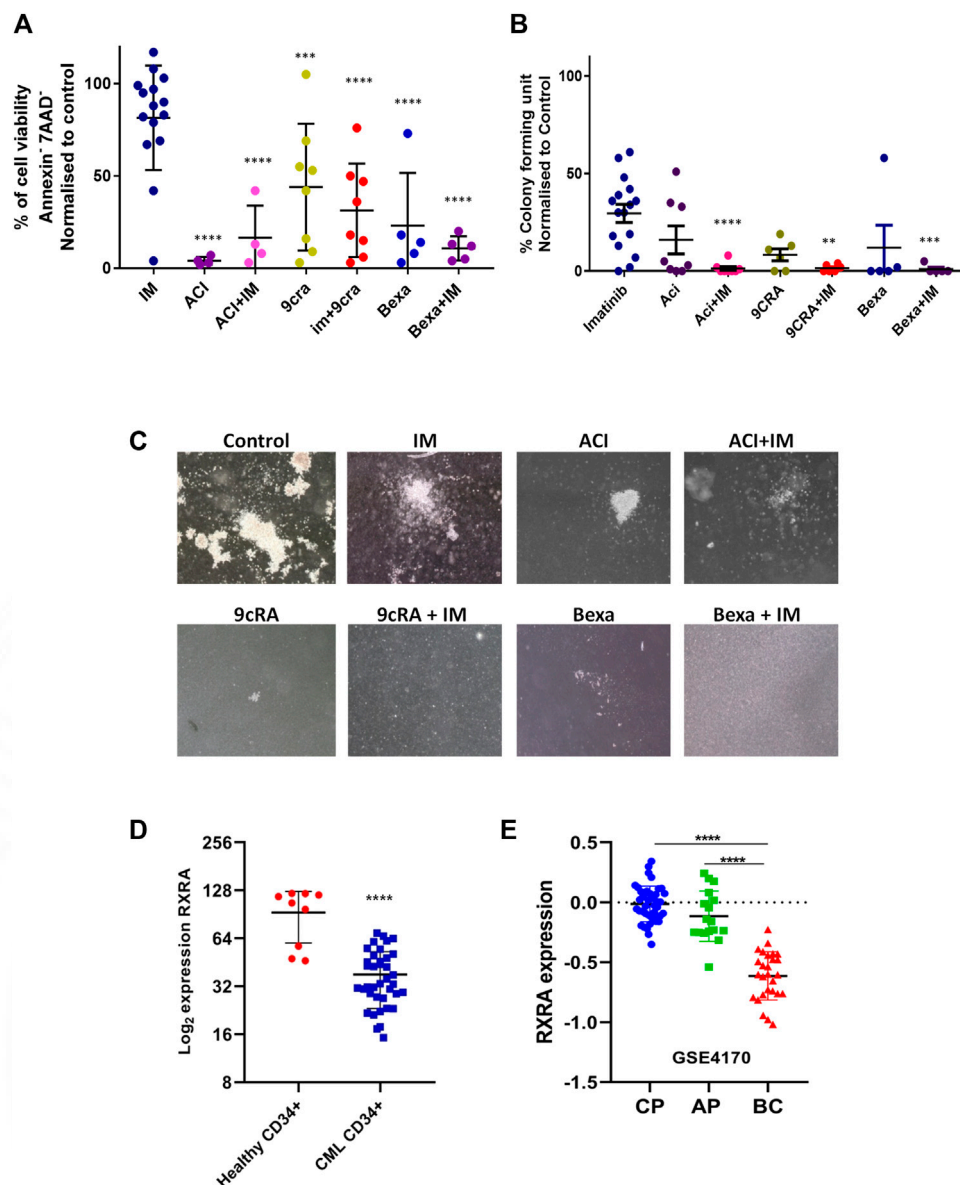
As IM treatment does not eliminate the CML LSCs, we next tested the effect of RXRA ligands alone and in combination with IM in purified primary CML CD34⁺ cells *in-vitro*, and the cell viability and colony-forming capacity were assessed. RXRA ligands, either alone or combined with IM, significantly decreased cell viability (Figure 3A) and colony-forming capacity (Figures 3B, C). Interestingly, acitretin alone treated CML CD34⁺ cells showed a significant reduction in cell viability compared to imatinib alone, 9cRA alone, or Bexa alone or in combination (Figure 3A). Treatment of healthy PBMCs with RXRA ligands 9cRA, bexarotene, and acitretin in combination with IM did not reduce cell viability compared to IM alone, suggesting that the cytotoxic effect of RXRA ligands in combination with imatinib is specific to CML cells (Supplementary Figures S4A–C). We then compared the expression of RXRA in CML CD34⁺ and HD CD34⁺ cells. RXRA expression was significantly lower in CML CD34⁺ cells than in HD CD34⁺ cells (Figure 3D). We also probed for RXRA expression across CML chronic phase (CP), accelerated phase (AP), and blast crisis (BC) patient samples from the GSE4170 dataset and found significantly reduced expression of RXRA in BC-CML samples compared to CP and AP (Figure 3E). These results suggest that either alone or combined with IM, RXRA ligands could selectively target CML CD34⁺ cells.

3.4 RXRA ligand treatment inhibits BCR-ABL downstream signaling and activates the apoptotic cascade in CML cells in combination with imatinib

RXRA forms a homodimer or heterodimerizes with other NHRs, such as RARA, VDR, and PPARG to regulate cellular proliferation, differentiation, and apoptosis (Gilardi and Desvergne, 2014). To identify the expression of other NHRs post RXRA ligand treatment in CML primary cells, RNA expression of *RARA*, *VDR*, and *PPARG* was tested. RXRA ligands treatment resulted in consistent upregulation of *RARA* and *VDR* and downregulation of *PPARG* (Supplementary Figure S5). RXRA ligand treatment also increased RXRA protein levels in the CML cell lines (Figure 4A).

We then checked the effect of these RXRA ligands on the BCR-ABL signaling pathways (p-CRKL as a marker of proliferation, p-AKT as a marker of survival, and p-STAT5 as a marker of quiescence) in CML cell lines and primary cells. RXRA ligands decreased p-CRKL, p-AKT, and p-STAT5 at the protein level in combination with IM (Figure 4B) in CML cell lines. Primary CML cells treated with RXRA ligands combined with IM also showed inhibition of downstream signaling pathway p-CRKL (Figure 4C).

Next, we examined the effect of these RXRA ligands on CML cells in inducing apoptosis. CML cell lines treated with RXRA

**FIGURE 3**

RXRA ligands in combination with imatinib decreases cell viability and colony-forming unit in primary CML CD34⁺ cells **(A)** Percentage cell viability in primary CML CD34⁺ cells treated with rexinoids (Aci $n = 4$, 9CRA $n = 8$ & Bexa $n = 5$) in combination with imatinib. Significance was calculated compared to imatinib-treated cells. p -value was calculated by Tukey's multiple comparison test. **(B)** Colony-forming capacity in primary CML CD34⁺ cells treated with rexinoids (Aci $n = 8$, 9CRA $n = 6$ & Bexa $n = 5$) combined with imatinib; colonies were scored on day 14. Significance was calculated compared to imatinib-treated cells. p -value was calculated by Tukey's multiple comparison test. **(C)** Representative images of the colony-forming units in primary CML CD34⁺ cells treated with imatinib alone, rexinoids alone, and in combination. **(D)** RXRA mRNA expression in healthy donor CD34⁺ cells ($n = 9$) and CML CD34⁺ ($n = 39$) cells analyzed using Nanostring nCounter. RXRA expression was normalized to ACTB, GAPDH & GUSB and presented in Log₂. **(E)** RXRA expression values from the GSE4170 dataset in CML chronic phase (CP), accelerated phase (AP), and blast crisis (BC) patients. p -value was calculated by the Mann-Whitney U test. Significance values: ** $p < 0.01$; *** $p < 0.001$.

ligands combined with IM showed a significant increase in cleaved caspase-3 and cleaved PARP expression (Figure 4D) and increased apoptosis combined with IM (Figure 4E) as well as with 2nd generation TKIs, dasatinib and nilotinib (Supplementary Figures S6A, B). These results indicate that RXRA ligands improve IM sensitivity by decreasing the BCR-ABL downstream signaling pathways and increasing apoptosis in CML primary cells and cell lines.

3.5 Molecular overexpression of RXRA in Imatinib resistant Lama84 cell line decreased proliferation, BCR-ABL downstream signaling, and OXPHOS resulting in improved imatinib sensitivity

As proof of principle, we evaluated if molecular overexpression of RXRA in the CML cell line would result in

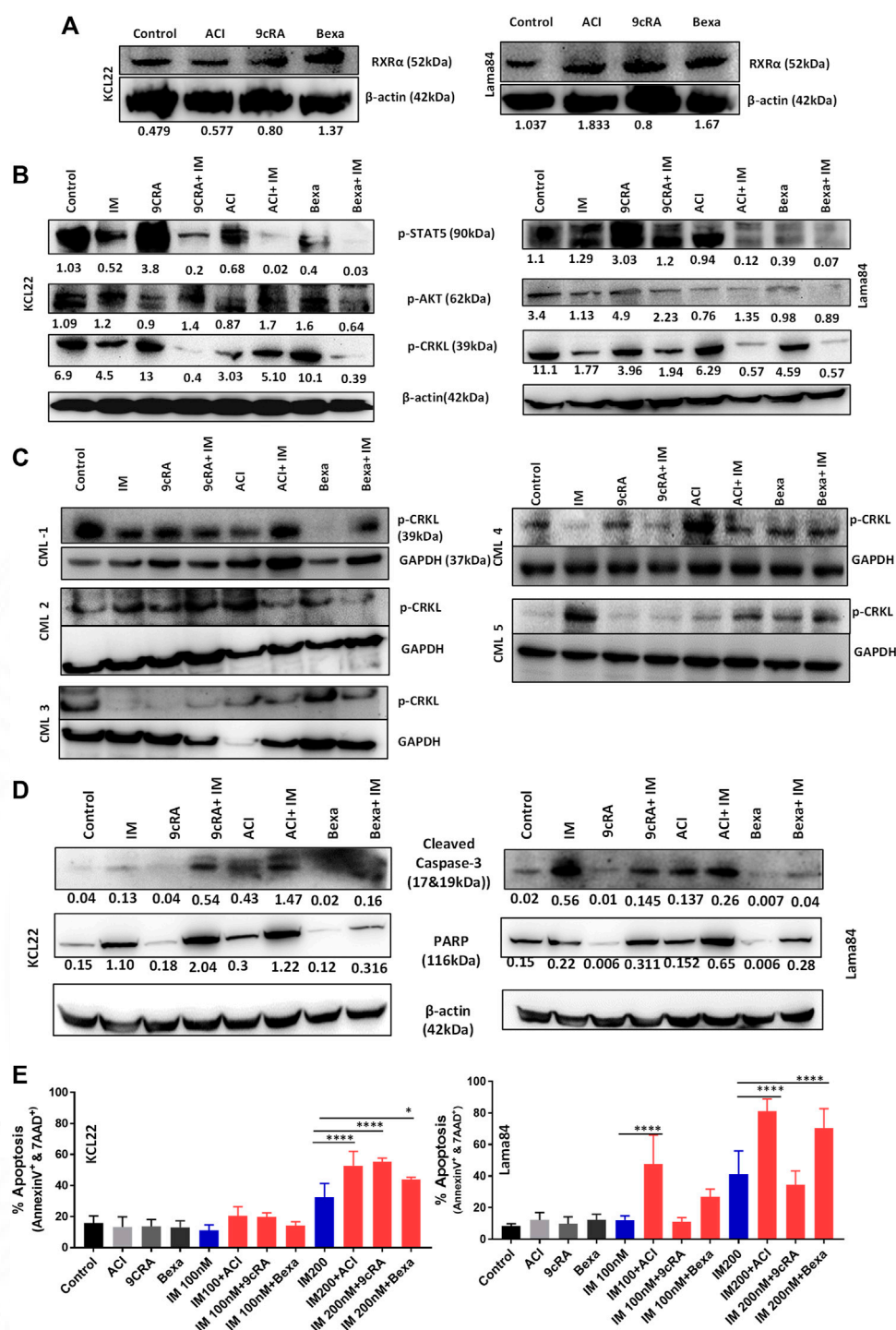


FIGURE 4

RXRA ligands decrease BCR-ABL signaling and activate the apoptosis pathway in CML cell lines (A) Western blot image showing increased RXRα protein expression in Lama84 and KCL22 cell lines treated with RXRα ligands AcI, 9cRA, and Bexa. β-actin was used as the loading control. (B) Western blot image showing the expression of BCR-ABL downstream signaling pathway proteins p-CRKL, p-AKT & p-STAT5 in KCL22, Lama84 cell lines, and (C) primary CML cells ($n = 5$) treated with rexinoids for 24 h, followed by imatinib treatment. β-actin/GAPDH was used as a loading control. (D) Western blot image showing the expression of apoptotic proteins of cleaved caspase-3 & PARP in CML cell lines treated with rexinoids followed by imatinib; β-actin was used as a loading control. (E) The percentage of apoptosis (Annexin-V and 7AAD positive cells) in CML cell lines treated with rexinoids with or without imatinib. p -value calculated by Tukey's multiple comparison test.

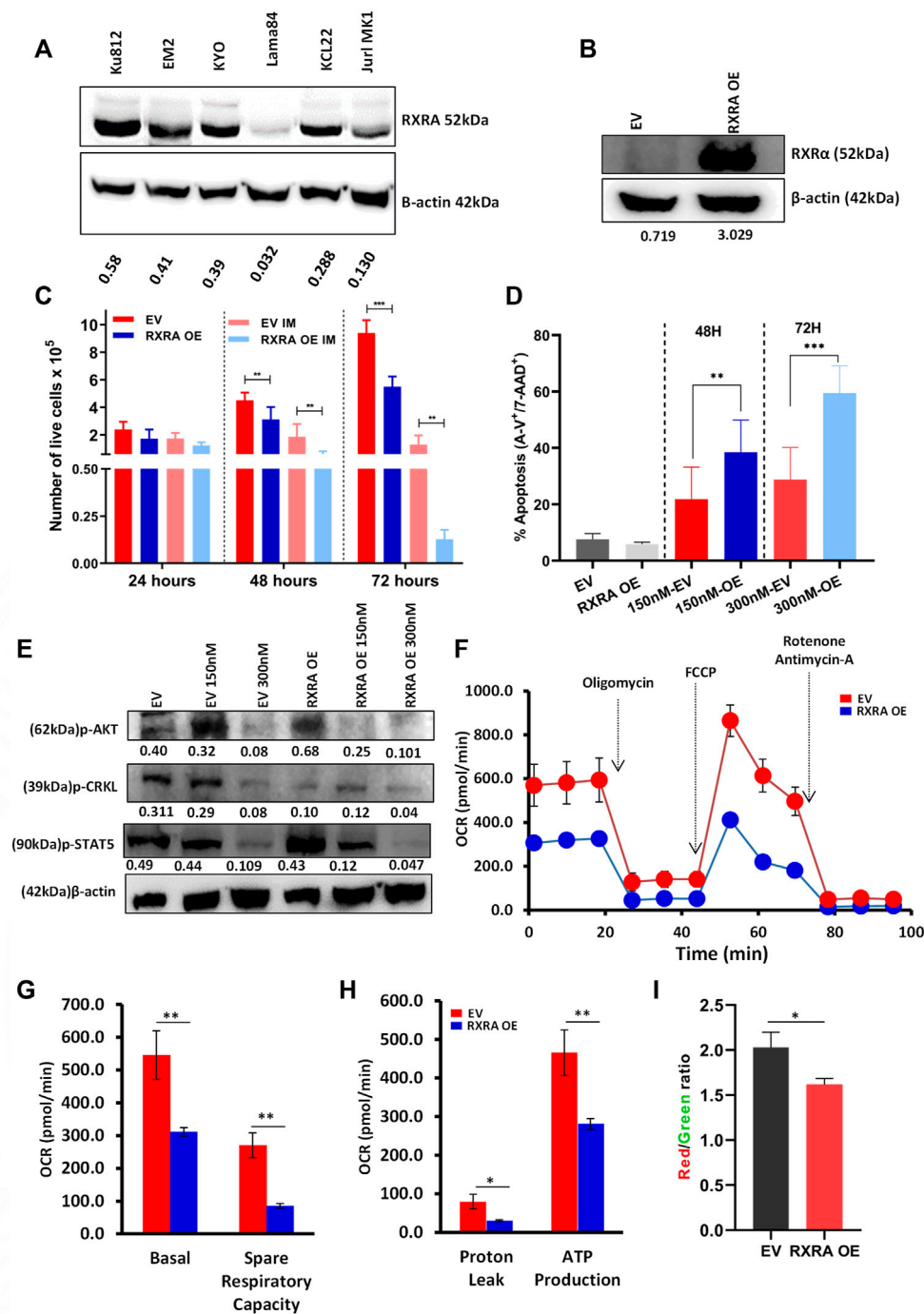


FIGURE 5

Molecular overexpression of RXRA improves imatinib sensitivity in the Lama84 cell line by inhibiting BCR-ABL signaling and oxidative phosphorylation (A) Basal RXRA expression in whole-cell lysate from CML cell lines KU812, EM2, Lama84, and KCL22 analyzed by western blot. Lama84 cell line with relatively low RXRA protein expression was transduced with RXRA OE plasmid, and overexpression was confirmed at the protein levels by western blotting (B). The number of viable cells in Lama84 RXRA OE vs. EV with and without imatinib treatment was assessed using trypan blue exclusion assay ($n = 3$) at three different time points. The doubling time was calculated using exponential curve analysis, and the p -value was calculated using the Mann-Whitney U test (C). EV and RXRA OE Lama84 cells were treated with 150 nM concentration of imatinib ($n = 3$), and the percentage of apoptosis was assessed by apoptosis assay (Annexin-V and 7AAD positive cells) at two different time points (D). The p -value was calculated by Tukey's multiple comparison test. Western blot image showing the expression of BCR-ABL downstream signaling proteins (p-CRKL, p-AKT, and p-STAT5) in RXRA OE cells and EV cells (E). Mito Stress Test for EV and RXRA OE cells using Sea horse extracellular flux analyzer (F). Quantitative assessment of Basal OCR rates, Spare respiratory capacity, proton leak, and ATP production (G,H) ($N = 4$). (I) The quantitative measure of mitochondrial membrane potential was analysed using the ratio of JC-1 dimer (Red) by JC-1 monomer (Green).

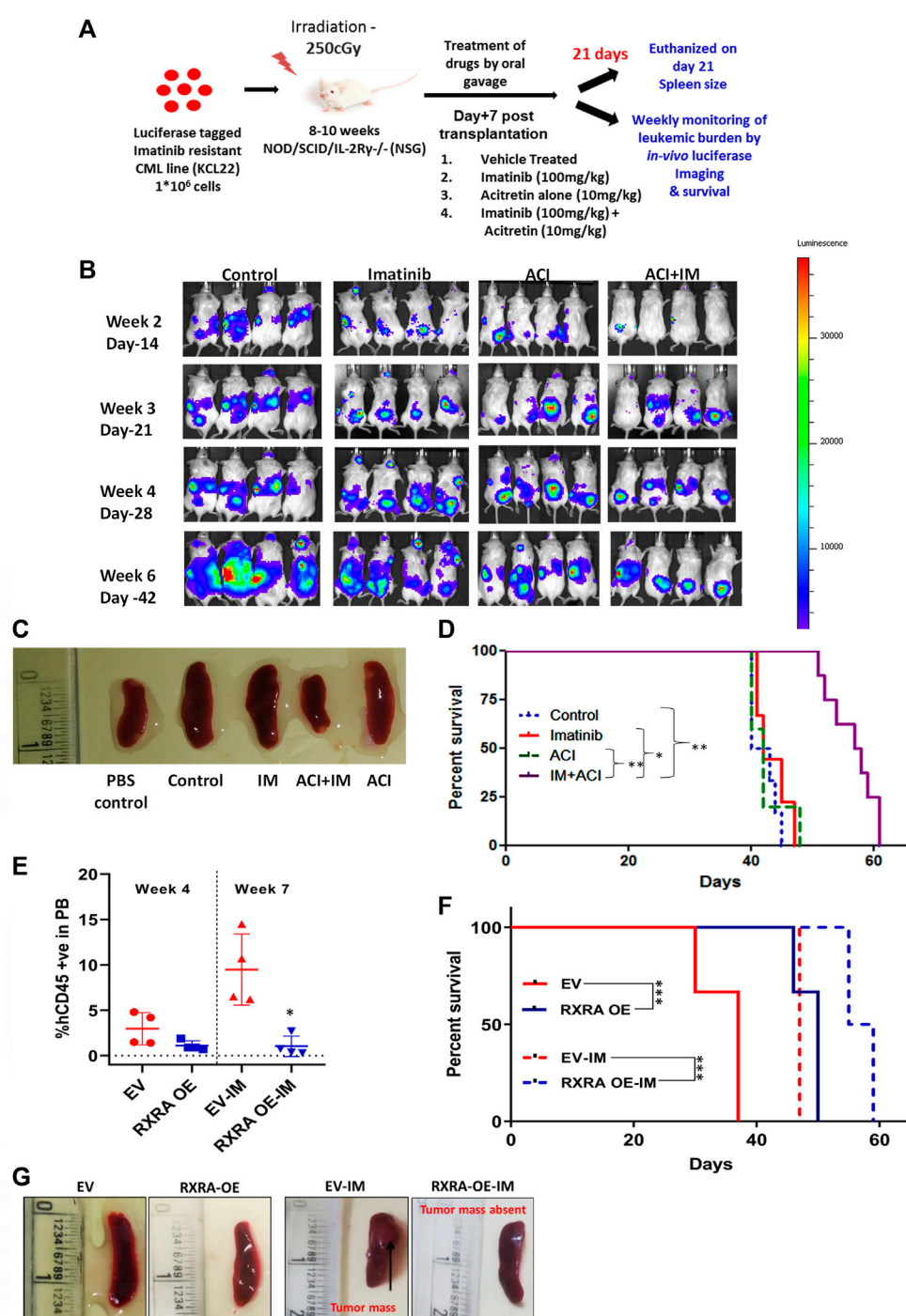


FIGURE 6

RXRA ligand acitretin in combination with imatinib decreases the leukemic burden and increases survival in the xenograft CML mice model (A) Outline of the CML cell line-derived (CDX) mouse model experiment. (B) Representative bioluminescence images showing *in-vivo* luciferase expression in CML mice treated with RXRA ligand with or without imatinib compared to the vehicle control mice. (C) Representative mice spleens in vehicle vs. treated animals. (D) Kaplan-Meier survival curve of NSG mice transplanted with KCL22 cell line and treated with IM alone ($n = 9$), ACI alone ($n = 5$), ACI + IM ($n = 8$), and vehicle control ($n = 6$). Percentage survival was calculated using the Kaplan-Meier analysis, and median survival was compared between treated groups vs. control groups by Dunn's multiple comparisons test. (E) Percentage of human CD45 cells from EV and RXRA OE injected mice after 4 weeks of transplantation and at week 7 between EV/RXRA OE treated with IM. (F) Kaplan-Meier survival curve of NSG mice transplanted with Lama84 EV ($n = 3$) and RXRA OE cell line ($n = 4$) (2×10^5 cells transplanted/mouse). Percentage survival was calculated using the Kaplan-Meier analysis, and median survival was compared between treated groups vs. control groups by Dunn's multiple comparisons test. (G) Representative spleens from mice injected with wild type vs. RXRA OE Lama84 cell line.

similar effects as that of ligand treatment. Overexpression (OE) of RXRA in the Lama84 cells with deficient basal protein expression (Figure 5A) of RXRA, resulted in significantly increased RXRA expression at the protein level (Figure 5B). Interestingly, RXRA OE alone considerably decreased the proliferative capacity of these cells and increased the sensitivity to imatinib in a time-dependent manner (Figure 5C; Supplementary Figure S7). We further validated the sensitivity to imatinib in RXRA OE cells by apoptosis and observed enhanced cytotoxicity to imatinib in these cells compared to the empty vector (EV) transduced (Figure 5D). We next assessed if the profound sensitivity to imatinib is due to inhibition of BCR-ABL downstream signaling pathways (p-CRKL, p-AKT & p-STAT5) as observed during ligand treatment. Immunoblotting of RXRA OE and EV cells revealed diminished p-CRKL signaling in the RXRA OE cells at the basal level. We observed complete inhibition of p-STAT5, p-CRKL, and p-AKT in a dose-dependent manner compared to EV, post-IM treatment in the RXRA OE cells (Figure 5E).

As leukemic cells rely on oxidative phosphorylation for energy demand and for withstanding TKI therapy, we assessed the bioenergetic profile of EV and RXRA OE cells. Intriguingly, RXRA OE markedly reduced the basal respiration rates indicative of restricted intrinsic ATP demand. RXRA OE significantly reduced ATP production, and the addition of mitochondrial uncoupler FCCP showed markedly reduced oxidative bursts compared to EV (Figures 5F–H). We further assessed the mitochondrial membrane potential (MMP) of EV and RXRA OE using JC-1 and found significantly reduced MMP in RXRA OE cells (Figure 5I). Interestingly, MMP measured post-RXRA ligand treatment in CML cell lines showed a significant decrease in MMP in the ACI-treated KCL22 cells, while there was no substantial change in Lama84 cells (Supplementary Figures S8A, B). These results collectively suggest that RXRA OE mimics the effect of RXRA ligand treatment in CML cells. Additionally, molecular and pharmacological activation of RXRA could potentially inhibit OXPHOS in CML cells improving sensitivity to IM.

3.6 RXRA ligand treatment and RXRA OE decreased leukemic burden and improved survival in cell-derived xenograft CML mice model by enhancing sensitivity to imatinib

As the *in-vitro* data suggested that RXRA ligands improved imatinib sensitivity and inhibited BCR-ABL downstream signaling in CML cell lines and primary CML cells, we investigated the *in-vivo* efficacy of acitretin combined with imatinib. A transplantable xenograft CML mouse model was developed by injecting luciferase-expressing KCL22 cells into sub-lethally irradiated NSG mice. Mice were treated with imatinib, acitretin, or a combination of both for 21 days (Figure 6A). Acitretin-treated mice showed ruffled fur coat and moderate weight loss. Intriguingly, mice treated with either imatinib alone or acitretin alone showed an increased incidence of hind leg paralysis compared to vehicle-treated mice and did not improve survival.

While the combination of acitretin and IM treatment reduced spleen size, decreased leukemic burden, and significantly increased median survival (Figures 6B–D). *In-vitro* treatment of the HD PBMNCs and CD34⁺ cells with acitretin in combination with imatinib did not significantly affect the viability of these cells as measured by apoptosis assay, suggesting that this effect is unique to CML cells (Supplementary Figures S9A, B).

Next, we evaluated the engraftment capacity of the RXRA OE and EV cells by transplanting an equal number of cells into sub-lethally irradiated NSG mice (Supplementary Figure S9C). RXRA OE hindered the engraftment capacity of the Lama84 cell line and improved survival. We also assessed the sensitivity to imatinib *in-vivo* in the RXRA OE/EV engrafted mice. Imatinib treatment for 21 days significantly reduced leukemic burden and prolonged survival in the RXRA OE cells transplanted mice compared to EV (Figures 6E, F; Supplementary Figure S9D). Imatinib treatment in EV-transplanted mice showed tumor mass formation but was absent in the RXRA OE mice treated with imatinib (Figure 6G). These comprehensive *in-vivo* results suggest that pharmacological and molecular approaches to activate RXRA could hinder leukemic cell engraftment, increase sensitivity to imatinib, and improve survival.

4 Discussion

Suboptimal response to TKI or TKI failure is associated with poor progression-free survival in patients with CML. Combining TKI with small molecules specific to pathways in which imatinib-resistant cells escape therapy could help eliminate the residual LSCs. In the present study, we observed differential expression of NHRs in imatinib sensitive vs. resistant CML cell lines when comprehensively screened for the expression of NHR and coregulators across CML cell lines. Upon treatment with ligands specific to the downregulated NHRs in the imatinib-resistant CML cell lines, only RXRA ligand treatment showed improved sensitivity to IM in both cell lines. Pioglitazone, a PPARG ligand, has been reported to eliminate CML LSCs but not the bulk primary CML cells (Prost et al., 2015; Wang et al., 2017). Combination treatment with Pioglitazone and imatinib induced complete molecular response (CMR) in patients who have not achieved CMR under long-term imatinib treatment in CML patients and remained in CMR after the withdrawal of imatinib (Prost et al., 2015).

Treating with ligands specific to the downregulated NHRs, including rosiglitazone (a PPARG ligand), showed a differential effect, sensitizing the KCL22 cell line but no impact on the Lama84 cell line in combination with IM. Similarly, THRA agonist T3 treatment sensitized Lama84 and did not affect KCL22. Although 17BE and stanozolol treatment improved viability in both cell lines, these ligands did not improve sensitivity to IM in bulk CML cells *ex-vivo*. As these ligands are present endogenously, it would be interesting to understand their effect on primary CML cells. Hence, we worked with RXRA ligands 9cRA, ACI, and bexarotene, which improved IM sensitivity in CML bulk and CML CD34⁺ cells but had minimal effect on HD CD34⁺ cells and PBMNCs. In addition, treatment with RXRA ligands

combined with imatinib decreased clonogenic potential in primary CML CD34⁺ cells.

Prost et al. (2015) showed inhibition of p-STAT5 led to clearance of CML LSCs after treatment with *PPARG* agonist pioglitazone. When we assessed the BCR-ABL downstream signaling pathways, we observed that RXRA agonist in combination with IM resulted in decreased p-CRKL, p-STAT5, and p-AKT along with activation of the apoptotic cascade in CML cell lines and primary cells.

Lentiviral-mediated overexpression of *RXRA* in imatinib-resistant Lama84 cell line showed decreased proliferation, decreased BCR-ABL downstream signaling, and improved imatinib sensitivity *in-vitro*. Di Martino et al. (2021a) showed that deleting endogenous *RXRA* in an MLL-AF9 AML mouse model increased leukemogenic potential and reduced survival. The same authors subsequently reported that a constitutively overexpressing mutant variant of *RXRA* resulted in myeloid maturation and prolonged survival in the murine model (Di Martino et al., 2021a; Di Martino et al., 2021b). In line with this, in our *RXRA* overexpressing CDX CML mice model, we observed decreased leukemic burden and improved survival compared to EV-transduced CDX. We also found a significant reduction in *RXRA* expression in CML CD34⁺ cells compared to HD CD34⁺ and a reduction in the expression of *RXRA* in blast crisis CML patients compared to chronic phase, accelerated phase CML from the GSE4170 dataset indicating a potential role of *RXRA* in hindering leukemogenic capacity in CML.

It is reported that CML CD34⁺ cells have a high oxidative capacity, which could be inhibited using tigecycline (Kuntz et al., 2017). Interestingly, we also identified that *RXRA* OE markedly inhibited the OXPHOS capacity of CML cells. Potentially *RXRA* ligands could mimic *RXRA* OE and have a disruptive effect on the oxidative potential of CML cells. In addition, *RXRA* OE and imatinib treatment significantly reduced leukemic cell growth *in-vivo* resulting in prolonged survival. Studies in solid tumors have demonstrated using *RXRA* agonists to increase sensitivity to chemotherapeutic drugs cisplatin and paclitaxel (Hermann et al., 2005; Blumenschein et al., 2008; Ramlau et al., 2008). Similar results were observed when the CML CDX model (KCL22 cell line) was treated with *RXRA* agonist acitretin combined with IM, improving survival. Interestingly, the agonist-treated arm had no survival benefit, unlike *RXRA* OE. The mechanisms of *RXRA* activation by pharmacological/molecular means may differ, and *RXRA* agonists (9cRA, ACI, and BEXA) may have a pleiotropic effect *in-vivo* compared to *RXRA* OE.

While our findings suggest the potential use of *RXRA* agonists in combination with IM in eliminating CML CD34⁺ *in-vitro* and sensitizing CML bulk cells highly resistant to IM (IC₅₀ > 53uM), a more robust *in-vivo* model to evaluate the therapeutic efficacy of this combination and anti-leukemogenic potential *RXRA* has on CML LSCs is needed. We are currently exploring the mechanism by which *RXRA* activation by both molecular/pharmacological means could orchestrate the downstream kinase activities.

We demonstrated differential NHR expression between imatinib-resistant and sensitive cell lines. The ligand-dependent activation of *RXRA* could be an effective CML target, affecting

BCR-ABL1 downstream signaling and apoptotic pathway. Our *in-vitro* and *in-vivo* results suggest that using *RXRA* ligands could be an effective combination therapy to improve the imatinib response in patients with CML.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, CMC, Vellore (IAEC No. 5/2019).

Author contributions

BR and RI designed the research, performed experiments, analyzed results, and wrote the manuscript. EB, BB, DJ, SD, AP, RV, AM, and SK performed experiments and analyzed the results. AA and VM contributed to the analysis and review of the manuscript. SV designed the research, performed experiments, contributed to analysis and review of the manuscript. PB designed the research, performed experiments, analyzed results, wrote the manuscript, and procured funding. All authors contributed to the article and approved the submitted version.

Funding

This study is supported by a Centre of Excellence grant from the Department of Biotechnology India: BT/COE/34/SP13432/2015 and the Indian Council of Medical Research Centre for Advanced Research grant 70/14/14-CAR to PB. This study is partly supported by CMC Fluid research grants: IRB No. 11169 & 12061 to PB and BR. SV and PB are supported by Wellcome DBT India Alliance (IA/S/17/1/503118 and IA/S/15/1/501842), respectively. BR, RI and DJ supported by ICMR SRF. SK and SD were supported by University Grants Commission, AP by DBT SRF, ESB by DST Inspire SRF, and RV by CSIR JRF.

Acknowledgments

We thank the staff of the animal facility and flow cytometry facility at the Centre for Stem Cell Research, Bagayam, Vellore, for their help. The help provided by Mr. Alex John, Ms. Mary Philomena, and Ms. Sangeetha in CML patient recruitment for the study is gratefully acknowledged. We thank Dr. Tamil Selvan from Anna University for providing access to the seahorse facility, supported by the Improvement of Science and Technology (S&T) Infrastructure grant (SR/FST/LSI-649/2015).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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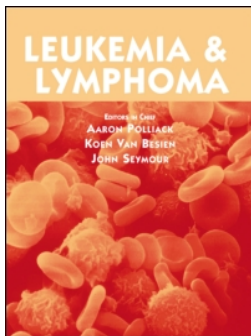
Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1187066/full#supplementary-material>

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***BCR-ABL1* kinase domain mutation analysis by next generation sequencing detected additional mutations in chronic myeloid leukemia patients with suboptimal response to imatinib**

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To cite this article: Esther Sathya Bama Benjamin, Niveditha Ravindra, Bharathi Murugan Rajamani, Senthamizhselvi Anandan, Bagavathi Kausalya, Vidya Veldore, Vikram Mathews, Shaji Ramachandran Velayudhan & Poonkuzhali Balasubramanian (2021): *BCR-ABL1* kinase domain mutation analysis by next generation sequencing detected additional mutations in chronic myeloid leukemia patients with suboptimal response to imatinib, *Leukemia & Lymphoma*, DOI: [10.1080/10428194.2021.1872074](https://doi.org/10.1080/10428194.2021.1872074)

To link to this article: <https://doi.org/10.1080/10428194.2021.1872074>



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LETTER TO THE EDITOR



***BCR-ABL1* kinase domain mutation analysis by next generation sequencing detected additional mutations in chronic myeloid leukemia patients with suboptimal response to imatinib**

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ARTICLE HISTORY Received 12 October 2020; accepted 28 December 2020

Mutations in the *BCR-ABL1* kinase domain (*BCR-ABL1* KD) is one of the most common determinants of tyrosine kinase inhibitor (TKI) resistance in patients with chronic myelogenous leukemia (CML) [1]. The emergence of *BCR-ABL1* kinase domain (KD) mutations contributes to 40–50% of all TKI resistant cases [2]. The current gold standard method for *BCR-ABL1* KD mutation detection is Sanger sequencing (SS), which has an analytical sensitivity limit of 10–20% [3]. SS cannot reliably discriminate between polyclonal (more than one mutant clone) and compound mutations [4]. Next generation sequencing (NGS) is increasingly being suggested as the method of choice for detecting *BCR-ABL1* KD mutations due to the increased sensitivity of this method to identify mutations at variant allele frequencies (VAFs) as low as 1–3% [3]. The presence of even low-level *BCR-ABL1* KD mutations have been shown to be significantly associated with poorer outcomes in patients receiving TKI for CML [3]. NGS based *BCR-ABL1* KD mutation testing at the time of suspected TKI failure could aid in subsequent therapy selection [3].

Although *BCR-ABL1* KD mutations explained TKI Sub-Optimal Response (SOR) to a certain extent in our patients (33% overall; 14% in Chronic phase (CP) CML [5]), the reasons for SOR in a majority of these patients remain unclear. Here we assessed whether NGS could identify *BCR-ABL1* KD mutations in CML patients with SOR to imatinib, but mutation negative by SS. We also attempted to demonstrate the kinetics of low-level mutations detected by NGS in a subset of these patients. This study is unique since we specifically analyzed whether NGS could detect any additional mutations in patients who had no detectable mutations by SS. All previous studies using NGS to detect *BCR-ABL1* KD mutations have tested all the imatinib failure patient samples by both SS and NGS [3,6,7].

Three cohorts of patients were included in this study (Figure 1). Cohort 1 included CML patients with *BCR-ABL1* KD mutation detected by SS for validation of the in-house bioinformatics pipeline ($n=5$). Cohort 2 included samples from CML patients with imatinib failure (*BCR-ABL1* transcript ratio of $>1\%$ at a median duration of 20 months post imatinib therapy (3–164 months) as per ELN guidelines [8] ($n=51$) collected between 2015 and 2017. Cohort 3 included CP-CML patients who did not achieve MMR (*BCR-ABL1* transcript ratio $>0.1\%$, defined as SOR) after 12-months of imatinib therapy ($n=41$), enrolled in a prospective study. Both cohorts 2 and 3 were negative for *BCR-ABL1* KD mutation by SS. Patients with clinically relevant *BCR-ABL1* KD mutation detected by NGS in cohorts 1 and 3 were also longitudinally followed-up by SS during subsequent time points at least once in 6 months. This study was approved by Institutional review board. The demographics of patients in all 3 cohorts is listed in Supplementary Table 1.

Total RNA was extracted from peripheral blood samples using Trizol reagent followed by cDNA synthesis using random hexamer and reverse transcriptase enzyme (High-Capacity c-DNA Reverse Transcription Kit, Thermo Scientific, Applied Biosystems). *BCR-ABL1* KD mutation screening by SS was performed as previously described [9,10].

BCR-ABL1 KD mutation by NGS was carried out as previously described [4]. Library preparation was carried out based on SureSelect QXT Library Preparation protocol (Agilent, Cat #G9683B). The Illumina-compatible sequencing library was quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA). Sequencing was performed in Illumina HiSeq and Miseq platforms. Analysis was carried out by creating a bioinformatic pipeline created and run on an open source, web-based platform (<https://usegalaxy.org/>). After checking the FASTQ files for quality

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 Supplemental data for this article can be accessed [here](#).

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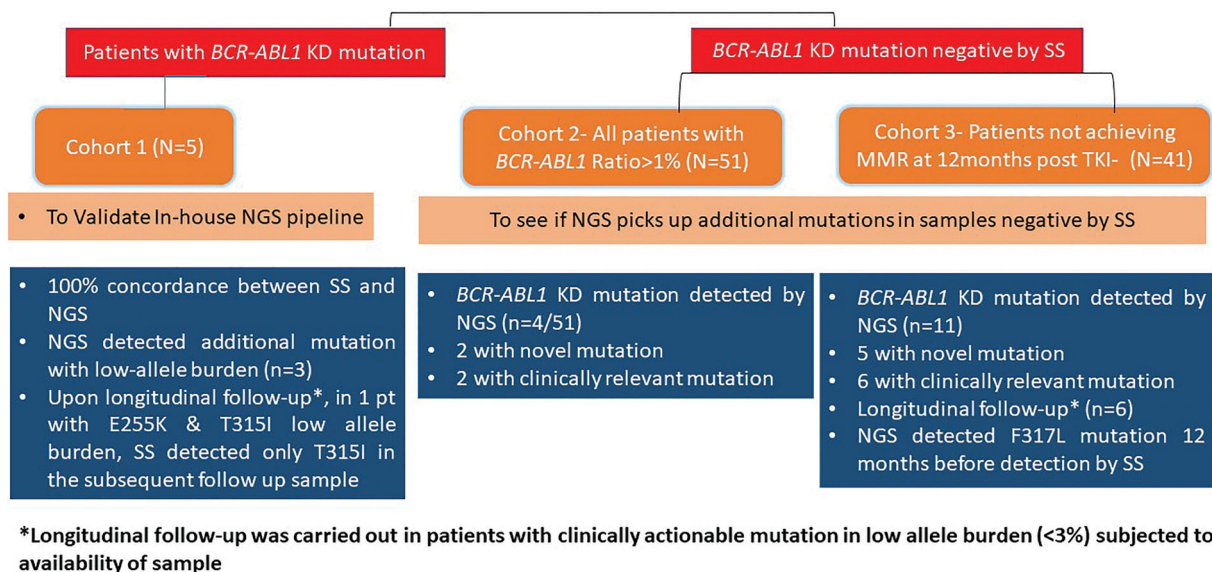


Figure 1. Study outline.

scores (FastQC Version 0.72 + galaxy1) and adaptor trimming by Trimmomatic (version 0.38.0), reads were aligned on GRCh37 human reference genome with the HISAT2 tool (Galaxy version 0.17.7.1). PCR duplicates and reads with low mapping quality were filtered out. The Varscan tool (Galaxy ver 2.4.2) was used to detect both single nucleotide variants (SNVs) and indels. VCF files were filtered and annotated by SnpSift Filter (version 4.3 + t.galaxy0) and SnpSift Annotate (version 4.3 + t.galaxy1). For Varscan analysis, parameters were set as follows: minimum read depth of eight and minimum supporting reads of two. The minimum VAF threshold for variant calling was set at 1%. Variants obtained were compared with the Catalogue of Somatic Mutations in Cancer (COSMIC) database (<https://cancer.sanger.ac.uk/cosmic>). Variants with VAF ~3% cutoff was considered true variants in all the samples as described by [11]. All variants obtained were visually checked on the Integrative Genomics Viewer. The 2-year failure free survival (as described in [5]) in cohort 3 patients with or without detectable mutations by NGS was compared by Kaplan-Meier Survival estimates and Fischer's Exact test using GraphPad Prism 6.

Five CML samples with detectable *BCR-ABL1* KD mutation by SS (cohort-1) were analyzed by NGS to validate the in-house Galaxy pipeline described above. NGS was able to detect all the mutations identified by SS (Supplementary Table 2) as well as 4 additional mutations with low allele burden (VAF >3 and <15%) in 3 of these samples. The in-house bioinformatics pipeline we developed was able to uncover clinically relevant mutations as well as polyclonal mutations missed by SS, which is one of its disadvantages [12].

When samples from CML patients with SOR to imatinib (3–164 months from the start of imatinib therapy; median: 20 months) with no detectable *BCR-ABL1* KD

mutation by SS (cohort-2) were assessed by NGS ($n = 51$), six mutations were identified in 4 of these patients (Supplementary Table 3). This is similar to a previous study in SS negative CML patients who lost MMR while on TKI therapy where NGS did not detect additional mutations [13]. Two of the mutations were previously reported to be resistant to Imatinib (T315I and F359V) and 2 were novel mutations: V304I (VAF 3.6%) and R332W (VAF 6.6%). These mutations were predicted to be 'damaging' by SIFT score and 'probably damaging' by PolyPhen-2. However, the impact of these mutations on TKI efficacy remains to be tested.

Of the 41 CML patients who did not achieve MMR at 12–18 months post imatinib therapy (cohort-3) screened for *BCR-ABL1* KD mutation by NGS, 20 mutations were detected in 12 patients. Eight clinically relevant mutations were present at low-allele burden (F317L, E355G, H396R, D276G, V289I, M244T, Y253F and F359L) (Supplementary Table 4) in seven patients. Eleven novel mutations were also detected in 7/11 patients at low VAF but the role of these mutations in TKI response is not known.

Several studies have shown that NGS can detect the presence of mutations 12–15 months before they are identifiable by SS [4]. To check if the mutant clones identified at low VAF by NGS get selected while on TKI therapy, longitudinal follow-up was carried out. SS was performed in patients with clinically relevant mutations at low VAF (detected by NGS), depending on the availability of follow-up samples (Cohort 1: $n = 1$; cohort 3: $n = 6$). In cohort 2, three of the 5 patients with detectable mutations were lost to follow-up and the other 2 patients had progressed to advanced disease (within 3 and 6 months respectively) (Supplementary Table 3). The range of follow-up duration of the 7 patients with longitudinal follow-up was 3–72 months from the time mutation was detected by NGS.

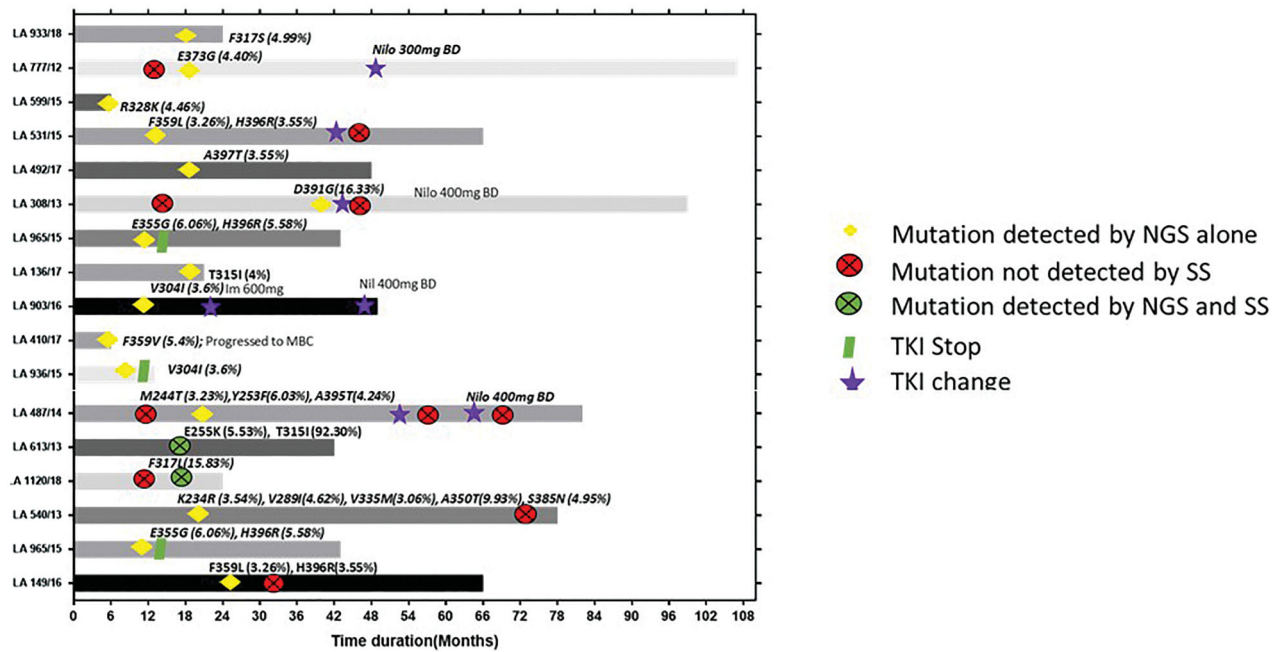


Figure 2. Kinetics of *BCR-ABL1* KD mutations detected by NGS in CML patients: Kinetics of mutations detected by NGS and Sanger sequencing (SS) during treatment in CML patients. Each line represents individual patients with time duration of mutation detected by NGS and SS.

In a patient with F317L mutation (VAF: 15.83%), SS detected the same mutation after 12 months whereas the rest of the low-level mutations were not identified in the subsequent follow-up samples in the remaining 6 patients. (Figure 2). F317L mutation is considered partially sensitive to imatinib but resistant to dasatinib [14]. In a patient with polyclonal mutations (E255K (VAF: 5.53%) + T315I (VAF: 92.30%)) detected by NGS, longitudinal follow-up by SS identified only T315I but not the mutation with low allele burden (Supplementary Table 5). Patients with other compound mutations (F359L + H396R, E355G + H396R and M244T + Y253F + A395T) did not have detectable mutational burden in the subsequent follow up by SS but showed a better response with therapy change (Figure 2). Both the patients with H396R mutation (partially sensitive to imatinib) received increased dose imatinib at the discretion of the treating physician which could be the reason why the mutant clone was not identified by SS [14]. The patient with Y253F mutation (which is partially sensitive to nilotinib [14]) underwent therapy switch to nilotinib and hence this mutation was not detected upon subsequent follow-up.

Recent studies [1,6] have shown that patients with even low-level of *BCR-ABL1* KD mutation detected by NGS have poor outcome compared to those without mutations. Comparison of 2-year FFS in mutation positive vs. negative cases (patients in cohort-3) showed no significant difference (13 of 23 mutation negative cases vs. 5 of 11 patients with detectable mutation showed failure to imatinib as defined previously [5]). This apparent divergence could at least be partly explained by the

differences in the selection of patients between previous studies and ours. Our study included only those cases with inadequate response to imatinib and were mutation negative by SS, while Kizilors et al. [6] have screened all patients' samples irrespective of their response to imatinib.

There is a lack of prospective randomized studies assessing the importance of low-level mutations detected by NGS for change of therapy and little data regarding the impact of low-level mutations in CML AP/BC and loss of MMR [11]. Based on our observations in a small set of samples, most of the low-level mutations detected by NGS were not selected in the subsequent follow-up. While the increased sensitivity of NGS based *BCR-ABL1* KD screening is well accepted, the usefulness of this tool in the context of patients with suboptimal response to imatinib warrants further investigation especially to aid in therapy selection in patients with novel mutations or clinically relevant mutations with low VAF.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Acknowledgements

RVS, VM and PB are supported by Wellcome DBT India Alliance (IA/S/17/1/503118, IA/CPHS/18/1/503930 and IA/S/15/1/501842) respectively.

ESB is supported by DST Inspire fellowship, Government of India.

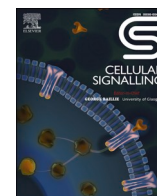
The help provided by Dr. Eunice S. Edison in managing the DNA sequencing core facility is gratefully acknowledged.
PB-Poonkuzhali Balasubramanian.
RVS- Shaji Ramachandran Velayudhan.
VM-Vikram Mathews.
ESB-Esther Sathya Bama Benjamin R.

Funding

This study is supported by a Centre of Excellence grant from Department of Biotechnology India: BT/COE/34/SP13432/2015 and Indian Council of Medical Research Centre for Advanced Research grant 70/14/14-CAR to Dr. Poonkuzhali Balasubramanian.

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Immortalised chronic myeloid leukemia (CML) derived mesenchymal stromal cells (MSCs) line retains the immunomodulatory and chemoprotective properties of CML patient-derived MSCs

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ARTICLE INFO

Keywords:

Chronic myeloid leukemia
Mesenchymal stromal cells
Leukemic stem cells
Tyrosine kinase inhibitors

ABSTRACT

Despite the success of Tyrosine kinase inhibitors (TKIs) in treating chronic myeloid leukemia (CML), leukemic stem cells (LSCs) persist, contributing to relapse and resistance. CML Mesenchymal Stromal Cells (MSCs) help in LSC maintenance and protection from TKIs. However, the limited passage and self-differentiation abilities of primary CML MSCs hinder extensive research. To overcome this, we generated and characterized an immortalised CML patient-derived MSC (iCML MSC) line and assessed its role in LSC maintenance. We also compared the immunophenotype and differentiation potential between primary CML MSCs at diagnosis, post-treatment, and with normal bone marrow MSCs. Notably, CML MSCs exhibited enhanced chondrogenic differentiation potential compared to normal MSCs. The iCML MSC line retained the trilineage differentiation potential and was genetically stable, enabling long-term investigations. Functional studies demonstrated that iCML MSCs protected CML CD34⁺ cells from imatinib-induced apoptosis, recapitulating the bone marrow microenvironment-mediated resistance observed in patients. iCML MSC-conditioned media enabled CML CD34⁺ and AML blast cells to proliferate rapidly, with no impact on healthy donor CD34⁺ cells. Gene expression profiling revealed dysregulated genes associated with calcium metabolism in CML CD34⁺ cells cocultured with iCML MSCs, providing insights into potential therapeutic targets. Further, cytokine profiling revealed that the primary CML MSC lines abundantly secreted 25 cytokines involved in immune regulation, supporting the hypothesis that CML MSCs create an immune modulatory microenvironment that promotes growth and protects against TKIs. Our study establishes the utility of iCML MSCs as a valuable model to investigate leukemic-stromal interactions and study candidate genes involved in mediating TKI resistance in CML LSCs.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm (MPN) of clonal origin that develops from altered primitive stem cells [1]. It is characterised by the BCR::ABL1 fusion gene, resulting in constitutive tyrosine kinase activity leading to atypical proliferation of both immature and mature myeloid cells [2]. Despite the impressive efficacy of TKIs in inducing remission and controlling the disease,

leukemic Stem Cells (LSCs) have emerged as a major obstacle to achieving treatment-free remission (TFR) [3–6]. The bone marrow mesenchymal stromal cells (BMMSCs) play a crucial role in leukemogenesis and the protection of LSC from TKIs [7,8]. Recent evidence suggests that the persistence of LSCs might be attributed to the sheltered environment provided by the BM niche. The BM hematopoietic microenvironment is replete with growth factors and cytokines that potentially offer survival signals to the residual CML cells [9–11]. It was

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<https://doi.org/10.1016/j.cellsig.2024.111067>

Received 6 November 2023; Received in revised form 24 January 2024; Accepted 26 January 2024

Available online 27 January 2024

0898-6568/© 2024 Published by Elsevier Inc.

reported previously that cytokines present in the bone marrow microenvironment (BMM) can stimulate leukemic cell growth and contribute to imatinib (IM) resistance in the mouse model of *BCR::ABL1*, Arf-null lymphoblastic leukemia [12]. Additionally, MSCs derived from IM-resistant CML patients exhibited protective effects against cell death induced by IM or Nilotinib (NI) in K562 and BV173 cells compared to MSCs from IM responders. This protection was attributed to elevated IL-7 secretion from the MSCs [11]. Furthermore, MSCs derived from CML patients displayed an enhanced capacity to support normal and CML BM CD34⁺CD38⁻ cells [13]. Despite these findings, there is limited knowledge of how the MSCs are altered due to their interaction with CML LSCs and further research is needed. It is challenging to conduct extensive research to study the alterations in CML patient-derived BMMSCs. Primary MSCs possess several drawbacks, including cell senescence and the loss of multipotent differentiation capacity. Immortalising primary MSCs offers several potential benefits and applications to overcome these limitations. Immortalised MSCs enable the generation of a consistent and abundant cell supply, provide a source for long-acting therapeutic cells, avoid cell senescence, and maintain differentiation capacity [14]. In the present study, we characterised bone marrow MSCs derived from CML patients at diagnosis, post-treatment, and from healthy volunteer bone marrow samples to assess alterations in their trilineage differentiation potential. Since primary CML MSCs have finite growth, and HS-5 (healthy donor-derived MSCs) cannot replicate the CML microenvironment, we generated and characterised immortalised MSCs from a CML patient (iCML MSCs), comparing them with primary CML MSCs. The iCML MSCs can be valuable for investigating the mechanisms underlying MSC-mediated protection in CML.

2. Materials and methods

2.1. Primary CML cells

After obtaining written informed consent, BM aspirates were collected from patients with chronic phase (CP) CML at diagnosis ($n = 4$), post-treatment ($n = 4$), and those with suspected lymphoma diagnosis ($n = 3$) (without bone marrow involvement) to be a source for normal MSCs (Supplementary Table 1). The institutional Ethics committee (IRB Min No. 14754 dated 27.07.2022) approved this study. The BM mononuclear cells (MNCs) were isolated using Lymphoprep (Stem Cell Technologies, Vancouver, Canada) by density gradient separation.

2.2. Generation of mesenchymal stromal cells from CML and Normal BM

BM-MNCs from normal and CML patients were resuspended in α -MEM containing 20% FBS and 1% penicillin-streptomycin and plated at an initial 5×10^5 cells/cm² density. After three days, the cells were washed with PBS, and the remaining adherent cells were cultured until the cells reached approximately 80% confluency. MSCs from passage one was cryopreserved and stored for RNA [15].

2.3. Enrichment of CD34⁺ cells

CD34⁺ cells were enriched immunomagnetically using an EasySep CD34 Positive Selection Kit (Stem Cell Technologies, Vancouver, Canada) following the manufacturer's protocol, and purity was confirmed by staining isolated cells with anti-CD34 antibody (BioLegend, San Diego, California) and analyzed using Accuri C6 flow cytometer (BD Biosciences). Cryopreserved CD34⁺ cells of the CML patients and the normal donor were thawed and cultured in CD34⁺ cell expansion medium constituting StemSpan™ SFEM II medium (Stem Cell Technologies, Vancouver, Canada) supplemented with CD34⁺ Expansion Supplement (Stem Cell Technologies, Vancouver, Canada) and UM729 (Stem Cell Technologies, Vancouver, Canada). The cells were cultured for 6–7 days with half-medium change every alternative day to assess the cell proliferation.

2.4. Immunophenotyping of CML MSCs

Normal BM and CML patient-derived MSCs were subjected to trypsinization and washed once with PBS. The cells were then incubated for 20 min with 1 μ l of anti-CD34-APC, anti-CD73-FITC, anti-CD90-PE, and anti-CD45-Pacific blue (BioLegend, San Diego, California). Flow cytometric data were analyzed using FlowJo software (BD Biosciences).

2.5. Generation of immortalised CML MSC line (iCML MSC)

Cryopreserved CML MSCs derived from CML patient were revived and used for immortalisation. Briefly, 0.2×10^6 cells were transduced with TRE-HEE-UbC-hKO1-rtTA lentiviruses encoding HPV16-E6/E7 (HEE) genes (a kind gift from Yukio Nakamura) by spinfection at 2250 rpm for 1.5 h. They were cultured in α -MEM supplemented with 20% FBS, with half-medium change on alternate days. The medium was supplemented with 1 μ g/ml of Doxycycline (dox) (Sigma-Aldrich, St. Louis, MO, USA) from the fourth day. The cells were maintained throughout the culture at a seeding density of $5\text{--}6 \times 10^5$ cells/ml at 37 °C in 5% CO₂ with complete medium change on alternative days. Single cells were sorted and characterized immunophenotypically for the MSC surface markers [14].

2.6. Trilineage differentiation

Trilineage differentiation for osteogenic, adipogenic, and chondrogenic lineage was evaluated in the primary CML MSCs, iCML MSC line, and HS-5 as control. Cells were seeded in 24-well culture dishes at 5000 cells/cm² for adipogenic differentiation, expanded to sub-confluence, and replaced with the adipogenic differentiation media (Stem Cell Technologies, Vancouver, Canada). Undifferentiated MSCs cultured on a stromal medium were used as a control. After 21 days, cells were fixed with 10% formalin, washed, and stained with 0.5% Oil Red O. All images were captured using the Leica DMIL microscope. The Oil Red O staining was eluted with 100% isopropanol for 15 min at room temperature, and dye concentration was quantified by absorbance measurement at 510 nm.

Chondrogenic and osteogenic differentiation was carried out in 3D culture. Briefly, 0.5×10^6 cells were loaded into 15 ml centrifuge tubes, spun at 400 g for 12 min, and left uninterrupted for 48 h to form 3D culture. The pellets were supplemented with chondrogenic /osteogenic medium every three days for four weeks. After incubation for four weeks, the resulting cell pellets were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 μ m sections. Osteogenic differentiation was assessed by 1% Alizarin Red S and Von kossa stain. To confirm the differentiation, the differentiated chondrogenic pellets were stained with 1% Alcian Blue, Safranin O fast green, Toluidine blue, and Picrosirius red [16].

2.7. Immunohistochemistry

2.7.1. Collagen type II

After chondrogenic differentiation, the sections were subjected to antigen retrieval by treatment with pronase (1 mg/ml) and hyaluronidase (2.5 mg/ml). The histopathology sections were then incubated with mouse monoclonal anti-collagen type II antibody (5 μ g/ml) (II-II6B3, DSHB) overnight at 4 °C. Subsequently, they were subjected to a 30-min incubation with a secondary antibody [HRP labeled goat anti-mouse immunoglobulin (31,430, Pierce, Wisconsin, USA)]. The stained sections were then treated with a 3, 3'-diaminobenzidine solution, followed by counterstaining with hematoxylin. Histological imaging of the stained sections was performed using an Olympus BX43F microscope.

2.7.2. Collagen type X

The osteogenic-differentiated paraffin pellet sections were subjected to antigen retrieval using chondroitinase ABC (0.1 units/ml) for 1 h (Cat

no. C3667, Sigma-Aldrich, St. Louis, MO, USA) and pepsin solution (Cat. R2283, Sigma-Aldrich, St. Louis, MO, USA). The sections were incubated with mouse monoclonal anti-collagen type X antibodies (ab49945, Abcam, Cambridge, UK) overnight at 4 °C, followed by a 30-min incubation with HRP-labeled goat anti-mouse immunoglobulin antibody (31,430, Pierce, Wisconsin, USA; 1:500 dilution). The sections were then further stained with a 3,3-diaminobenzidine solution and counterstained with hematoxylin. Slides were dehydrated, cleared with xylene, and mounted with DPX. Histological imaging of the stained sections was performed using an Olympus BX43F microscope [16].

2.8. Real-time quantitative PCR

Total RNA from 5×10^5 cells was extracted using a Total RNA extraction purification plus kit (Norgen Biotek Corp, Ontario, Canada) per the manufacturer's protocol. Following the manufacturer's instructions, 180 ng of total RNA was converted to cDNA using a High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and stored at -20 °C for further downstream applications. cDNA conversion was confirmed by PCR amplification of the housekeeping gene β -glucuronidase (*GUS*). Quantitative RT-PCR was set up with SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Shiga, Japan) using specific primers (Supplementary Table 2) and analyzed with Quant Studio 6 Flex real-time PCR systems (Applied Biosystems, Carlsbad, CA, USA). The program was 95 °C (30s), followed by 40 cycles of 95 °C (5 s) and then 60 °C (34 s). The expression of genes specific to osteogenic (*RUNX2*), adipogenic (*PPARG*), and chondrogenic (*ACAN*) differentiation were estimated after trilineage differentiation. The results were normalized to *GAPDH* as the housekeeping gene [16].

2.9. NanoString nCounter Plexset custom panel

We made a custom gene panel consisting of 21 target genes associated with cell surface markers, transporters, cell adhesion, and immune regulation such as *CTSG*, *S100A8*, *S100A9*, *ELANE*, *RHOA*, *SPC24*, *KALRN*, *PRTG*, *CDH2*, *PCDH17*, *ARHGAP6*, *CYBB*, *PPARG*, *CTTN*, *ABCC3*, *RXRA*, *HIST1H2BJ*, *HIST1H2BN*, *CD82*, *CD276* based on previous publications [17–24]. The expression of these 21 genes were examined in a cohort of CML CD34⁺ ($N = 10$) with and without iCML MSC coculture and hypoxia. Read counts were normalized using a positive control and three housekeeping genes (*ACTB*, *GAPDH*, and *GUSB*).

2.10. iCML MSCs functional analysis

For coculture experiments to show the interaction between CML CD34⁺ cells and the MSC stromal layer, the stromal cells were seeded in tissue culture dishes at 20,000 cells/cm² concentration. After 12 h, CML CD34⁺ cells were added to the stromal layer at a ratio of 1:10 (stromal cell: leukemic cells). The protective effect was evaluated by measuring apoptosis after 48 h of treatment with or without imatinib at concentration 10 μ M and 20 μ M using Annexin V/7AAD (BD biosciences) [25]. 10 mg of Imatinib mesylate was dissolved in 1 ml of DMSO and stored at -20 °C.

2.11. Collection of conditioning media from the MSC

Immortalised CML MSCs and CML MSCs from 3 different CML patient samples were seeded in tissue culture-treated plates in standard growth media and allowed to grow to 60–70% confluency. After the cells reached confluency, fresh, complete media was added to the cells. The conditioned media (CM) containing secreted factors was collected after 24 h centrifuged to remove cell debris and filtered through a 0.2 μ m filter. CM was aliquoted and stored at -20 °C until further use. CM media from the 3 primary CML MSCs were pooled for assessment in the cytokine array [8].

2.12. Cytokine array

Human Cytokine Antibody Array C Series 1000 (RayBiotech, Norcross, GA) allows us to detect 274 cytokines, was used per the manufacturer's instructions. Membranes with pre-spotted capture antibodies were blocked using 1 $\times$ blocking buffer for two hours and then incubated with a conditioning medium for 3 h at 25 °C. Chemokines were detected by incubation with biotinylated detection antibodies for 2 h at 25 °C. Following the addition of horseradish peroxidase conjugated streptavidin and overnight incubation at 4 °C, signals were revealed by enhanced chemiluminescence (Westar Supernova, Cyanogen, Bologna, Italy). Signals were captured by a 3–5 s exposure on a Chemidoc (Biorad, Richmond, California, USA) [26].

2.13. Semi-quantification of cytokine antibody array data

Chemiluminescent cytokine array data were semi-quantified with ImageJ software (developed by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, MD). The intensity of each cytokine spot was measured based on spot signal density. For every spot, the net density gray level was determined by normalizing with the negative controls from the measured density gray levels. The density gray level of six positive controls was used to normalize the results from different membranes. The density for each cytokine was then averaged over duplicated spot signals.

2.14. Statistical analysis

Two-tailed Student's *t*-test for unpaired samples with unequal variance or 1-way ANOVA with Tukey's correction for multiple comparisons were applied using GraphPad Prism version 8 (GraphPad Software, La Jolla, USA). Differences between various treatment groups were analysed by 2-way ANOVA, with Tukey's correction for multiple comparisons, using GraphPad Prism version 8 (GraphPad Software). *p* values <0.05 were considered statistically significant.

3. Results

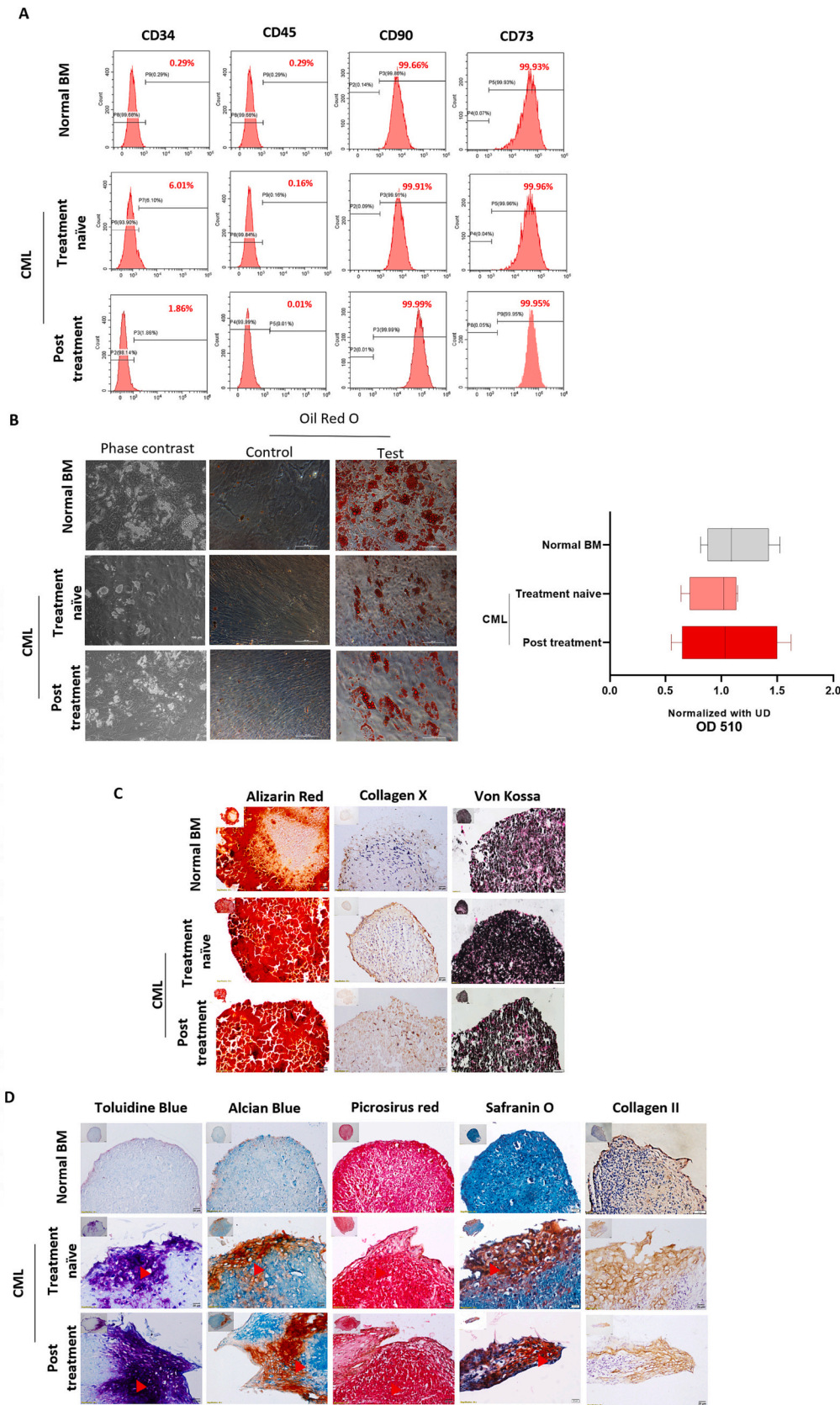
3.1. Comparison of BM MSCs from CML patients vs. Normal BM

To understand the specific changes incurred by the stromal cells in CML, we compared the characteristics of the MSCs from the bone marrow of CML-CP patients at diagnosis and post-treatment, with that of normal bone marrow (NBM) cells. The CML and NBM-derived MSCs were spindle-shaped and reached confluency approximately in 2–3 weeks. Immunophenotypically, both NBM and primary CML MSCs were positive for stromal markers CD73 and CD90 and negative for hematopoietic lineage CD45 and CD34, suggesting phenotypic similarity (Fig. 1A and Fig. S1A–D).

To assess the alteration in the differentiation capacity of MSCs derived from CML patients at diagnosis, post-treatment, and NBM, we differentiated the MSCs into adipocytes, osteocytes, and chondrocytes by supplementing specific differentiation media. Both CML and NBM-derived MSCs could be differentiated into all three lineages (Fig. 1B–D). Although there was no significant difference in the adipogenic (Fig. 1B–C) or osteogenic differentiation potential between CML MSCs vs. NBM MSCs, the chondrogenic differentiation potential was enhanced in CML MSCs (Fig. 1D). There was no difference in the trilineage differentiation potential between the primary CML MSCs derived from treatment naïve and post-treatment BM.

3.2. Immortalised CML MSCs (iCML MSC) retained the characteristics of primary CML MSCs

HS-5 has been extensively used as a model to investigate the interplay between the leukemic and stromal cells. However, it has been



(caption on next page)

Fig. 1. Propagation and characterization of CML and healthy donor-derived MSCs. A) Immunophenotypic characteristics of CML ($n = 8$); Treatment naïve ($n = 4$) and Post treatment ($n = 4$) vs. Healthy MSCs ($n = 3$). MSCs were positive for CD73 and CD90 and negative for hematopoietic lineage markers CD45 and CD34. Representative image of B) adipogenic differentiation (after oil Red O stain) and Quantification of in vitro adipogenic differentiation potential of NBM and CML MSC C) Osteogenic differentiation by Alizarin Red stain and immunohistochemistry of collagen X, and D) Chondrogenic differentiation potential (by Toluidine blue, Alcian Blue, Picrosirius red, Safranin O and Collagen II protein expression). Scale bar: 50 μ M, 10 $\times$ Magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reported that HS-5 cells lack the ability to differentiate into chondrocytes [27]. Although primary CML MSCs showed enhanced chondrogenic differentiation potential compared to NBM-derived MSCs and HS-5, they have a limited lifespan and tend to undergo senescence, losing their functional abilities after a few passages (typically 2–3). Hence, we immortalised CML MSC derived from treatment naïve CML patient who was treated with dasatinib and achieved major molecular response at 12 months. We transduced the primary CML MSCs with HEE lentivirus carrying Human Kusabira Orange (huKO⁺) fluorescent protein, using dox to induce the expression of HEE proteins. After four days of dox induction, huKO⁺^{bright} cells were single-cell-sorted and then characterised further. After isolating single cell expressing the huKO⁺ fluorescent protein, we carefully examined their characteristics

(Fig. 2A). Among these clones, we chose a clone that displayed spindle-shaped morphology and was positive for MSC markers CD73 and CD90 and negative for hematopoietic markers CD45 and CD34 (Fig. 2B). To assess further the genetic stability of the immortalised CML MSC clone, we carried out a karyotyping analysis, which showed no additional chromosomal abnormalities after the immortalisation process compared to the primary CML MSC (Fig. 2C). The iCML MSC line we developed could be cultured up to 25 passages, allowing for long-term investigations.

The iCML MSCs retained the capacity for trilineage differentiation at early passage (p-12) and late passage 25, closely resembling the differentiation potential observed in primary CML MSCs (Fig. 3A–C and Fig. S2A–C), while HS-5 displayed limited differentiation potential

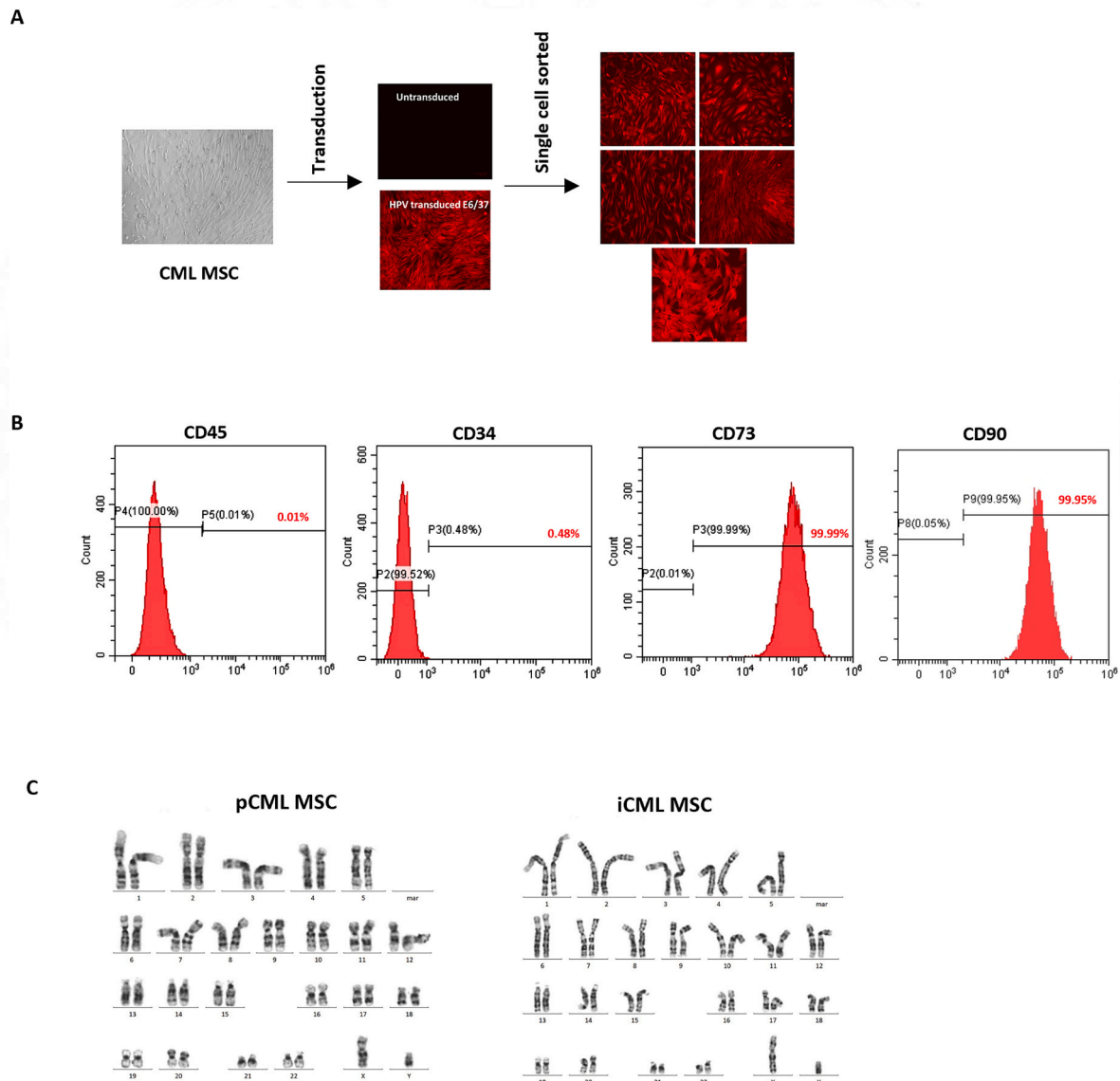


Fig. 2. Generation and characterisation of immortalised CML MSCs. A) Representative image of MSCs transduced with HEE lentivirus expressing huKO⁺ after dox induction. Scale bar: 50 μ M B) Immunophenotypic characterisation of immortalised CML MSCs expressing MSC markers and C) Karyotypic analysis of primary CML MSCs before and after immortalisation, and negative for hematopoietic lineage markers. 10 $\times$ Magnification.

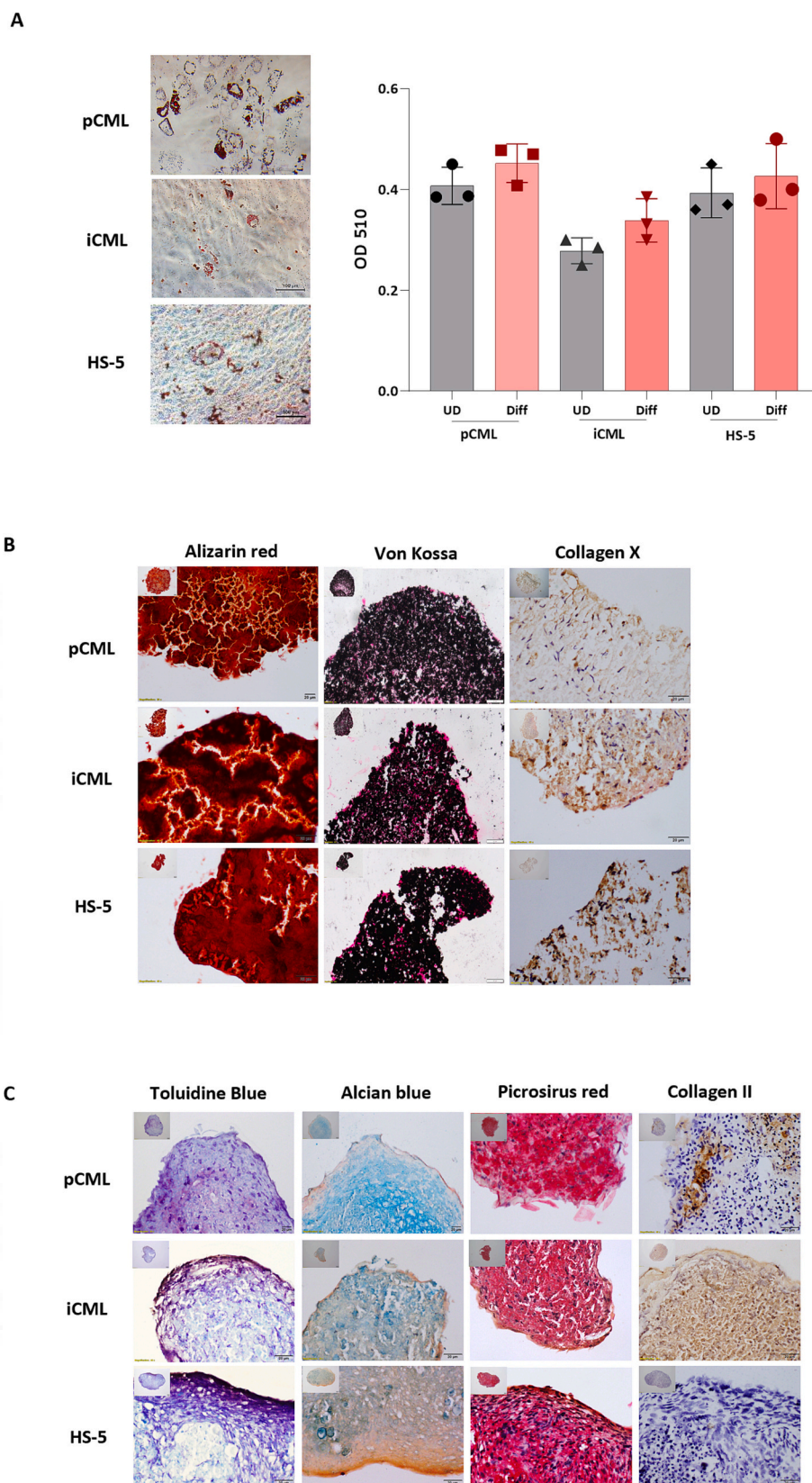


Fig. 3. iCML MSC retained trilineage differentiation potential. Representative image of A) Oil Red O stain and absorbance at OD540 ($n = 3$) showing adipogenic differentiation B) osteogenic differentiation by Alizarin Red stain, Von Kossa stain and immunohistochemistry of Collagen-X, and C) Chondrogenic differentiation by Toluidine blue, Alcian blue, Picrosirius red, and Collagen II in p-CML, iCML MSCs at passage 12, and HS-5 after adipogenic differentiation. pCML – Primary CML MSCs, iCML MSC – immortalised CML MSCs, and HS-5 – Healthy donor-derived immortalised MSCs. Scale bar: 50 μ m, 10 $\times$ Magnification.

towards the chondrogenic lineage (Fig. 3C), similar to a previous study [27]. The expression of *PPARG*, *RUNX2*, and *ACAN* genes were evaluated as markers of adipogenic, osteogenic, and chondrogenic differentiation, respectively in primary CML MSCs, iCML MSCs, and HS-5. Compared to undifferentiated cells, we observed increased *PPARG* and *RUNX2* expression in adipo and osteo-differentiated cells in all these MSCs. iCML MSCs showed decreased *PPARG* and increased *RUNX2* expression compared to primary CML MSCs (Fig. S3 A-B). As expected, *ACAN* expression was higher in primary and iCML MSCs than HS-5, confirming the enhanced chondrogenic differentiation potential of CML MSCs (Fig. S3C) [3–6]. The iCML MSCs started to lose their spindle shape, forming enlarged and more granular morphology after passage 25, after which proliferation of the cells reduced over time (Fig. S4A and B). The iCML MSCs retained the chondrogenic differentiation potential as the primary CML MSCs, suggesting that the iCML MSC clone can be an appropriate model for studying the interaction between BMM and CML cells.

3.3. iCML MSC reduce imatinib-induced apoptosis of primary CML CD34⁺ cells

We then studied the functional role of iCML MSCs in regulating the growth of CML CD34⁺ cells and their protective effect against imatinib. MSCs interact with the leukemia cells through their secreted factors/direct cell contact to regulate the growth of leukemic cells [9] and chemoprotect the leukemic cells from therapeutic insult [11]. To confirm the iCML MSCs' role in mediating the protection of CML LSCs, we cocultured primary CML CD34⁺ cells with iCML MSCs and then treated them with IM, as described in Fig. 4A. CML CD34⁺ cells cocultured with iCML MSC showed a significant increase in viable cells compared to those cultured alone (132% vs. 100%, $p = 0.019$). Further, CML-CD34⁺ cells cocultured with iCML MSC and maintained in hypoxia showed a similar increase in viable cells compared to those cultured alone (137% vs. 100%, $p = 0.025$). We also observed that CML-CD34⁺ cells cultured with MSCs and then treated with IM (20 μ M) showed reduced cell death in comparison to those cultured alone (85% vs 53%, $p < 0.0001$) (Fig. 4B). These results suggest that iCML MSC retained their functional property and protected the CML CD34⁺ cells from IM treatment. We then tested if the secretory factors released from the iCML MSC line support the growth of leukemic cells. Conditioning media was collected, and the CML CD34⁺, AML blast and healthy donor CD34⁺ cells were cultured with and without the conditioning medium. The proliferation and percentage of CD34⁺ cells were measured sequentially. The growth of leukemic cells increased gradually by 15 times when cultured in the presence of conditioning media (Fig. 4C—D and S5A—B) unlike the cells cultured in basal media. In contrast, healthy donor CD34⁺ cells cultured in conditioning media did not show enhanced proliferation as leukemic cells. These results suggest that the secretory factors released from the iCML MSC may selectively support the growth and proliferation of leukemic cells.

3.4. Coculture of CML CD34⁺ with iCML MSC showed dysregulation of genes associated with calcium metabolism and cell adhesion

To identify the genes related to bone marrow microenvironment-mediated protection in CML LSCs, we cocultured CML CD34⁺ cells with iCML MSCs and tested the expression of selected genes using NanoString technology, as described in Fig. 5A. We observed upregulation of *CDH2* and *CD276* (Fig. 5B and C) and downregulation of *S100A8*, *S100A9*, *ARHGAP6*, *GP1BB*, *CTSG* and *HIST1H2BJ* in CML CD34⁺ co-cultured with iCML MSCs (Fig. 5D-I). None of the other genes tested showed any significant change in expression with and without coculture. These genes are found to be associated with cell adhesion, calcium metabolism, and stemness. Investigating the functional role of these genes may provide valuable insights into identifying novel therapeutic targets for eradicating CML CD34⁺ cells (Fig. S6A-L).

3.5. Cytokine profiling of iCML MSCs

Soluble factors and cytokines secreted by MSCs play a direct role in maintaining leukemic cells through cell-extrinsic mechanisms. To investigate this, we performed cytokine profiling for 274 cytokines and chemokines using conditioned media obtained from pooled primary CML MSCs ($n = 3$) and iCML MSCs ($n = 1$). We found that 207 cytokines were detected in pooled CML MSCs, while 83 were detected in iCML MSCs (Fig. 6A-B). Importantly, all cytokines detected in iCML MSCs were also present in the pooled primary CML MSCs (Fig. 6C). Notably, the cytokines abundantly secreted by the pooled CML MSCs were also secreted by iCML MSCs, albeit in lower quantities (Fig. 6D). These included cytokines such as MCP-1, TIMP1, TIMP2, IL-6, PAI-1, IGFBP-6, IL-1Ra, CCL5, Nidogen, Ferritin, Beta-2-M, Beta IG-H3, Angiogenin, BDNF, and Follistatin. MCP-1, also known as Monocyte chemoattractant protein-1 or Chemokine (CC-motif) ligand 2 (CCL2), is a key chemokine that regulates the migration and infiltration of monocytes/macrophages [28,29]. TIMP1 and TIMP2, which are tissue inhibitors of metalloproteinases, regulate the activity of MMPs (Matrix Metalloproteinases) involved in tissue remodelling and degradation [30]. Additionally, Follistatin and Beta IG-H3, regulated by TGF-beta, are involved in various cellular processes, including cell adhesion, migration, and extracellular matrix remodelling [31,32].

4. Discussion

Like normal hematopoietic progenitors, leukemic cells physically interact with the BM niche and deliver signals capable of reprogramming MSCs and endothelial cells (ECs), consequently altering the hematopoietic niche [33]. The present study shows that CML MSCs have morphology and immunophenotypes like NBM MSCs. In contrast to a previous study, we have observed that CML MSCs exhibited enhanced chondrogenic differentiation compared to NBM MSCs [13]. This could probably be due to the heterogeneity in the patient samples from which these MSCs were derived. In normal hematopoiesis, chondrocytes play an essential role in the secretion of inflammatory cytokines, and the increased chondrogenic differentiation potential in CML MSCs might replicate an inflammatory microenvironment in CML patients [34]. There was no difference in the trilineage differentiation potential between CML patients at diagnosis and post-treatment, indicating IM treatment has little or no effect on the stromal cells. These results are in agreement with a previous study showing no difference in the trilineage differentiation potential [35].

HS-5 is extensively utilised as a model for exploring the interaction between leukemia and stromal cells. However, HS-5 lacks chondrogenic differentiation ability. Given the higher chondrogenic potential in CML MSCs, HS-5 may not be ideal for investigating BMM-mediated protection in CML leukemic stem cells (LSCs) [27]. Meanwhile, primary CML MSCs, despite their relevance, undergo senescence after a few passages (typically 2–3), leading to a loss of functional abilities. We developed an immortalised CML MSC line to overcome this challenge. Immortalisation enables indefinite culturing, facilitating long-term investigations and hence the ability to be used for screening molecules capable of targeting CML LSCs and their interactions with the bone marrow microenvironment. iCML MSCs and the primary CML MSCs were karyotypically similar.

Both early and late passages of iCML MSCs retained the trilineage differentiation potential as the primary CML MSCs. This characteristic makes the iCML MSC an appropriate model for studying the interaction between BMM and CML cells. iCML MSCs also protected CML CD34⁺ cells from IM treatment because of the secretory factors released from the iCML MSCs. Genes associated with calcium metabolism were decreased in CML CD34⁺ cells cocultured with iCML MSCs. Investigating the functional role of these genes may provide valuable insights into identifying novel therapeutic targets for eradicating CML CD34⁺ cells. Previous studies in other diseases generated immortalised MSC

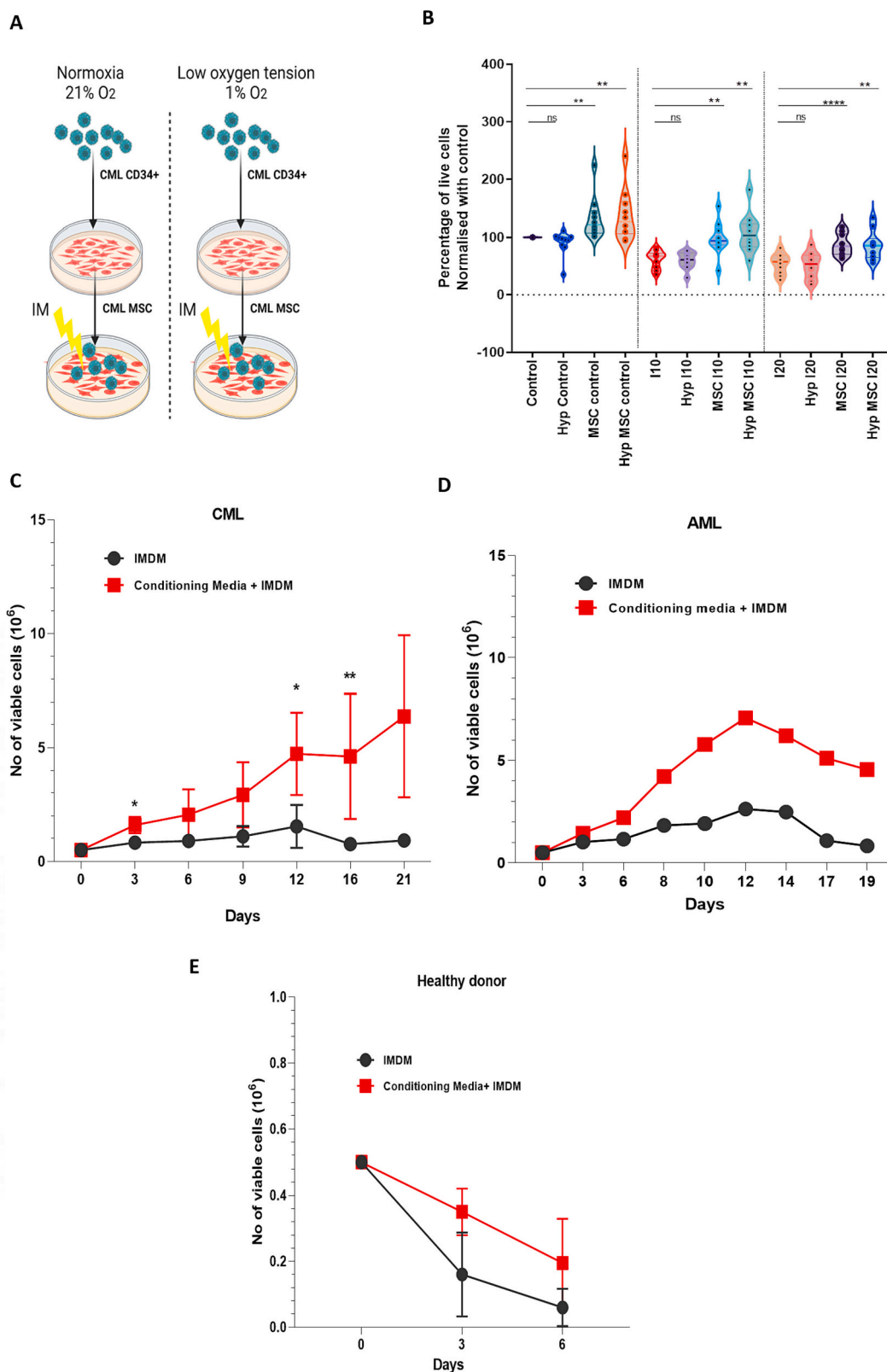


Fig. 4. iCML MSC protected the CML CD34⁺ cells from TKI treatment and selectively increased the growth of CD34⁺ cells in ex vivo. A) Experimental outline of iCML MSC coculture with CML CD34⁺ (n = 10) B) Violin plot showing the percentage of live cells with and without IM treatment. Growth rate of C) CML CD34⁺ cells (n = 5) D) AML blast cells (n = 1) and E) Healthy donor CD34⁺ cells (n = 2) cultured with and without conditioning medium. CM: Conditioning medium.

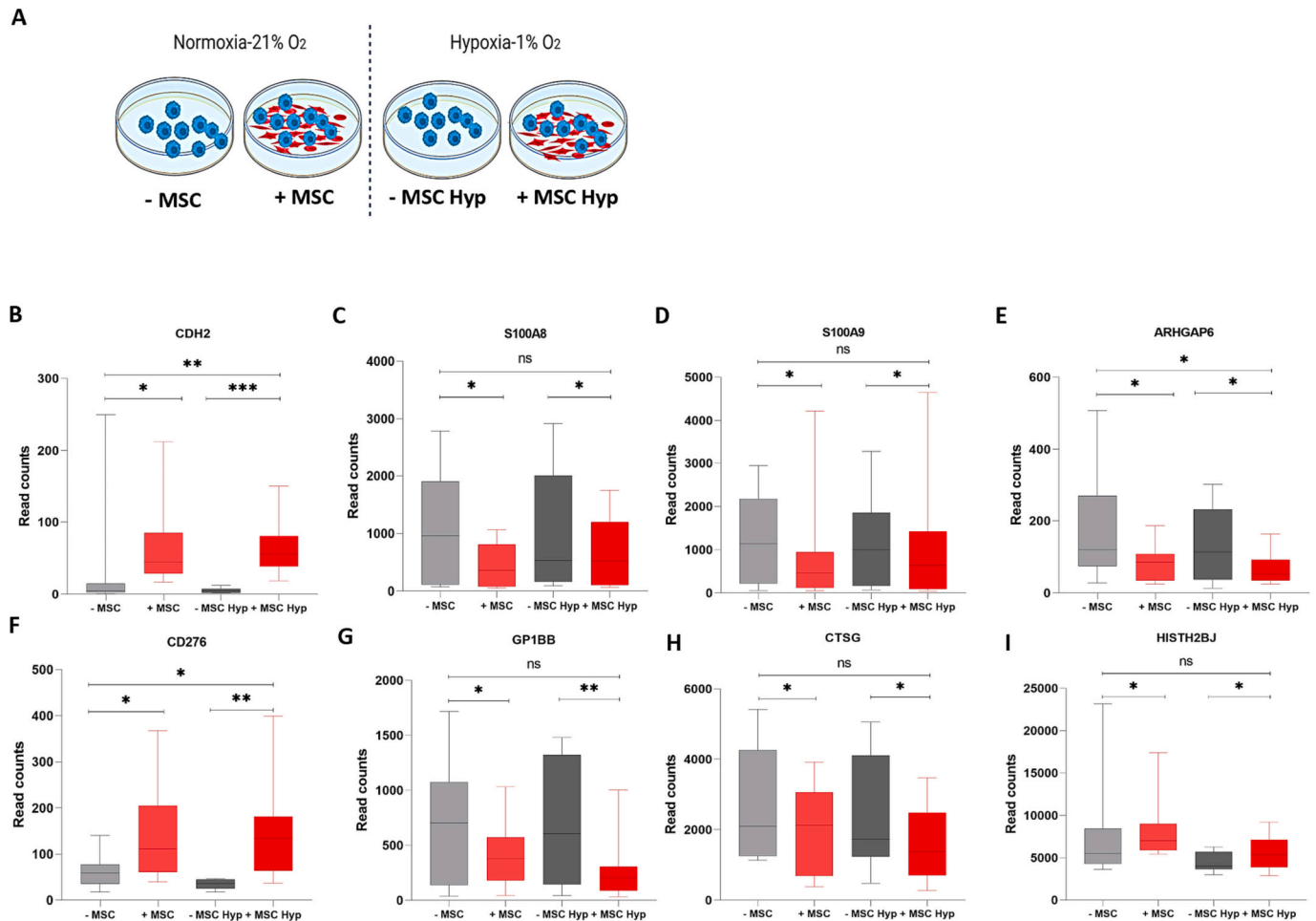


Fig. 5. Genes associated with cell adhesion and calcium metabolism are dysregulated in CML CD34⁺ cocultured with iCML MSCs. A) Experimental outline of coculture system B) Significant downregulation of *S100A8*, *S100A9* and upregulation *CDH2* in CML-CD34⁺ ($n = 10$) cocultured with MSCs.

lines have shown consistent phenotype and function similar to the respective primary MSCs [36,37]. Our findings suggest that iCML MSCs showed enhanced chondrogenic potential compared to primary CML MSC, probably because of clonal selection. The fact that the iCML MSCs retained the trilineage differentiation potential may be beneficial in studying leukemia-stromal cell interaction, whilst the role of enhanced chondrogenicity in mediating BMM resistance remains unclear.

Further cytokine profiling of CML MSCs revealed that 20 cytokines and 5 chemokines associated with immune response and inflammation were abundantly secreted by CML MSCs present. Among the cytokines secreted, we identified Insulin-like Growth Factor Binding Protein-6 (IGFBP6), which plays an essential role in proliferation, differentiation, migration and cell survival. High expression of IGFBP6 was observed in primary myelofibrosis compared to the healthy donors. The primary myelofibrosis microenvironment and that IGFBP-6 may play a central role in activating sonic hedgehog (SHH) pathway during the fibrotic process [38]. In CML, pretreatment with IGFBP-6 and/or PMO (an inducer of SHH) restored LAMA-84 cell viability after treatment with dasatinib, suggesting that both IGFBP-6 and SHH are involved in the resistance mechanisms induced by the modulation of TLR-4 (*Toll-like receptor 4*) [39]. Additionally in murine CML model, MIP-1 α , MIP-1 β , IL-1 α , IL-1 β , and TNF- α supported the growth of BCR::ABL⁺ LTHSC compared to the healthy LTHSC [8]. MIP-1 α and MIP-1 β have been reported to inhibit the growth of hematopoietic progenitors [40]. Interestingly, we observed that MIP-1 β was secreted by CML MSCs, and conditioning media from these MSCs exclusively supported the growth of CML CD34⁺ cells. Notably, cytokines associated with cell adhesion

(BMP-4, Nidogen-1, TIMP1 and TIMP-2), TGF- β signalling (beta IG-H3 and Follistatin) and inflammation (RANTES, Angiogenin, PAI-I, Osteoprotegerin) were secreted by CML MSCs [30,31,41–47] which might play a significant role in immune regulation, supporting the hypothesis that CML MSCs create an immunomodulatory microenvironment that promotes cell growth and protects against TKIs [48–51]. Further studies are warranted to confirm these results.

In summary, the immortalised CML cell line maintains the main characteristics observed in primary CML MSCs, thus making this a valuable model for studying leukemic-stromal interaction and functional studies of candidate genes involved in mediating TKI resistance in CML LSCs.

5. Conclusion

CML MSCs, while sharing similarities with normal bone marrow MSCs, exhibited an enhanced chondrogenic differentiation potential. To overcome challenges related to the limited lifespan of primary CML MSCs, we developed an iCML MSC which retained its trilineage differentiation potential and protected the CML CD34⁺ cells from IM treatment. Cytokine profiling of CML MSCs highlighted the secretion of numerous factors associated with immune response and inflammation, thereby creating an immunomodulatory microenvironment that promotes selective growth of CML CD34⁺ cells and protection against TKIs.

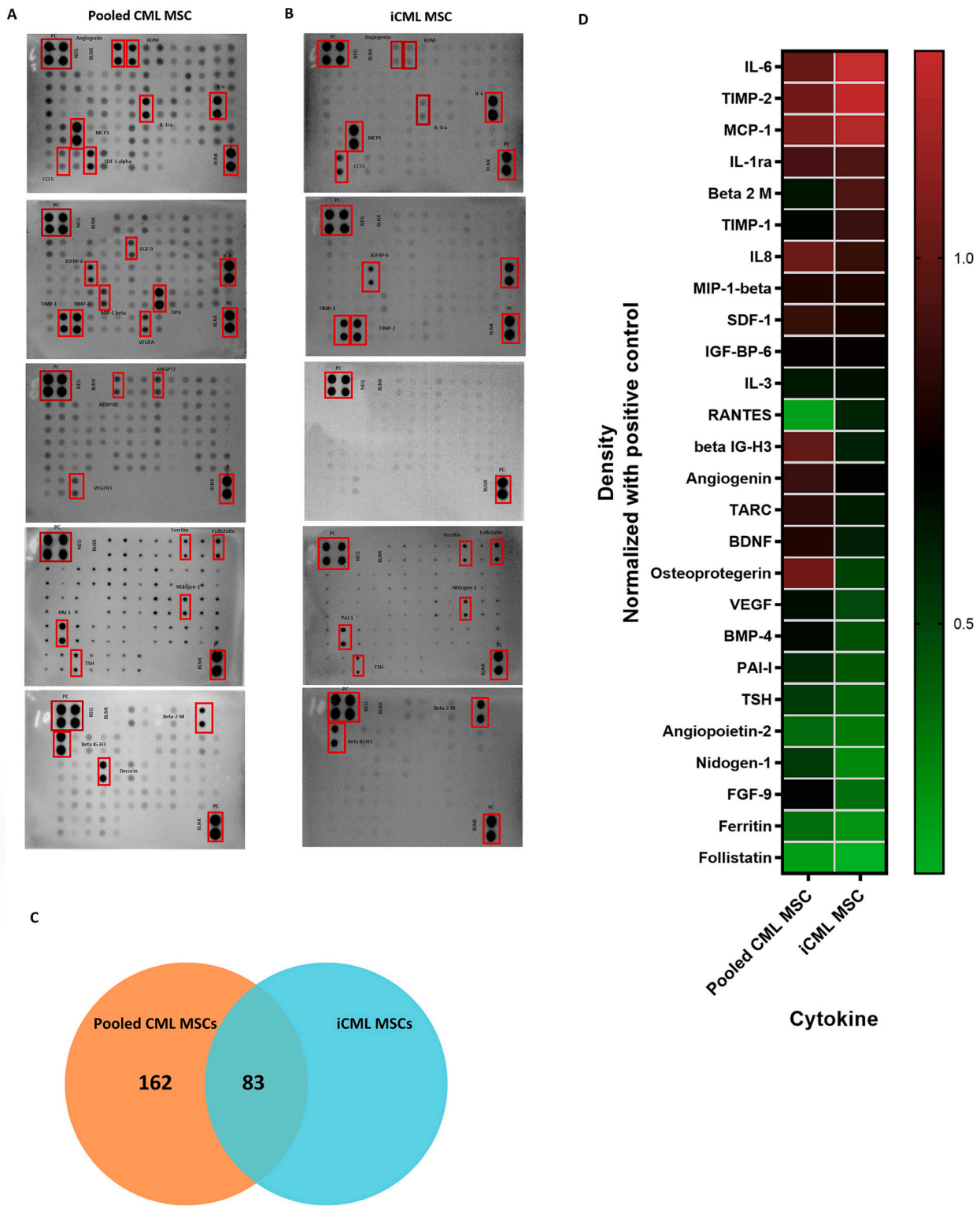


Fig. 6. Cytokine profiling of iCML MSC. ELISA array showing the cytokines detected from the supernatant of A) Pooled CML and B) iCML MSCs, C) Comparison of cytokines secreted by HS-5, Pooled CML, and iCML MSC, and D) Relative expression of secreted factors detected using the cytokine array.PC-Positive control, BLNK-Blank, NEG-Negative control.

CRedit authorship contribution statement

Esther Sathya Bama Benjamin: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Elizabeth Vinod:** Investigation, Methodology, Resources,

Supervision, Visualization, Writing – review & editing. **Raveen Stephen Stallon Illangeswaran:** Conceptualization, Methodology, Visualization, Writing – review & editing, Data curation. **Bharathi M. Rajamani:** Data curation, Methodology, Writing – review & editing. **Rakhi Thalayattu Vidhyadharan:** Data curation, Methodology. **Abhirup Bagchi:** Resources, Writing – review & editing. **Arnab Maity:** Methodology.

Ajith Mohan: Data curation, Methodology. **Ganesh Parasuraman:** Data curation, Methodology, Visualization, Writing – review & editing. **Soosai Manickam Amirtham:** Methodology. **Aby Abraham:** Investigation, Resources, Writing – review & editing. **Shaji R Velayudhan:** Investigation, Resources, Supervision, Writing – review & editing. **Poonkuzhali Balasubramanian:** Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Poonkuzhali Balasubramanian reports financial support was provided by India Ministry of Science & Technology Department of Biotechnology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study is supported by a Centre of Excellence grant from Department of Biotechnology India: BT/COE/34/SP13432/2015 to BP and by a CMC Fluid Research grant (IRB Min No. 14754, dated 27.07.2022).

RVS and PB are supported by Wellcome DBT India Alliance (IA/S/17/1/503118, and IA/S/21/2/505932), respectively.

ESB is supported by DST-INSPIRE fellowship, Government of India. RSSI and BMR are supported by ICMR (Indian Council of Medical Research) SRF. RTV by CSIR and AM by UGC. Technical assistance provided by Ms. Sangeetha is gratefully acknowledged. We thank the staff of the histopathology and flow cytometry facility of the Centre for Stem Cell Research, Vellore, India for their help.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellsig.2024.111067>.

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Synergistic effect of co-treatment with all-*trans* retinoic acid and 9-*cis* retinoic acid on human lung cancer cell line at molecular level

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Received: 3 March 2018 / Accepted: 24 March 2019
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Abstract

The major challenge in treating cancers with ATRA is the limited availability inside the cell and resistance developed in prolonged treatment. We made an attempt for co-treatment of human NSCLC cell lines (A549) with ATRA and its isomeric precursor (9*cis*RA). In this study, the growth inhibitory effect of ATRA, 9*cis*RA and combination of both were tested in A549 cells by MTT and Trypan blue assays. As the effects of retinoid are mediated through their receptors, their gene expression levels were analyzed by RT-PCR. The target gene receptor, RAR- β protein expression, was analyzed by immunocytochemistry. The cancer cell (A549) growth inhibitory effect was significantly ($p \leq 0.001$) enhanced in combination treatment when compared with the result of individual treatments. The mRNA expression levels of both RAR- β and RXR- β were found to be increased in co-treatment (band density of 0.75 and 0.806, respectively) when compared with 9*cis*RA treatment (0.25 and 0.112) and ATRA treatment (0.01 and 0.081). A concomitant enhancement in the target RAR- β protein expression was observed in co-treated cells when compared with individual treatments. We thus conclude that the co-treatment had increased the availability of ATRA, by isomerization of the 9*cis*RA which then resulted in an increased expression of both RAR- β and RXR- β receptors and the target protein RAR- β which in turn inhibited lung cancer cell growth. Our study results have explored the mechanism of synergistic effect of co-treatment with ATRA and 9*cis*RA and further preclinical studies are necessary to validate the application of co-treatment of retinoid in clinical use.

Keywords 9-*Cis* retinoic acid · ATRA · Retinoic acid receptor · Retinoid X receptor · RT-PCR · Immunocytochemistry

Introduction

Lung cancer is developed by the unregulated proliferation of cells present in the lung. It is one of the deadliest diseases that affects men mostly. It is the second most cause of cancer death in women. One of the major causes of lung cancer is smoking tobacco and about 80–90% of lung cancer is due to cigarette smoking. However, only 11% of heavy cigarette smoking ultimately develops lung cancer, suggesting that there might be genetic factors which predispose to lung cancer risk. Despite improvements in therapy, 90% of lung cancer patients die (Minna et al. 2002). It urged the researchers across the globe to develop new strategies like molecular therapy to treat or at least to control the cell multiplication

in lung cancer. One of the promising approaches is to regulate the gene expression, which is involved in cell growth homeostasis. Tumorigenesis is a multistep process which begins with a mutation in genes leading to the transformation of malignant derivative genes either gaining a dominant function or recessive loss of function (oncogene) of tumor suppressor genes (Hanahan and Weinberg 2000; Stewart and Wild 2017; Jemal et al. 2011).

Most of the lung cancers are caused due to mutation in various genes such as tumor suppressor gene, proto-oncogenes and by certain other epigenetic factors which are involved in the regulation of cell proliferation, apoptosis, and differentiation (Cheung et al. 2003; Moison et al. 2013). The epigenetic factors are signal and ligand-dependent molecules which are able to modulate the expression level of target genes involved in cell regulation. Certain natural compounds act by regulating proliferation and differentiation of cells. Among them, retinoid is one that is naturally occurring ligand that controls the growth of various cell types, most commonly it acts by interacting with the nuclear receptor.

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Natural retinoids and their synthetic derivatives act as chemoprevention agents. They are used for various cancer types and acute promyelocytic leukemia. One of the challenges faced in the chemotherapies is to develop a strategy that can control malignant disease. These retinoids treatment aims at the future therapeutic effect for the management of metastases (Dragnev et al. 2000; Pettersson et al. 2002).

Retinoids act specifically by interacting with the nuclear retinoid receptor belonging to the superfamily of steroid/thyroid hormone receptor. These receptors are retinoic acid receptors (RARs) and retinoid X receptors (RXR), each comprising 3 subtypes which are α , β , and γ . RARs and RXRs heterodimers bind to a specific DNA sequence, the RA response element, in the promoter regions of target genes to modify their expression of target genes. Several retinoid-regulated genes carrying these response elements in their regulatory regions have been identified. Each subtype of nuclear retinoid receptor is thought to regulate the expression of distinct genes and specific patterns of expression during embryonic development and different distributions in adult tissues (Dragnev et al. 2000).

All-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9*cis*RA) are the important metabolic products of Vitamin A that plays a significant role in the expression of various genes, including Retinoic Acid Receptor- β (RAR β) and Retinoid X Receptor- β (RXR- β) which play an important role in the regulation of cell cycle (Rhinn and Dollé 2012; Lotan 1999). Both ATRA and 9*cis*RA are reported to have therapeutic effects (Zuccari et al. 2005; Han et al. 2010; Wen et al. 2005). ATRA binds RAR only with high affinity, whereas 9*cis*RA binds both RAR and RXR. 9*cis*RA exhibited 40-fold more affinity to RXR than the RAR. However, the heterodimerization of RAR–RXR is essential for the action of retinoid (Buskohl et al. 2012). So not only ATRA but also 9*cis*RA are necessarily needed for efficient therapeutic effects (Bushue and Wan 2010). Currently, the ATRA therapy is in clinical use for APL and under clinical trials for other cancers. The major drawback faced in ATRA therapy is the resistance developed over the long-term treatment. This may be due to the internal regulatory mechanism through the binding proteins such as CRABP-I and II which direct the excess ATRA for degradation. Since the 9*cis*RA can be converted into ATRA inside the cells to meet the demand for ATRA, we intended to study the therapeutic efficacy of combinational treatment of both ATRA and 9*cis*RA on human lung cancer A549 cells. Several conclusions indeed recommend the therapeutic potential of combinational treatment of retinoids with chemotherapeutic agents, arsenic trioxide or drugs targeting epigenetic enzymes (Di Masi et al. 2015). The expression of RAR- β as well as RXR- β was reported to be downregulated in non-small cell lung cancer, which enabled the cancer cell to evade apoptosis and it was also reported that the downregulation of RAR- β expression has

led to increase lung cancer risk. These receptors are suggested to act significantly in the prognosis of lung cancer and the biological aggressiveness of non-small cell lung cancer (Brabender et al. 2005). Maintaining the levels of RAR and RXR was reported to inhibit the growth of cancerous cell by regulating the cell cycle proliferation (Mi et al. 2007). It was also reported that the downregulation of RAR- β expression has led to increase in lung cancer risk (Bogos et al. 2008). The RAR- β is also known as tumor suppressor and the major target gene of retinoid action (Lei and de Thé 2003). Previous studies have noted abnormalities in RAR- β gene and its expression was depleted in human lung cancer (Soria et al. 2003).

The current investigation has been, therefore, undertaken to evaluate the synergistic therapeutic action of ATRA and 9*cis*RA treatments in lung adenocarcinoma cell lines and their molecular action on the expression of RAR- β and RXR- β genes by RT-PCR. The RAR- β protein expression was also analyzed in the treated cell line by immunocytochemistry using antibody. This will eventually aid to formulate an effective drug for lung cancer that can act at the molecular level, as the existing chemotherapy was reported to be ineffective to this non-small cell lung cancer. Hence, this investigation on the combinatorial drug effect on A549 cell line will lead to the development of an effective combinational drug.

Materials and methods

Chemicals and cell line

Non-small cell lung cancer cell line (A549) was obtained from NCCS, Pune and cultured in DMEM (Hi-media) containing 10% fetal bovine serum and antibiotics at 37 °C and CO₂ level was maintained at 5% (Lotan 1999). All-*trans* retinoic acid and 9*cis*RA were purchased from Sigma-Aldrich, Inc.

Experimental groups

- Group I: A549 cell line without treatment.
- Group II: A549 cell line treated with ATRA and 9*cis*RA.
- Group III: A549 cell line treated with 9*cis*RA alone.
- Group IV: A549 cell line treated with ATRA alone.

Treatment of cell line

Non-small cell lung cancer (A549) cell line was seeded in 96-well flat-bottom plates (5000 cells/ well) and allowed to adhere for 24 h. Then, the cells were treated with ATRA and 9*cis*RA individually as well as in combination at a total

concentration of 1 μM as given in the experimental groups for 24, 48 and 72 h (Szabó et al. 2014).

MTT assay

Cells were seeded in 96-well flat-bottom plates (5×10^3 cells/well) and allowed to adhere for 24 h at 37 °C in 5% CO_2 atmosphere. Treatment was done as mentioned above and incubated further for different time intervals such as 24, 48 and 72 h. After incubation time, 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml) was added. Then, 100 μl of DMSO was added and the absorbance was measured at 570 nm (Siddikuzzaman and Grace 2012). The percentage viability was calculated using the following formula:

$$\% \text{ viability} = (\text{OD of treated}) / (\text{OD of control}) \times 100.$$

Trypan blue dye exclusion method for cytotoxicity

The cells were treated as mentioned above in the experimental group for various time intervals such as 24, 48 and 72 h. Then, the cells were diluted with 0.4% trypan blue dye solution which was then counted using hemocytometer, under microscopic observation. The non-viable cells were stained blue and the viable cells remained unstained (Ramya et al. 2012). The percent viable cells were calculated as follows:

Percent viable cells

$$= (\text{No of viable cells}) / (\text{Sum of viable cells and non - viable cells}) \times 100.$$

RNA isolation and quality analysis

RNA was extracted from the treated cell line using TRIzol (Invitrogen) as mentioned in the manufacturer's instructions. The quality of the RNA was analyzed by 1% agarose gel electrophoresis and spectrophotometer readings. cDNA was generated from RNA using Superscript First-Strand cDNA Synthesis System (Invitrogen). After inactivation of the reverse transcription (RT) enzyme (15 min at 75 °C), the RNA template was hydrolyzed with 50U of RNase H for 1 h at 37 °C (Moison et al. 2013).

Retinoid receptor gene expression analysis

The synthesized cDNA was amplified using Taq polymerase (NEB, Ipswich, MA, USA) with specific primers designed for RAR- β : F-5'TCCGAAAGCTCACCAGGAAA-3', R-5'GGCCAGTTCACCTGAATTTGTCC-3'. RXR- β : F-5'-GCA GCCAAATGACCCTGT-3', R-5'CCCGCAGCAATATGACCTGA-3' and beta-actin (internal control) F-5'CATGTACGTTGCTATCCAGG, R-5'CTCCTTAATGTCACGCAC

GAT'3. The PCR was carried out for 30 cycles: 94 °C for 20 s, 60–64 °C for 20 s, 72 °C for 45 s and then 10 min at 72 °C. The PCR product was then analyzed by agarose gel (1%) electrophoresis (Cheung et al. 2003). The bandwidth in the gel was measured using the software GelQuant.Net—version 1.8.2.

Immunocytochemical staining for RAR β protein expression

Coverslips were sterilized using 95% ethanol and then kept in 6 well plate. Cells were grown on a coverslip in a 6-well plate at a density of 5×10^4 cells/well and allowed to reach confluency. Cells were treated as mentioned above in the experimental group for 72 h. After the treatment, the cells were fixed using 4% paraformaldehyde and incubated overnight with primary antibody, anti-RAR β (Santa Cruz biotechnology, USA). It was incubated with IgG-HRP labeled secondary antibody (Santa Cruz biotechnology, USA) for 2 h, which was then incubated in peroxidase substrate and the cells were counter stained with Hematoxylin. The cells were then mounted on the glass slide using DPX mountant and observed under light microscope (Cras et al. 2007).

Statistical analysis

All data were expressed as mean $\pm$ SD. The statistical analysis was done using one-way analysis of variance (ANOVA) followed by a Dunnett's test (Graph pad InStat version 3.00 for Windows 10; Graph Pad Software, Inc., La Jolla, CA, USA). A p value ≤ 0.05 is considered significant.

Results

Growth inhibitory activity of retinoid by MTT assay

The growth inhibitory activity of 1 μM ATRA, 9*cis*RA and their combinational treatment on A549 cell line were expressed in terms of percent viable cells in Fig. 1a. The percent viability was found to be decreased upon increasing treatment period from 24 to 72 h in all the 3 groups. Moreover, the combination therapy of ATRA and 9*cis*RA has shown a significantly lower cell viability ($p \leq 0.001$) when compared with individual treatments. From the observations, it was clear that A549 cells were sensitive to combinational treatment of ATRA and 9*cis*RA and their synergistic effect increased with the increase in treatment time period.

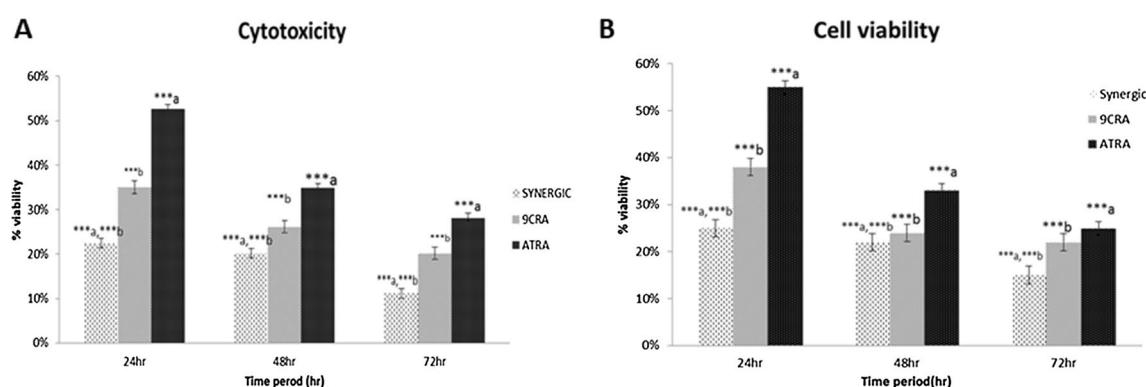


Fig. 1 **a** Growth inhibitory effects of ATRA, 9-cis RA and its combination in A549 cell line. Values are expressed in mean $\pm$ SD ($n=6$). ^aATRA vs combined drug ($a=***p\leq 0.001$); ^b9cisRA vs combined drug ($b=***p\leq 0.001$). **b** Cytotoxicity effects of ATRA, 9-cis

RA and its combination in A549 cell line. Values are expressed in mean $\pm$ SD ($n=6$). ^aATRA vs combined drug ($a=***p\leq 0.001$); ^b9cisRA vs combined drug ($b=***p\leq 0.001$)

Cytotoxicity level by trypan blue assay

Cytotoxic effect of 1 μ M ATRA, 9cisRA and its combinational treatment on A549 cell line were expressed in terms of percent viable cells in Fig. 1b. The toxic effect of retinoids is time dependent. From the result, it was observed that the combinational treatment exhibited maximum toxicity towards A549 cells as indicated by significantly lower percent viability of 15% after treated for 72 h, which was considerably less in comparison to the individual treatments.

Effect of retinoid treatment on expression levels of RAR- β and RXR- β genes

The gene expression was evaluated from the band seen at the expected molecular size of 125 bp for RAR- β and 132 bp for

RXR- β for their specific primers. The RAR- β and RXR- β gene expressions were found to be upregulated in treated A549 cells, when compared with untreated control cell lines as shown in Figs. 2 and 3, respectively. Relatively, a high level of expression was found in combinatorial treatment which was indicated by the bandwidth (Figs. 2, 3). The bandwidth measurement was carried out by GelQuant.Net software and the bandwidth was given as volume percent (%) unit.

RAR- β protein expression in retinoid-treated cell lines

The expression of RAR- β protein was visualized in the nucleus as well as in the cytoplasmic region through microscope after immunocytochemistry staining in A549 cells. The expression levels correlated well with the sensitivity

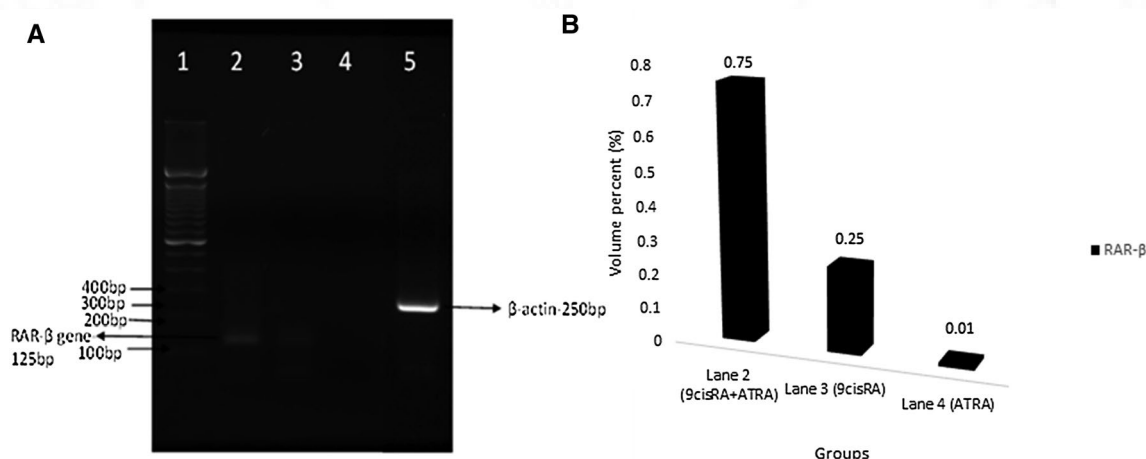


Fig. 2 Reverse transcriptase PCR observation. **a** RAR- β gene expression. Lane 1 shows 100 bp DNA marker, Lane 2 corresponds to the upregulation in the expression of RAR- β in A549 cell line after combinational treatment in comparison with individual treatment 9cisRA

(Lane 3) and ATRA (Lane 4) alone. Lane 5: β -actin gene expression. **b** RAR- β gene band density for combination treatment (0.75), 9 cis RA treatment (0.25) and ATRA treatment (0.01)

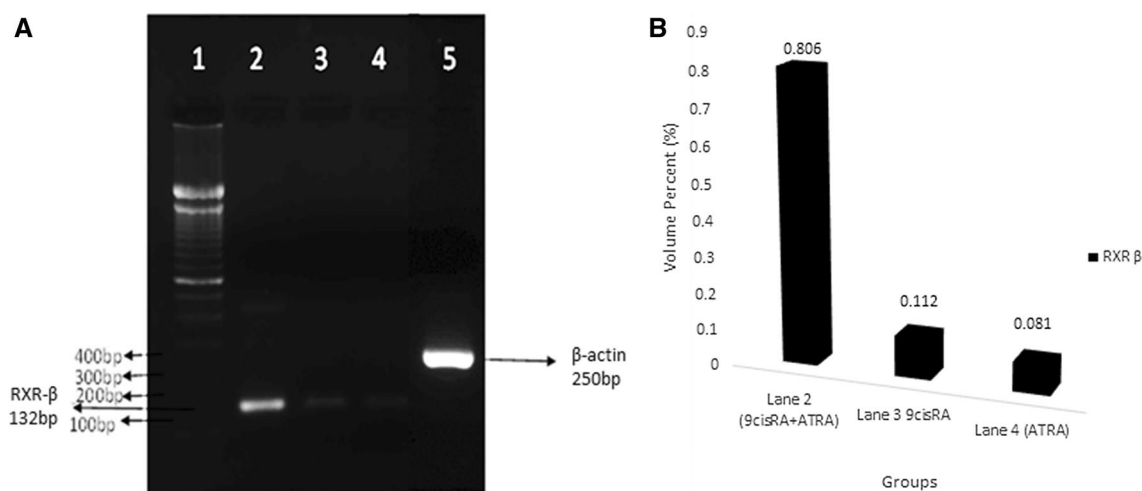


Fig. 3 Reverse transcriptase PCR observation. **a** RXR- β gene expression. Lane 1 corresponds to 100 bp DNA marker, Lane 2 indicates the enhanced expression of RXR- β gene in combinatorial treatment with ATRA and 9cisRA when compared with the individual treat-

ment with 9cisRA (Lane 3) and ATRA alone (Lane 4). Lane 5: β -actin gene expression. **b** RXR- β gene band density for combination treatment (0.806), 9 *cis* RA treatment (0.112) and ATRA treatment (0.081)

of the cell to RA treatment. The expression was found to be more in the co-treatment (ATRA and 9cisRA) as shown in Fig. 4 when compared with individual treatments.

Discussions

The retinoids are a class of natural and synthetic vitamin A analogs, and some retinoids are known to play major roles in cell growth regulation and in the differentiation of normal, benign, and malignant cell type (Dragnev et al. 2000). We have already reported a promising anti-cancer effects of ATRA alone in *in vivo* lung cancer mice models (Siddikuz-zaman and Grace 2012; Ramya et al. 2012). These retinoids act by binding to RARE via RAR/RXR heterodimer formation and eventually follows the classical pathway by which it triggers apoptosis and regulates NF-Kb, TNF- β , MAPK and chromatin remodeling. In the cancerous condition, the

classical pathway is downregulated and thereby resulted in uncontrolled growth of cell. This downregulation is caused due to the lack of expression in certain types of RAR isoforms (RAR- α , β and γ) and RXR (RXR- α , β and γ) isoforms. Among the various isoforms, RAR- β and RXR- β are of importance because of their primary role as tumor suppressor (Pavan et al. 2008; Altucci et al. 2007). Altered RAR and RXR expression level was observed in premalignant oropharyngeal lesions, APL, breast, prostate, lung cancer in comparison with the normal cell. Prolonged treatment with ATRA has made the cancer cells insensitive to this drug. Hence, we made an attempt to study its therapeutic effect and its molecular response levels when co-treated with its isomeric form 9cisRA which is relatively unstable and can be converted to ATRA at demand. RAR- β protein has been reported as the responsive target of ATRA therapy by various studies (Sun et al. 2000).

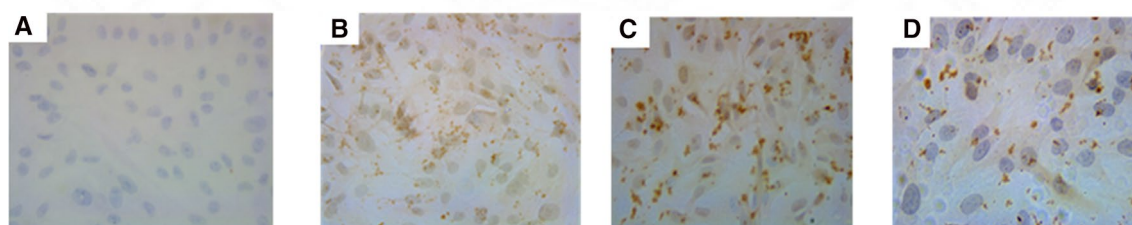


Fig. 4 Immunocytochemical observation of RAR- β protein expression in A549 cell line. **a** Cancer control without retinoid treatment showing very less RAR- β protein expression, **b** A549 cell line treated with the combination of ATRA and 9cisRA showing an upregulated

expression of RAR- β protein. **c, d** 9cisRA and ATRA-treated cells showing enhanced RAR- β protein but in lesser level when compared with the combinatorial drug treatment

In the present study, a comparative study on the effect of 1 μ M ATRA, 9*cis*RA and its synergistic effect on non-small cell lung cancer in A549 cell line is done. As lung cancer is one of the causes of cancer death worldwide, the improvements in diagnostics and treatments are urgently needed for non-small cell lung cancer since it is referred as insensitive to chemotherapies with poor survival rate. In addition, the expression of RAR and RXR is down-regulated in non-small cell lung cancer. Our data showed that ATRA, 9*cis*RA, and its combinational treatment could inhibit the growth of A549 cells, but the growth inhibitory effect of combinational treatment is found to be significantly ($p \leq 0.001$) higher than the individual treatments. The gene expression study carried out in this research revealed that the combination treatment could enhance the mRNA expression levels of both RAR- β and RXR- β than the individual treatments did. Furthermore, the target of RAR- β protein expression has been enhanced significantly by the synergistic effect of all-*trans* retinoic acid and 9-*cis* retinoic acid treatments in our study. This RAR- β is known for its tumor suppressor activity in many cancers (Moison et al. 2013; Cras et al. 2007; Altucci et al. 2007). This combinational treatment would thus enable the treatment of retinoic acid resistant cancer cell by optimizing the levels of retinoids internally in cancer cells. However, further gene expression study is necessary to analyze the role of RAR- β protein in the lung cancer cells.

On combinatorial treatment, the RAR- β protein expression was upregulated and visualized by immunocytochemistry. We thus suggest that the 9*cis*RA might have been converted to ATRA by isomerization. This conversion facilitated more attachment of ATRA to RAR- β and also the 9*cis*RA binding to both RAR- β and RXR- β . These binding dynamics efficiently enhanced the level of RAR- β tumor suppressor protein which in turn resulted in an effective therapeutic action against human lung cancer cells.

Conclusion

The results have shown that the combinational treatment of ATRA and 9*cis*RA has the most impressive activity in non-small cell lung cancer cell by inducing the expression of their receptors such as RAR- β and RXR- β . This dynamic interactions lead to an enhanced level of RAR- β protein which in turn exhibited its growth inhibitory tumor suppressor action of human lung cancer cell line. Thus, the combination therapy may be proposed as an alternative molecular chemotherapy, which is efficient even in cancers known to be insensitive to ATRA. Detailed study on the mechanism of action of retinoic acid is necessary to further validate the application of co-treatment of retinoid in clinical studies.

Acknowledgements The authors would like to acknowledge the Karunya Institute of Technology and Sciences for the financial support given through Karunya Short-Term Project Grant. We also acknowledge partial financial support shared from DST-SERB and DBT, Govt. of India. We would also like to acknowledge the valuable technical support of Dr. C. Vani, Assistant Professor and Ms. Jissin Mathew, Research Scholar, Department of Biotechnology, Karunya Institute of Technology and Sciences, Coimbatore.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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NUDT15 c.415C>T Polymorphism Predicts 6-MP Induced Early Myelotoxicity in Patients with Acute Lymphoblastic Leukemia Undergoing Maintenance Therapy

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Purpose: Severe myelosuppression in patients with acute lymphoblastic leukemia (ALL) undergoing 6-MP-based maintenance therapy is attributed to *TPMT* gene polymorphisms, which is rare in Asian populations. This study aims to evaluate the role of selected polymorphisms in *NUDT15*, *ITPA*, and *MRP4* genes in addition to *TPMT* in predicting 6-MP intolerance during ALL maintenance therapy.

Patients and Methods: We screened for the presence of *NUDT15**3 (c.415 C>T, rs116855232); *MRP4* c.2269 C>T (rs3765534), *ITPA* c.94 C>A (rs1127354) polymorphisms in addition to *TPMT* *2 (rs1800462), *3A (*3B and *3C; rs1800460 and rs1142345) in ALL patients with documented severe neutropenia (cohort-1; n=42). These polymorphisms were then screened in a prospective cohort of ALL patients (cohort-2; n=133) and compared with 6-MP dose reduction, early/late myelotoxicity.

Results: Nineteen (45%) patients in cohort-1 and 18 (14%) in cohort-2 had *NUDT15* c.415 C>T variant while 4 (3%) patients in cohort-2 had *TPMT**3C variant. Five (12%) in cohort-1 and 30 (24%) in cohort-2 had *ITPA* c.94 C>A variant while 9 (22%) and 15 (12%) had *MRP4* c.2269 C>T variant in cohorts-1 and 2, respectively. All in cohort-1 and 36 (27%) in cohort-2 had severe myelotoxicity. Twenty-eight patients (66.6%) in cohort-1 and 40 (30%) patients in cohort-2 had significant 6-MP dose reduction. *NUDT15* c.415 C>T variant explained severe myelotoxicity in 63% and 33% in cohort 1 and 2. *TPMT**3C and *ITPA* c.94 C>A variants also explained myelotoxicity in cohort-2 (Median ANC: 376 vs 1014 mm³; p=0.04 and 776 vs 1023 mm³; p=0.04 respectively). *NUDT15* c.415 C>T polymorphism explained significant myelotoxicity (507 vs 1298 mm³; p<0.0001) in the multivariate analysis as well (β =−0.314, p<0.0001).

Conclusion: *NUDT15* c.415 C>T (15*3), *TPMT**3C, as well as *ITPA* c.94 C>A and *MRP4* c.2269 C>T polymorphisms explain hematotoxicities. Preemptive genotype-based (*NUDT15**3, *TPMT*, *ITPA* c.94 C>A) 6-MP dosing could improve the outcome after maintenance therapy.

Keywords: leukemia, mercaptopurine, myelotoxicity, pharmacogenomics

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer and also occurs in adults.¹ The last phase of the treatment of ALL involves maintenance therapy with daily oral 6-mercaptopurine (6-MP) and weekly methotrexate (MTX) for nearly 2 years in most treatment protocols. However, the significant dose-limiting toxicity is life-threatening myelosuppression² owing to the narrow

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therapeutic indices of these drugs and a wide inter-individual variation in drug response. These toxicities often lead to extended hospitalization resulting in increased cost of treatment. Genetic polymorphisms in drug-metabolizing enzymes and transporters contribute significantly to this variability in response to drugs. It is now well acknowledged that polymorphisms in Thiopurine Methyltransferase (*TPMT*)³ and Nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*)^{4–6} genes explain 6-MP mediated cytopenia.

We have reported previously that *TPMT* variants are rare in our population and do not fully explain the severe myelosuppression occurring in patients with ALL undergoing maintenance therapy.⁷ Further screening for *NUDT15* c.415 C>T polymorphism in a cohort of patients with severe myelosuppression (but lacking *TPMT* variants) showed that only ~53% of these patients carried *NUDT15* c.415 C>T⁸ suggesting that additional genetic factors could play a role. Polymorphisms in Multidrug resistance protein-4 (*MRP4* c.2269 C>T)^{9,10} and Inosine triphosphate pyrophosphatase (*ITPA* c.94 C>A)^{11–13} have also been shown to explain toxicity to 6-MP in specific ethnic populations. We have previously reported that *TPMT* polymorphisms are rare in our populations and do not entirely explain the variation in 6-MP toxicity.⁷ We have recently reported that *NUDT15* c.415C>T polymorphism is a significant determinant of myelosuppression related to the intake of thiopurines in patients with Immune thrombocytopenic purpura and autoimmune hemolytic anemia.¹⁴ A recent report from the Clinical Pharmacogenetics Implementation Consortium (CPIC) has recommended preemptive genotyping of *NUDT15* apart from *TPMT* variant testing before 6-MP dosing.⁶

Although previous studies in the Indian population have highlighted the role of *NUDT15* c.415 C>T polymorphism associated with 6-MP toxicity,^{15–17} there are no comprehensive reports on all the identified genetic polymorphisms related to 6-MP intolerance and early hematological toxicities in patients with ALL. This study aims to evaluate the role of selected polymorphisms in *ITPA*, and *MRP4* genes in addition to CPIC recommended *NUDT15* and *TPMT* testing in predicting 6-MP intolerance/6-MP dose reduction and toxicities during ALL maintenance therapy.

Patients and Methods

Patients

Patients with ALL who experienced severe clinical thiopurine-related myelotoxicity requiring dose-reduction

referred for *TPMT* genetic testing from 2009 to 2017 were included (cohort-1). In addition, all consecutive patients diagnosed with ALL undergoing maintenance therapy between September 2018 and March 2020 in the Department of Hematology, Christian Medical College, Vellore, India, were prospectively enrolled (Cohort-2). The purpose of retrospective cohort 1 is to identify genetic polymorphisms (in addition to *TPMT*) that could explain myelotoxicity, while prospective cohort-2 is to study the influence of genetic polymorphisms on 6-MP intolerance, toxicities, and survival. Patients with less than six-month follow-up or lost follow-up, who underwent consolidation therapy with 6-MP, and those who refused to consent, were excluded from the analysis. Written informed consent was obtained from the patients/parents. This study was approved by the Institutional Review Board [IRB (EC)-ER-1-23-07-2014]. The initial doses of 6-MP and MTX for maintenance therapy were 50 mg/m² daily and 20 mg/m² weekly, respectively. The treating physicians adjusted the 6-MP/MTX doses to maintain a white blood cell (WBC) count of $3.0 \times 10^9/L$ and avoid infections and hepatotoxicity.

Genotyping

Before starting maintenance therapy, peripheral blood was collected in EDTA tubes, and DNA was extracted using the Qiagen Gentra kit. The samples were screened for selected polymorphisms: *NUDT15**3 (c.415 C>T, rs116855232, p.Arg139Cys), *ITPA* (c.94 C>A, rs1127354, p.Pro32Thr) and *MRP4* (c.2269 G>A, rs3765534, p.Glu757Lys) by bidirectional sanger sequencing¹⁴ and *TPMT**3A [*3B (460 G>A, rs1800460, p.Ala154Thr) and *3C (719 A>G, rs1142345, p.Tyr240Cys)] by Restriction Fragment Length Polymorphism (RFLP) and [*2(238G>C, rs1800462, p.Ala80Pro)] by PCR using allele-specific oligonucleotides (ASO) as reported previously.⁷ Additionally, polymorphisms in *NUDT15* exon1 were screened by Sanger sequencing as reported previously.¹⁸

Clinical Outcomes

Clinical data such as dose reduction, WBC/ANC counts, and ALT/AST levels were monitored and documented longitudinally throughout the maintenance therapy. %6-MP dose intensity was defined as the ratio between clinician prescribed 6-MP dose to protocol dose (%) and was captured monthly for the first 6-months since the start of 6-MP. We calculated the average 6-MP dose intensity (up to 6 months) for association analysis in the present study. In addition, information on the total direct hospital costs incurred during the first 6 months of

maintenance therapy was obtained for all the patients. Therapy interruption was defined as the cessation of medicine administration resulting from cytopenia, infections, or hepatotoxicity. Hepatotoxicity (Grade 3 and above) was defined based on the Common Terminology Criteria for Adverse Events version 5.0) (CTCAE5.0)¹⁹ (ALT/AST levels >5.0 – $20.0 \times$ ULN if the baseline was normal; >5.0 – $20.0 \times$ baseline if the baseline was abnormal at any time point during maintenance therapy). Severe myelotoxicity/neutropenia was defined as ANC (Absolute Neutrophil Count) below $500/\text{mm}^3$. Early and late myelotoxicity were documented during 1–3 and 4–6 months, respectively, after maintenance therapy. Overall survival (OS) was calculated from the start of maintenance therapy to the date of death or the last follow-up as applicable. Event Free Survival (EFS) was defined as the percentage of patients who were alive without relapse or death at last follow-up through maintenance therapy. Relapse Free Survival (RFS) was defined as the percentage of patients who had no relapse event at last follow-up from the start of maintenance therapy. Relapse and survival were documented for the prospective cohort only.

Statistics

All statistical analyses were performed using SPSS (IBM SPSS statistics version 21.0, Armonk, NY) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA); p -value <0.05 was used for significance testing. Comparison of clinical response indices such as myelotoxicity (ANC), leukotoxicity (WBC), hepatotoxicity (ALT/AST levels), and %6-MP dose intensities with genotype was done using one-way ANOVA or Mann–Whitney U -test. Associations between genotypes and clinical responses were evaluated using a linear regression model, and multivariate analysis was performed with significant covariates from univariate linear regression model. Haplotype analyses for all genes were performed using SNPStats programs (Institut Català d'Oncologia, Barcelona, Spain). We used Firth logistic regression to test the association between polymorphism and outcomes (R Statistical software version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria). Log-rank Cox regression was used for the survival analysis, and the Kaplan–Meier curves were generated for OS, EFS, and RFS.

Results

Patient Demographics

The baseline characteristics of patients included in cohort-1 ($n=42$) are listed in Table 1. During the study period, 241 patients with ALL underwent maintenance therapy in our

center. Of these, patients who gave consent to participate in the study and those with regular follow-up were prospectively enrolled (cohort-2; $n=133$). The study design is illustrated in Figure 1. There was no significant difference in demographics between the patients enrolled in the study and those not enrolled (Table S1). Most of the patients had B-ALL (82.5%) and belonged to intermediate cytogenetic risk (58%) based on stratification reported previously.^{20,21}

Genotype

None of the patients carried *TPMT* polymorphisms in cohort-1, while four patients (3%) were heterozygous for the *TPMT*3C* variant in cohort-2. Nineteen patients in cohort-1 had *NUDT15 c.415C>T* variant (14 heterozygous and five homozygous), while 18 in cohort-2 were heterozygous for this variant. None of the patients included in the study had polymorphisms in exon 1 of the *NUDT15* gene. The allelic and genotypic frequencies are tabulated in Table S2.

6-MP Dose

In cohort-1, eight patients (19%) required drastic 6-MP/MTX dose reduction (less than 50%), 20 (47.6%) received 50–80% of the total dose, and 14 (33.4%) received more than 80% of the planned dose of 6-MP.

The majority of the patients in cohort-2 ($n=93$; 70%) received more than 80% of the planned dose while 36 (27%) received 50–80% of the full dose, and four (3%) patients received less than 50% of the total planned dose of 6-MP.

Incidence of Myelotoxicity and Hepatotoxicity

Although all the 42 patients in cohort-1 had severe myelotoxicity, 16 patients (38%) had severe neutropenic episodes [median ANC <500 (range 32–504/ mm^3)], while remaining 26 (62%) had a median ANC of 1100 (range: 528–1760/ mm^3) during the study period. Three patients (7%) had a high ALT level (Grade 3), and one patient (2%) had a high AST level (Grade 3) based on NCI CTCAE criteria.

Thirty-six patients (27%) in cohort-2 had severe neutropenic episodes [median ANC <500 (range: 36–507/ mm^3)]. Twelve patients (9%) had high ALT levels (Grade 3), and two patients (2%) had high AST levels (Grade 3).

Further outcome analysis was carried out only in cohort-2, where 21 patients (16%) relapsed during or after maintenance therapy, and the 3-year RFS in this cohort was 84.2% at a median follow-up of 18 (6–38) months. One hundred and twenty-eight patients were

Table 1 Baseline Characteristics of Patients

Patient Parameters	Cohort-1 (n=42) N (%)	Cohort-2 (n=133) N (%)
Median Age at Diagnosis (range)	16.5 (2–56)	17 (1–63)
Sex		
Male	35 (83.3)	88 (66)
Female	07 (16.7)	45 (34)
BSA (m²)	1.5 (0.5–2.2)	1.5 (0.4–2.1)
Immunophenotype		
B cell	31 (73.8)	111 (83.4)
T cell	09 (21.4)	20 (15)
MP-ALL	–	1 (1)
NA	2 (4.7)	1 (1)
Cytogenetics Risk		
Favourable	6 (14.2)	25 (18.7)
Intermediate	25 (59.5)	81 (61)
Poor	7 (16.7)	7 (5.3)
NA	4 (9.6)	20 (15)
ALL Risk		
Standard Risk	17 (40.4)	20 (15)
Intermediate Risk	20 (47.6)	70 (52.6)
High Risk	5 (12)	5 (3.8)
NA	–	38 (28.6)
Polymorphisms		
TPMT variants		
*2A		
Homozygous reference	42 (100)	130 (100)
Not available	0	3 (–)
*3A (*3B&C)		
Homozygous reference	39 (100)	123 (96.8)
Heterozygous variant	–	4 (3.2)
Not available	3 (–)	6 (–)
ITPA c.94 C>A		
Homozygous reference	37 (88)	97 (76.4)
Heterozygous variant	5 (12)	28 (22)
Homozygous variant	–	2 (1.6)
Not available	–	6 (–)
MRP4 c.2269G>A		
Homozygous reference	32 (76.2)	107 (80)
Heterozygous variant	9 (21.4)	13 (10)
Homozygous variant	1 (2.4)	2 (2)
Not available	–	11 (8)
NUDT15 c.415C>T		
Homozygous reference	23 (54.7)	115 (86)
Heterozygous variant	14 (33.3)	18 (14)
Homozygous variant	5 (12)	–

alive at the time of the last follow-up. The 3-year OS and EFS were 96.2 and 83.5%, respectively, at a median follow-up of 18 (6–38) months.

NUDT15, TPMT, ITPA, MRP4 Polymorphisms, and 6-MP Dose Reduction/Therapy Intervention Cohort-1

Although all the patients in cohort-1 had neutropenic episodes and required dose reduction, the *NUDT15* c.415C>T was identified only in 19 patients and *ITPA* c.94C>A in 3 and *MRP4* c.2269G>A in 2 patients, respectively. While five patients homozygous for *NUDT15* c.415C>T polymorphism were highly sensitive to 6-MP and tolerated only 25% of the median dose intensity (range 14–34% of the protocol dose), those with heterozygous (n=14) or wild-type genotype (n=23), tolerated an average dose intensity of 58% (31–94) and 77% (46–96), $p=0.040$ and 0.0005 , respectively (Figure 2A).

With respect to therapy intervention, patients in cohort-1 who were homozygous for *NUDT15* c.415C>T polymorphism had the maximum duration of cessation of 6-MP therapy [median: 80 (25–94) days] compared to patients who were heterozygous or wild type [21 (7–54) and 28 (7–70) days, $p=0.080$ and 0.009 , respectively] (Figure 2B).

Cohort-2

None of the patients in cohort-2 were homozygous for *NUDT15* c.415C>T polymorphism. Patients heterozygous (n=18 (14%)) for *NUDT15* c.415C>T polymorphism were sensitive to 6-MP compared to those with wild type (n=115) genotype [%6-MP dose intensity: 82% (45–96) vs 90% (27–100), $p=0.034$] (Figure 2C).

None of the other polymorphisms screened (*TPMT**3C, *ITPA* c.94 C>A, and *MRP4* c.2269 G>A) were significantly associated with 6-MP dose intensity. With respect to therapy intervention, patients who were heterozygous for *NUDT15* c.415C>T polymorphism had the maximum duration of cessation of 6-MP therapy compared to those who were wild type [23 (0–67) vs 14 (0–67) days, respectively, $p=0.033$] in cohort-2 (Figure 2D).

In the multivariate analysis, there was no significant association between *NUDT15* c.415C>T polymorphism and 6-MP dose reduction (Table 2).

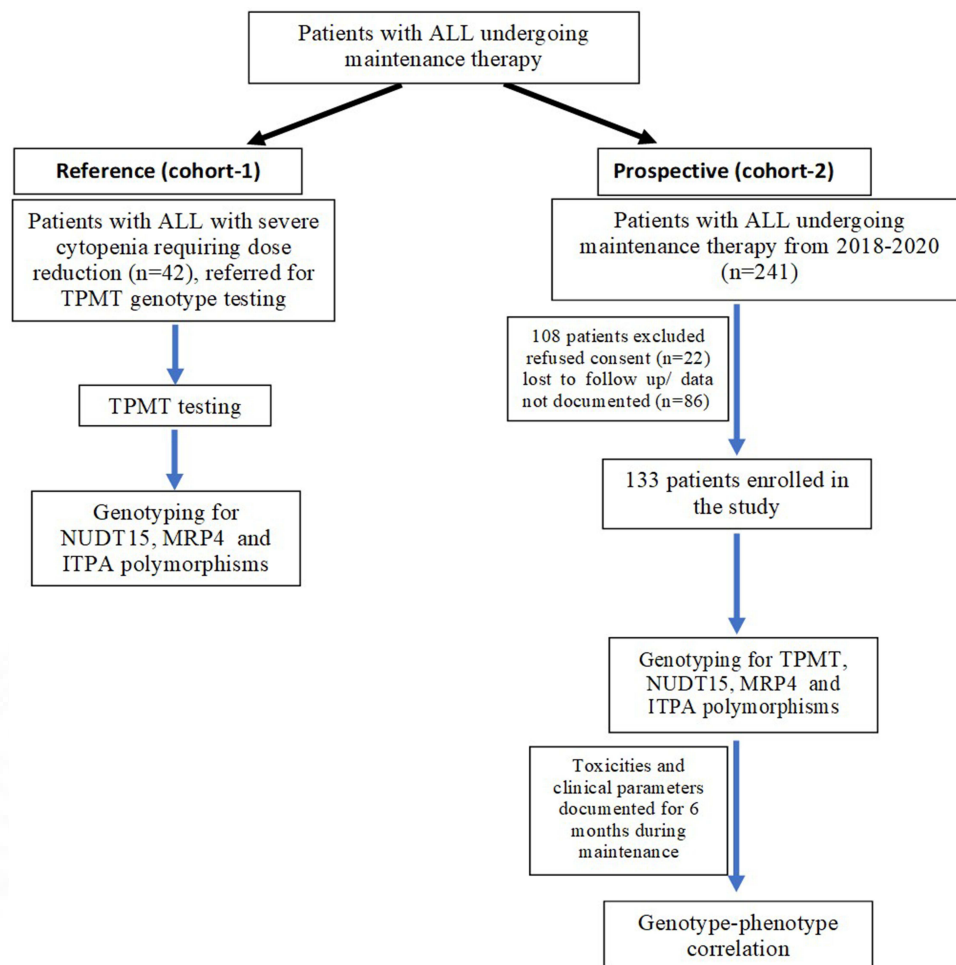


Figure 1 Study design.

NUDT15, ITPA, and TPMT Polymorphisms Explain Myelotoxicity but Not Hepatotoxicity

We then evaluated the association between these polymorphisms and myelotoxicity, hepatotoxicity, relapse, and survival. In cohort-1, patients who were homozygous/heterozygous for *NUDT15* c.415C>T polymorphism had significantly lower ANC [Median ANC-504 (32–1443) mm³] compared to patients with wild-type genotype [988 (280–1760) mm³, $p=0.006$].

In cohort-2, patients who were heterozygous for *NUDT15* c.415C>T polymorphism had significantly higher early (Median ANC: 507 vs 1298 mm³; $p<0.0001$) and late myelotoxicity (Median ANC: 982 vs 1517 mm³; $p=0.015$) compared to patients carrying wild-type genotype (Figure 3). Additionally, *ITPA* c.94C>A and *TPMT**3C polymorphisms also contributed to myelotoxicity; patients who had risk allele for *TPMT**3C and *ITPA* c.94C>A polymorphism had

significantly lower ANC (Median ANC: 376 vs 1014 mm³; $p=0.04$ and 776 vs 1023 mm³; $p=0.04$ respectively) during maintenance therapy (Figure S1). Upon multivariate analysis (cohort-2), although *NUDT15* c.415C>T, and *TPMT**3C ($\beta=-0.209$, $p=0.02$) were significantly associated with myelotoxicity, *NUDT15* c.415C>T was the most significant variable associated with both early ($\beta=-0.314$, $p<0.0001$) and late myelotoxicity ($\beta=-0.197$, $p=0.018$) (Table 2). None of the polymorphisms tested, including *NUDT15*, was associated with hepatotoxicity, relapse (RFS), and survival (OS & EFS) (Figure S2).

Interestingly, a significant proportion of patients (10 of the 36 patients with severe myelotoxicity in cohort 2 (28%)) carried no variants for any of the screened polymorphisms but still had severe neutropenia [Median ANC-214 (88–420 mm³)]. Also, 53% of patients ($n=21$) who received $\leq 80\%$ of the planned dose of 6-MP [Median % 6-MP intensity-67 (45–79)] did not bear any genetic polymorphisms.

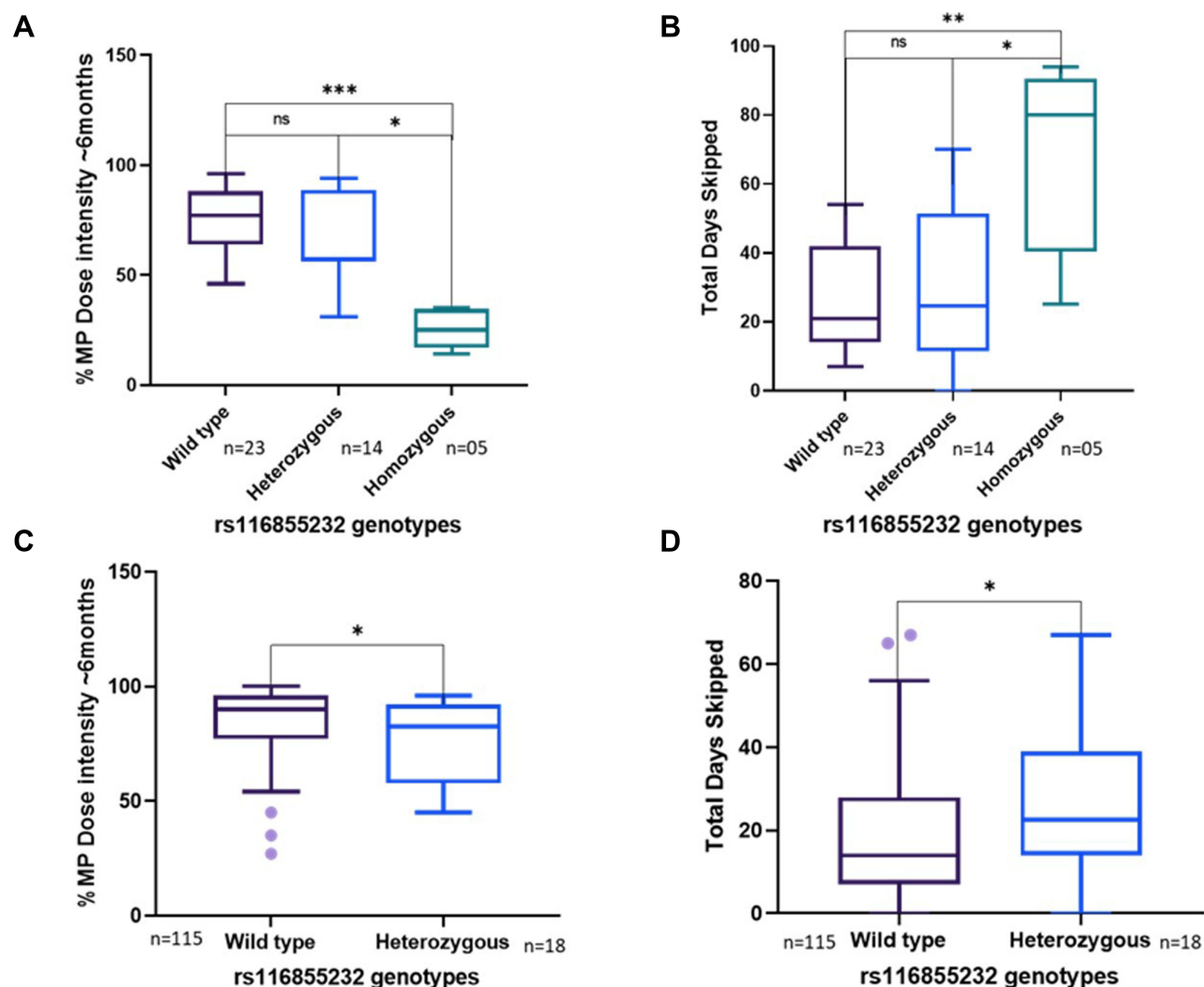


Figure 2 Associations between *NUDT15* c.415C>T polymorphism (rs116855232) and % 6-MP dose intensity (**A**) and therapy interruption (**B**) in cohort-1 and cohort-2 (**C**) and (**D**). *Asterisks indicate the level of the significance (p-value); *Means $p < 0.05$, **Means $p < 0.01$, ***Means $p < 0.001$ and ns-not significant.

Combined Effect of Genotypes on 6-MP Intolerance and Toxicities

To evaluate the combined effects of *TPMT*, *NUDT15*, *ITPA*, and *MRP4* variants on 6-MP intolerance and toxicities, the patients in cohort-2 with different genotype combinations were grouped-

Group 1- patients wild type for *NUDT15*, *TPMT*, *ITPA*, and *MRP4* (n=66);

Group 2- patients with *MRP4* c.2269G>A alone (n=11);

Group 3- patients with *ITPA* c.94 C>A alone (n=21) and

Group 4- patients with *NUDT15* c.415C>T alone (n=12).

Patients carrying the risk allele for *NUDT15* c.415C>T polymorphism alone (Group 4) had increased myelotoxicity (OR-7.33; 95% CI-0.72–3.33; $p=0.002$) compared to Group 1. Similarly, patients harboring the risk allele for *ITPA* c.94 C>A polymorphism alone (Group 3) also had

increased myelotoxicity (OR-3.49; 95% CI-0.12–2.31; $p=0.030$) compared to patients in Group 1.

Cost Analysis

We then compared the total direct hospital costs incurred during the first 6 months of maintenance therapy between patients with wild-type genotype and those with heterozygous or homozygous for *NUDT15* c.415C>T polymorphism among all patients (Cohort-1 + Cohort-2). The median total direct hospital cost incurred during the first 6 months of maintenance therapy was higher for patients with heterozygous or homozygous compared to patients with wild-type genotype for *NUDT15* c.415C>T polymorphism [20245 (IQR: 12192 to 28746 INR) vs 14356 (IQR: 9939 to 19894 INR), $p=0.07$]

Table 2 Association Analysis of Genetic Variants and Clinical Response in Prospective Patients with ALL Undergoing Maintenance Therapy

Clinical Response/ Toxicities	Association Analysis*			
Covariates	Univariate Linear Regression		Multivariate Regression	
	β	p value	β	p value
% MP Dose intensity				
Age	-0.195	0.063	-0.044	0.760
ALL risk group	-0.228	0.027	-0.169	0.134
BSA	-0.191	0.028	-0.166	0.265
<i>MRP4</i> c.2269G>A	-0.178	0.050	-0.029	0.789
<i>NUDT15</i> c.415C>T	-0.173	0.047	0.138	0.203
<i>ITPA</i> c.94 C>A	0.155	0.081	0.137	0.200
Myelotoxicity				
Early				
Age	0.162	0.063	-0.062	0.590
BSA	0.245	0.005	0.263	0.024
<i>NUDT15</i> c.415C>T	-0.328	<0.001	-0.314	<0.001
Late				
<i>TPMT</i> *3A	-0.181	0.042	-0.209	0.025
<i>NUDT15</i> c.415C>T	-0.191	0.028	-0.197	0.018

Notes: *Amongst covariates tested in both univariate and multivariate regression, only significant variables are listed above. Bold values denote statistical significance at the $p \leq 0.05$ level.

Discussion

The present CPIC guidelines⁶ recommend *NUDT15* and *TPMT* testing in patients before thiopurine therapy to prevent dramatic myelotoxicity. The frequency of these

genetic polymorphisms is noticeably lower in the Indian population than in other populations (Table S3). Therefore, we aimed to explore the role of additional genetic variants over and above CPIC recommended genetic variants. This is the first single centre study to explore the association between *NUDT15* (c.415C>T), *TPMT* (G238C, G460A & A719G), *ITPA* (c.94C>A), and *MRP4* (2269 G>A) polymorphisms vs early and late myelotoxicity to 6-MP in Indian patients with ALL on final maintenance therapy.

Similar to previous reports from other Asian studies²² and ours,⁷ except for the rare occurrence of the *TPMT**3C variant, other *TPMT* polymorphisms were not identified in this cohort. The frequency of the *NUDT15* c.415T allele in the present study is similar to previous studies in India,^{15–17} but higher than that of the West Asian population,²³ and lower than East Asian population^{24–26} (Table S3).

Patients belonging to Asian ancestry with *NUDT15* c.415C>T polymorphism have been shown to be poor metabolizers of 6-MP and may show intolerance to the drug.²⁷ Patients homozygous for *NUDT15* c.415C>T polymorphism in the present study tolerated ~25% of the total planned 6-MP dose, which is higher than the previously reported studies^{4,5} and the current recommended CPIC guidelines on 6-MP dosing.^{4–6} This discrepancy in 6-MP dose intensity (25% in the present study vs 8%^{4,5} as per previous reports) can be attributed to different treatment practices. Apart from the *NUDT15**3, we failed to identify additional polymorphisms in *NUDT15* exon 1 that has been reported to play role in 6-MP intolerance.^{28,29} It is possible that additional confounding genetic/non-genetic factors could also contribute to increased tolerance,

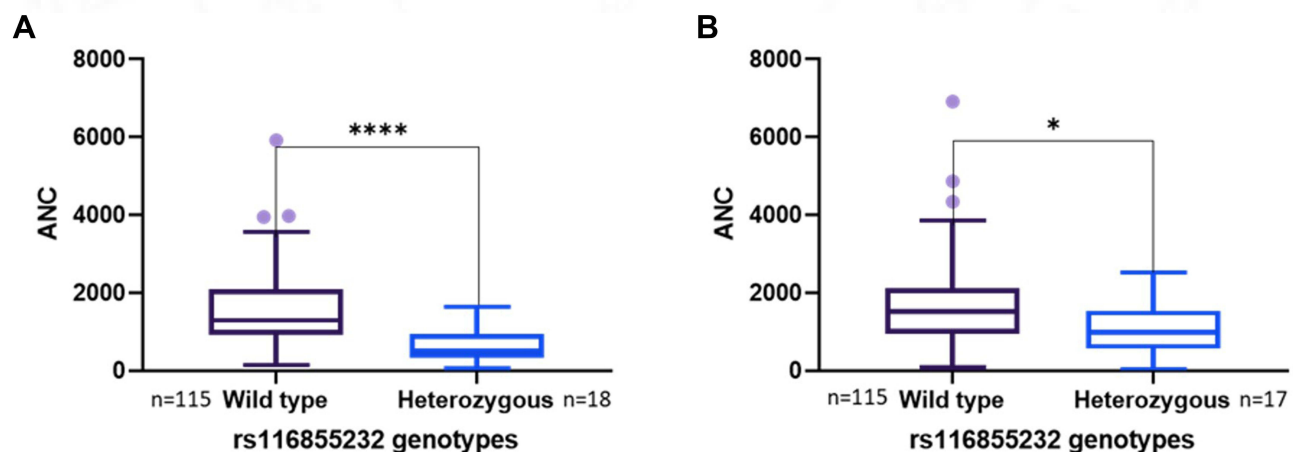


Figure 3 Associations between *NUDT15* c.415C>T polymorphism (rs116855232) and (A) Early, (B) Late Myelotoxicity. *Asterisks indicate the level of the significance (p-value); *Means $p < 0.05$ and ****Means $p < 0.0001$.

Abbreviation: ANC, absolute neutrophil count.

especially in patients homozygous for *NUDT15* c.415C>T polymorphism in the Indian population compared to other ethnicities, which needs to be further evaluated.

Myelotoxicity is a common adverse drug reaction during 6-MP maintenance therapy that may result in frequent febrile neutropenic episodes affecting treatment outcomes, risk of relapse, and quality of life.³⁰ Similar to previous studies,^{15,25–27,31,32} patients who were homozygous for *NUDT15* c.415C>T polymorphism experienced a significantly higher incidence of marrow toxicities and, as a result, experienced considerably more therapy interruptions/6-MP dose reduction in comparison to patients who were wild-type or heterozygous for this polymorphism (cohort-1). Although there were no patients with homozygous genotype for *NUDT15* c.415C>T polymorphism in the prospective cohort, patients with heterozygous genotype experienced moderate/severe neutropenia leading to considerable dose reduction (Table 3). Although the *TPMT* polymorphisms were rare in our study cohort, patients who were heterozygous for *3C polymorphism experienced significantly increased neutropenic episodes similar to previous studies,^{33–35} but no significant 6-MP dose reduction.

ITPA is another crucial enzyme in thiopurine detoxification whose enzyme activity is genetically determined.¹¹ A missense variant (c.94 C>A) was observed to be a significant determinant of mercaptopurine metabolism and severe febrile neutropenia.^{35–37} Consistent with previous reports,^{9,10,22} we observed that patients with variant genotype for *ITPA* c.94 C>A polymorphism experienced neutropenic episodes due to low ANC. Unlike previous studies^{13,38} that showed *ITPA* c.94 C>A polymorphism to

be associated with lower EFS, we did not find this association in the present study.

A missense polymorphism (c.2269G>A) identified in the *MRP4*/ATP binding cassette subfamily C member-4 (*ABCC4*) has been shown to dramatically enhance 6-MP sensitivity, especially in the Asian population.³⁹ Tanaka et al¹⁰ have reported a high frequency of 6-MP dose reduction in Japanese children with ALL bearing homozygous variant allele for this polymorphism. These authors subsequently reported a higher incidence of leukopenia in children with risk allele for *MRP4* c.2269G>A polymorphism but not with %6-MP dose intensity.²⁴ Similar to these reports,^{10,24} we observed that patients carrying risk allele for *MRP4* c.2269G>A polymorphism experienced leukopenia (data not shown), albeit no 6-MP dose reduction. Our data suggest the limited clinical utility of *MRP4* pharmacogenetics as compared to *NUDT15* /*TPMT* in ALL.

In addition, we also identified a significant proportion of patients with severe neutropenia yet did not bear any genetic variants screened in this study, suggesting that additional genetic polymorphisms related/unrelated to the thiopurine metabolic pathway could explain 6-MP intolerance/toxicities. It is now well acknowledged that severe neutropenia can also be attributable to methotrexate intolerance^{15,40} during maintenance therapy, which was not evaluated in the present study. An exploratory approach to identify additional genetic variants in patients who required dose reduction due to toxicities with no polymorphisms in *NUDT15*, *ITPA*, *TPMT*, or *MRP4* genes is ongoing in our laboratory.

Table 3 Influence of *NUDT15* c.415C>T Polymorphism in Early 6-MP Induced Toxicities (Cohort-2)

Clinical Response	Patients with <i>NUDT15</i> c.415C>T Polymorphism		
	Wild Type, N=115 (%)	Heterozygous, N=18 (%)	Odds Ratio (95% CI), p-value*
MP dose reduced (<100%)	99 (86)	18 (100)	1.6 (–0.56–1.46), 0.361
% MP Dose intensity	>80%	11 (61)	
	50–80%	6 (33)	
	<50%	1 (6)	
Neutropenia (ANC <1500 mm³)	84 (73)	18 (100)	13.79 (0.57–7.48), 0.005
Severe neutropenia (ANC <500 mm³)	24 (21)	12 (67)	7.18 (0.95–3.07), 0.0001
Dose interruption	98 (85)	17 (94)	2.07 (–0.75–2.97), 0.373

Notes: *ORs and p-values were calculated using penalized likelihood test-Firth logistic regression method using R. Bold values denote statistical significance at the p ≤ 0.05 level.

Neutropenia and other thiopurine-induced toxicities culminate in increased cost of maintenance therapy. We observed that patients with heterozygous or homozygous *NUDT15* c.415C>T polymorphism had higher maintenance therapy-related costs. Therefore, pre-emptive genotype-based (*NUDT15* c.415C>T, *TPMT*) dosing could benefit Indian patients by decreasing the cost of maintenance therapy, minimizing hospital visits and therapeutic interventions.

The major limitation of the study is its retrospective nature, wherein none of the patients received tailored dosing based on genotype, including *NUDT15* c.415C>T polymorphism. Other limitations include small sample size, wide window of the study period, and lack of validation cohorts. MTX is administered along with 6-MP, which also possesses similar myelotoxicity and hepatotoxicity.⁴¹ However, the present study did not address the role of MTX pharmacogenetics, which again is a major limitation.

Conclusion

Our results suggest that *NUDT15**3, *TPMT**3C, as well as *ITPA* c.94 C>A and *MRP4* c.2269 C>T polymorphisms explain hematological toxicity to 6-MP in patients with ALL undergoing maintenance therapy. Genotyping for *NUDT15*, *TPMT*, and *ITPA* is routinely performed in ALL patients undergoing maintenance therapy as well as in patients on thioguanine therapy for other conditions including AHA, ITP, inflammatory bowel disease (IBD), and autoimmune disorders at our centre. Preemptive genotype based (*NUDT15**3, *TPMT*, *ITPA* c.94 C>A) 6-MP dosing could improve the outcome after maintenance therapy. Further whole-genome sequencing in patients with severe myelotoxicity but not carrying *TPMT* or *NUDT15* variants is ongoing to identify potential genetic risk for intolerance to maintenance therapy in ALL.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

Approval was obtained from the Institutional Review Board [IRB (EC)-ER-1-23-07-2014] of Christian Medical College, Vellore, India. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Informed Consent

Informed consent was obtained from all individual participants/legal guardians included in the study.

Acknowledgments

We thank Dr Partha Majumdar (NIBMG, Kalyani) and Dr Ram Prasad (MedGenome Laboratories, Bengaluru) for providing the variant allele frequency data of the selected polymorphisms in the Indian population. Technical assistance provided by Ms Sangeetha is gratefully acknowledged. We thank all the medical and nursing staff who managed the outpatients.

Author Contributions

All authors contributed to data analysis, drafting, and revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

This study is funded by the Indian Council of Medical Research Centre for Advanced Research grant 70/14/14-CAR to Dr Poonkuzhali Balasubramanian. AAP is supported by a Senior Research Fellowship from DBT, India. VM and PB are supported by Wellcome DBT India Alliance (IA/CPHS/18/1/503930 and IA/S/15/1/501842), respectively. UK is supported by an early career fellowship program of Wellcome DBT India Alliance (IA/CPHE/17/1/503351).

Disclosure

The authors report no conflicts of interest in this work.

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