FIBRIN BASED MATRIX DIRECTED DIFFERENTIATION OF ADIPOSE DERIVED MESENCHYMAL STEM CELLS INTO OLIGODENDROCYTES FOR CELL REPLACEMENT THERAPY IN SPINAL CORD INJURY

KRISHNAPRIYA CHANDRABABU

Ph.D. THESIS

2020

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM
INDIA

FIBRIN BASED MATRIX DIRECTED DIFFERENTIATION OF ADIPOSE DERIVED MESENCHYMAL STEM CELLS INTO OLIGODENDROCYTES FOR CELL REPLACEMENT THERAPY IN SPINAL CORD INJURY

A THESIS PRESENTED BY

KRISHNAPRIYA CHANDRABABU

TO

SREE CHITRA TIRUNAL INSTITUTE

FOR

MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM

INDIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2020

CERTIFICATE

I, **Krishnapriya Chandrababu**, hereby certify that I had personally carried out the work depicted in the thesis entitled, ***"Fibrin based matrix directed differentiation of adipose derived mesenchymal stem cells into oligodendrocytes for cell replacement therapy in spinal cord injury"***, except where due acknowledgement has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Thiruvananthapuram

Krishnapriya Chandrababu

Reg.No: 2016/PhD/03

Dr. Lissy K. Krishnan

Scientist G (Senior Grade)

Division of Thrombosis Research,

Department of Applied Biology,

BMT Wing, SCTIMST, Thiruvananthapuram

This is to certify that Ms. Krishnapriya Chandrababu, in the Division of Thrombosis Research of this Institute has fulfilled the requirements prescribed for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The thesis entitled, ***"Fibrin based matrix directed differentiation of adipose derived mesenchymal stem cells into oligodendrocytes for cell replacement therapy in spinal cord injury"***, was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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

Thiruvananthapuram

Dr. Lissy K. Krishnan

(Research Supervisor)

The thesis entitled

"Fibrin based matrix directed differentiation of adipose derived mesenchymal stem cells into oligodendrocytes for cell replacement therapy in spinal cord injury"

Submitted by

Krishnapriya Chandrababu

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY THIRUVANANTHAPURAM - 695011

Is evaluated and approved by

12.8.2020 .... 13.12-8-20

Dr. Lissy K Krishnan (Research Supervisor)

Thesis Examiner

This thesis is dedicated to

*My loving **parents** for giving me dreams to look forward to...*

*My dearest **sister** for her support and love…*

*My wonderful **husband** for making my world a better one …*

Family, Teachers, and friends…

Everyone who stood beside me…

***The Almighty God**…*

ACKNOWLEDGEMENT

With a deep sense of gratitude, I take this opportunity with great pleasure to thank all those who contributed in one or the other way for the success of this study.

I am extremely thankful to the Director, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, and the Head, BMT Wing for all the support and excellent facilities provided during the course of my work.

I express my deep sense of gratitude to the Dean, Associate Dean, the Deputy Registrar, and all members of Division of Academic Affairs, Director's office, and Head BMT Wing office for their valuable assistance.

It was a privilege and honor to have had the opportunity to carry out my doctoral studies in the Division of Thrombosis Research, SCTIMST.

I express my deepest sense of gratitude to my supervisor Dr. Lissy K. Krishnan for the constant encouragement, guidance, and support provided to me throughout the course of the study. It was the timely advice and great effort that she put into for training me in the scientific field that made this thesis happen. She has always been a pillar of strength and support during times of crisis.

I thank the members of the doctoral advisory committee, Dr. Anoopkumar T, Scientist F, Division of Molecular Medicine, Dr. Easwer H.V, Professor, Neurosurgery department and Dr. Syam K, Additional professor, Neurology department for their invaluable inputs which helped in the improvement of the quality of this work. I also take this opportunity to express my gratitude to all the teaching faculty of my Ph.D. course work.

I thank the Department of Biotechnology, India for the fellowship provided during the doctoral program. I also acknowledge the travel fellowship provided by Stem cell Society Singapore for attending the international conference in Singapore. I also thank International Brain Research Organization (IBRO) and the IBRO-APRC school coordinator Dr. Kamalesh K Gulia for giving me an opportunity to attend IBRO-APRC school, SCTIMST.

I express my earnest gratitude to Dr. Harikrishnan V.S for his effort and patience in developing the animal model of SCI and for helping with the transplantation studies.

I thank Dr. Arya Anil and Dr. Aiswarya Lekshman for helping with the surgical procedures during animal experiments and timely suggestions. I thank all members of DLAS, especially Mr. Sarath, Mr. Manoj, Mr. Sunil, and Ms. Jolly for their assistance during the animal experiments.

I sincerely appreciate the efforts of Dr. Sabareeswaran and all staff of Division of Histopathology for helping with the histological analysis of tissue sections, for training and providing the cryostat facility, and for the staining, procedures conducted during animal experiments.

I thank Dr. R.S Jayasree for extending the facilities for In vivo imaging of animal tissue and fluorescence microscope. I also express my sincere thanks to Ms. Reshmi, Mr. Jibin, and Ms. Hema for their timely help in acquiring the tissue images.

I sincerely thank Mr. Ranjith S, Ms. Priyanka, Mr. Anil Kumar, and Ms. Deepa S from the Division of Thrombosis Research for providing the fibrin sealant components.

I thank all the staff of the Department of Cardiovascular and Thoracic Surgery, SCTIMST, and Department of plastic surgery, KIMS Hospital, Thiruvananthapuram for providing adipose tissue samples during my research work.

I express my sincere gratitude to the Director of RGCB, TVM for providing the flow cytometry facility. I also thank Ms. Indu and Ms. Arya of RGCB for the technical assistance in performing flow cytometry.

I express my sincere gratitude to Dr. Anoop Kumar T, Scientist F and all staff of Division of Molecular Medicine for nanodrop facility and all staff of Division of Tissue Culture for all the help they have provided.

I am thankful to Dr. Nishad K V, Bioceramic laboratory for technical assistance in eSEM analysis.

I am deeply indebted to all staff and colleagues at the Division of Thrombosis Research (TRU). My sincere thanks to Dr. Anugya Bhatt for the flow cytometry analysis, her advice, and support. I place my heartfelt appreciation to my seniors Dr. Tara S, Ms. Subha S, and Ms. Renu Ramesh for guiding and training me on various techniques of stem cell isolation and cell culture. I express my deepest gratitude to Ms. Priyanka Manoj, Mr. Ranjith S, Mr. Anilkumar V, Dr. Ranjith P Nair, Ms. Amita Ajit, Ms. Deepa S, Ms. Rashmi R, Ms. Karthika, Ms. Hima, Ms. Silpa, Ms. Safeena, Ms. Rakhi, Mr. Mejo, Ms. Gayathri, Ms. Athira, Ms. Lakshmi for their support and friendship. I also thank Ms. Usha, Ms. Bindu, Mr.Hari Mr. Shibu, and Mr. Arun for helping me in maintaining the aseptic conditions in cell culture facility.

I express my sincere gratitude to all my fellow students from other departments of our campus for their support and friendship. I thank the staff of the administrative department, library, and cleaning staff for their assistance.

I owe a huge debt of gratitude to all my teachers. I cannot end without acknowledging my whole family for their endless support, love, and prayers. Finally, I am indebted to the Divine Power who has been a guiding force throughout my life.

TABLE OF CONTENTS

Certificate...... i

Certificate of guide ii

Approval of thesis iii

Acknowledgement v

List of figures xiii

List of tables xv

Abbreviations xvi

Annotations xix

Synopsis xx

CHAPTER 1 1

1.INTRODUCTION......1

1.1 Nervous system 2

1.2 Extracellular matrix of the nervous system......4

1.3 Signaling Mechanisms in development and regeneration......6

1.4 Injury of the nervous system 7

1.5 Spinal cord injury 8

1.6 Current research approaches in SCI 11

1.7 Advances in Cell Replacement Research......11

1.8 Stem Cell Sources......13

1.9 Gap Area 15

1.10 Definition of the problem......15

1.11 Prerequisite for Niche 17

1.12 Current developments 18

1.13 Hypothesis......19

1.14 Objectives......19

1.15 Rationale of the study 19

CHAPTER 2 20

2. LITERATURE REVIEW 20

2.1 Central Nervous System and SCI 20

2.1.1 SCI types 21

2.1.2 SCI pathophysiology 22

2.2 Inflammatory responses in SCI 23

2.2.1 Role of microglia & astroglia 23

2.2.2 Glial Scar 25

2.2.3 SCI: Timeline of injury responses 26

2.3 Oligodendrocytes 26

2.3.1 Embryonic development of Oligodendrocytes 27

2.3.2 Oligodendrocyte development: Role of the notch and wnt signaling 28

2.3.3 Oligodendrocytes: dual role in SCI 30

2.3.4 Myelin-associated inhibitors 30

2.3.5 Regeneration and re-myelination: Role OL in SCI 31

2.3.6 Oligodendrocyte: Proliferation and survival signaling in SCI 32

2.3.7 Growth factor Signaling 33

2.4 ECM: SCI regeneration 34

2.4.1 Role of fibrinogen in SCI 35

2.4.2 OPC: replacement therapy 35

2.4.3 Pre-differentiation of stem cells to OPCs 38

2.5 *In vivo* studies in SCI 39

2.5.1 Behavioural testing 41

2.6 Clinical trials in SCI 42

2.7 ADMSCs in cell therapy 43

2.7.1 In vivo ADMSC niche and their maintenance 44

2.7.2 ADMSC isolation and expansion in culture 45

2.7.3 ADMSC characterization 45

2.7.4 Multipotency of ADMSCs 46

2.7.5 Mechanisms of ADMSC maintenance in culture 47

2.7.6. Signaling Mechanisms in ADMSC Proliferation in vitro 48

2.7.7 *In vitro* differentiation of ADMSCs to neural lineage 49

2.7.8 ADMSC based therapies in SCI 50

CHAPTER 3 .... **52**

3. MATERIALS AND METHODS **52**

3.1 Human ADMSC Isolation from lipoaspirate samples 52

3.2 ADMSC Characterization 53

3.2.1 Flowcytometric analysis 53

3.2.2 Trilineage Differentiation 53

3.3 Preparation of fibrin matrix coated TCPS for induction studies 54

3.4. Induction of OL from ADMSC 54

3.4.1 Neurosphere induction 55

3.4.2 Reseeding of NS and Secondary NS formation assay 55

3.4.3 Neural progenitor and Glial progenitor induction 55

3.4.4 Neuronal induction 56

3.4.5 Terminal differentiation of OPC 56

3.4.6 Co-culture of induced neurons and OPCs 56

3.5 Signalling study 57

3.5.1 MTT Assay 57

3.5.2 Effect of notch & wnt inhibitor on Neurosphere induction 58

3.5.3 Effect of notch & wnt inhibitor on OPC induction 58

3.6 Analysis of differentiation 59

3.6.1 RNA Isolation and cDNA preparation 59

3.6.2 Real-time PCR (qPCR) 59

3.6.3 Immunocytochemistry (ICC) 61

3.6.4 Flow cytometry 64

3.7 *In vivo* cell transplantation studies in SCI rat model 65

3.7.1 Rat ADMSC isolation 66

3.7.2 Rat ADMSC induction to neural progenitors 66

3.7.3 Allocation of animals 67

3.7.4 Labelling of cells for transplantation and tracking 68

3.7.5 SCI: Surgery, Transplantation, and Post-operative care 68

3.7.6 Tracking of transplanted cells 69

3.7.7 Analysis of the SCI site using Immunohistochemistry 69

3.8 Statistical analysis 70

CHAPTER 4 71

4. RESULTS 71

4.1 ADMSC isolation and characterization 71

4.1.1 ADMSC Isolation 71

4.1.2 ADMSC Characterization 72

4.1.3 Effect of fibrin matrix on hADMSC to NS conversion 74

4.1.4 Effect of Fibrin in the formation of secondary NS 77

4.1.5 Induction of NS to proliferating NPCs 80

4.1.6 Effect of Fibrin matrix on NPCs to OPCs conversion 82

4.1.7 Comparison of fibrin & bare TCPS on OPC maturation 84

4.1.8 Fibrin niche induced differentiation of NPCs to Neurons 86

4.1.9 Co-culture of Neurons and OPCs on Fibrin 89

4.2 Signaling cascades elicited by fibrin-based niche 91

4.2.1 MTT assay to evaluate the cytotoxicity of the inhibitors 91

4.2.2 Action of inhibitors of Notch and Wnt on NS formation 92

4.2.3 Effect of Notch and Wnt inhibitors on Nestin gene expression. 93

4.2.4 Effect of inhibitor on Notch downstream molecules. 94

4.2.5 Identification of notch and wnt downstream molecules 95

4.2.6 Action of Wnt/Notch inhibitors on NPC to OPC differentiation 99

4.2.7 Action of inhibitors on Proliferation and OPC gene expression 100

4.2.8 Effect of signaling inhibitor on notch downstream molecules. 101

4.2.9 Identification of notch and wnt downstream molecules 102

4.3 Transplantation of rADMSC derived NPCs & OPCs in rat SCI models 106

4.3.1 rADMSC isolation and induction to NS 106

4.3.2 Reseeding of rADMSC derived NS 108

4.3.3 Induction of rat NS to NPCs 109

4.3.4 Induction of rat NPCs to OPCs 110

4.3.5 Terminal differentiation of OPCs to OLGs in the niche. 112

4.3.6 Creation of SCI model and *In vivo* Cell Transplantation 114

4.3.7 Clinical symptom development in SCI animals 114

4.3.8 Survival & differentiation of transplanted cells 115

4.3.9 Clinical outcome of cell transplantation in SCI 118

4.3.10 Histopathological Evaluation of the Tissue 121

CHAPTER 5 127

5. DISCUSSION.. 127

5.1 Influence of niche on hADMSC differentiation to neural cells 127

5.2 Biomimetic signaling by fibrin-based niche 133

5.3 Survival of transplanted progenitors in rat SCI... 135

5.4 Limitations of the study 139

CHAPTER 6 140

6. SUMMARY AND CONCLUSION 140

6.1. Summary of Results:.. 141

6.2 Conclusion.. 144

6.3 Future perspective... 145

BIBLIOGRAPHY.. 146

LIST OF PUBLICATIONS.. 165

CURRICULUM VITAE.. 166

APPENDIX 168

LIST OF FIGURES

1. ADMSC Isolation 72
2. Micrograph depicting multipotency of hADMSCs 73
3. Histograms showing flowcytometric analysis of hADMSCs 73
4. Phase-contrast Micrographs of neurospheres derived from hADMSC 74
5. Environmental Scanning Electron Micrograph of Fibrin niche and NS 75
6. Graphical representation showing PCR analysis of NES gene expression 76
7. Immunofluorescence analysis of NS using ICC & Flow cytometry 77
8. 2^0 sphere formation potential of induced NS,Maintenance of Nes positivity 79
9. NPC morphology and gene expression after induction on fibrin niche 80
10. Immunostaining of induced NPCs with NES, TUJ & PCNA antibodies 81
11. Flowcytometric analysis of NPCs 82
12. Phase contrast micrograph of OPC induced from NPCs 82
13. OPC marker expression in induced cultures 83
14. Flow cytometric analysis of induced OPCs using OLIG 2 marker 84
15. Morphology and gene expression analysis of OPCs induced to OLGs 85
16. Immunostaining of OLG markers 86
17. Morphology and specific marker expression of the induced neurons 87
18. The fluorescence micrograh showing neuronal markers in induced cells 88
19. Coculture of OPCs and neurons 90
20. Graphical representation MTT assay 91
21. Morphological effect of signal inhibitors in NS induction 92
22. Graphical representation of qRT-PCR analysis of nestin marker 93
23. qRT-PCR analysis of notch downstream markers 94
24. Fluorescence micrograph of cells immunostained with Notch antibody 95
25. Fluorescence micrograph of cells immunostained with HES 1 antibody 96
26. Fluorescence micrograph of cells immunostained with wnt 3a antibody 97
27. Fluorescence micrograph of cells immunostained with βcatenin antibody 98
28. Morphological effect of signal inhibitors in OPC induction 99
29. qRT-PCR analysis of OPC markers 100

30. qRT-PCR analysis of notch downstream markers ... 101
31. Fluorescence micrograph of OPC immunostained with Notch antibody ... 102
32. Fluorescence micrograph of induced OPCs immunostained with HES 1 ... 103
33. Fluorescence micrograph of cells immunostained with wnt3a antibody ... 104
34. Fluorescence micrograph of cells immunostained with βcatenin antibody ... 105
35. Analysis of rat NS derived from rADMSC ... 107
36. Expression of Nestin in rat NS ... 108
37. Phase contrast micrograph of NS formation and reseeding ... 109
38. Induction of rat NS to NPCs in fibrin niche ... 109
39. Histograms showing TUJ1^{+ve} population in NPCs induced from rat NS ... 110
40. Induction of rat NPC to OPCs in fibrin niche ... 111
41. Phase contrast micrograph of OLGs derived from induced OPCs ... 112
42. Analysis of OLGs derived from induced OPCs in fibrin niche ... 113
43. SCI contusion model development ... 114
44. Clinical symptoms developed in SCI animals ... 115
45. IVIS imaging of the transplanted cells ... 116
46. Fluorescence micrographs tissue showing cells transplanted at SCI ... 117
47. Immunohistochemistry of the SCI contusion site ... 118
48. Change in body weight over 4 weeks after SCI induction in rats ... 119
49. BBB scoring of the SCI animals ... 120
50. Gross image of rat spinal cord after 28 days of observation ... 121
51. Micrographs showing H & E staining of the injured tissue ... 122
52. Micrographs showing CD 68 staining of the injured tissue ... 124
53. Micrographs showing GFAP staining of the injured tissue ... 126

LIST OF TABLES

I. Growth factors and molecules used for pre-differentiation to OL lineage 39

II. Stem cell therapy in SCI- clinical trials & outcome 43

III. Primer sequences for human genes 60

IV. Primer sequences for rat genes 61

V. List of primary antibodies 63

VI. List of Seconady antibodies.. 64

VI. Allocation of animals for *in vivo* studies 67

ABBREVIATIONS

ABAM	: Antibiotic Antimycotic
ADMSC	: Adipose Derived Mesenchymal Stem Cell
AGE	: Agarose Gel Electrophoresis
ANOVA	: Analysis of variance
ASIA	: American Spinal Cord Injury Association
BBB	: Basso Beattie Bresnahan
BDNF	: Brain derived neurotrophic factor
bFGF	: Basic FGF
BMMSC	: Bone marrow derived Mesenchymal Stem Cell
BSA	: Bovine Serum Albumin
Ca^{2+}	: Calcium
CD	: Cluster of Differentiation
cDNA	: Complimentary Deoxy ribo Nucleic Acid
CNS	: Central Nervous System
CSF	: Cerebrospinal Fluid
CSPG	: Chondroitin sulphate proteoglycan
DAPI	: 4’, 6-diamidino-2-phenylindole
DNA	: Deoxyribonucleic acid
dNTP	: deoxy Nucleotide Tri Phosphate
DMEM	: Dulbecco's Modified Eagle Medium
DPX	: Dibutylphthalate Polystyrene Xylene
ECM	: Extracellular matrix
EDTA	: Ethylene Diamine Tetra Acetic Acid
ESC	: Embryonic Stem Cell
FACS	: Fluorescence Activated Cell Sorter
FBS	: Fetal Bovine Serum
FGF	: Fibroblast Growth Factor
Fib	: Fibrin
FN	: Fibronectin
GAPDH	: Glyceraldehyde 3- Phosphate Dehydrogenase
GF	: Growth Factor
GFAP	: Glial Fibrillary Acidic Protein
H & E	: Hematoxylin and Eosin
HBSS	: Hank’s Balanced Salt Solution
HC	: Higher Concentration
HLA	: Human Leukocyte Antigen
HSC	: Hematopoietic Stem Cell

IAEC	: Institutional Animal Ethics Committee
IEC	: Institutional Ethics Committee
IGF-1	: Insulin-like growth factor 1
IL	: Interleukin
INB	: Induced on bare
INF	: Induced on fibrin
iPSC	: Induced Pluripotent Stem Cell
ISCT	: International Society for Cellular Therapy
KCL	: Potassium Chloride
LC	: Lower Concentration
MAG	: Myelin Associated Glycoprotein
MAI	: Myelin Associated Inhibitor
MAP 2	: Micro Tubule Associated Protein 2
MBP	: Myelin Basic Protein
MSC	: Mesenchymal Stem Cell
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Nes	: Nestin
NK	: Natural Killer
Nkx	: NK2 transcription – factor related
NPC	: Neural Progenitor Cell
NS	: Neurosphere
NSE	: Neuron Specific Enolase
NSC	: Neural Stem Cell
NT	: Neurotropin
OLG	: Oligodendrocyte
OPC	: Oligodendrocyte Progenitor Cell
OSP	: Oligodendrocyte Specific Protein
PBMNC	: Peripheral Blood Mononuclear Cell
PBS	: Phosphate Buffered Saline
PCNA	: Proliferating Cell Nuclear Antigen
PDGF	: Platelet-derived growth factor
PNN	: Perineuronal nets
PNS	: Peripheral Nervous System
qRT-PCR	: Quantitative Real Time Polymerase Chain Reaction
RA	: Retinoic acid
RNA	: Ribo Nucleic Acid
ROS	: Reactive Oxygen Species
SCI	: Spinal Cord Injury
SD	: Standard Deviation
SEM	: Standard Error of Mean

SFM	: Serum Free Medium
SGZ	: Sub Granular Zone
SHH	: Sonic Hedge Hog
SVF	: Stromal Vascular Fraction
SVZ	: Sub Ventricular Zone
Syn	: Synaptophysin
T3	: Triiodothyronine
T10	: Thoracic 10
TCPS	: Tissue Culture Polystyrene
TF	: Transcription Factor
TH	: Tyrosine Hydrolase
TNF	: Tumor Necrosis Factor
Tn-R	: Tenascin R
TUJ 1	: Neuron-specific class III beta-tubulin
VN	: Vitronectin
WHO	: World Health Organization
Wnt	: Wingless
β-III tub	: Beta-3-Tubulin

ANNOTATIONS

%	: Percentage
<	: Less than
>	: Greater than
µg	: Microgram
µl	: Microliter
µm	: Micromolar
d	: Days
h	: hours
IU	: International Unit
mA	: Milli Ampere
min	: Minutes
mM	: Milli Molar
ng	: Nanogram
nm	: Nanometers
pmol	: Picomole

SYNOPSIS

Adult stem cells have attracted researchers for potential use in cell-based therapy due to their extensive multiplication potential and ability to differentiate into multiple lineages. Adult stem cell-based therapies offer a great promise for treating various disease conditions that have no pharmacological remedies. Human adipose-derived mesenchymal stem cell (hADMSC) gains attention in regenerative medicine due to wide availability, ease of isolation/expansion in cultures, non-immunogenic, and immune-modulatory properties. The multipotency of hADMSC may be exploited to obtain different types of cells of diseased tissue in the context of regeneration. At the same time, multipotency poses a concern because undesired *in vivo* differentiation upon cell transplantation can lead to adverse effects. Therefore, pre-differentiation of hADMSCs into required cell lineages is considered to reduce the chances of undesired differentiation of cells post-transplantation. Extensive differentiation inversely affects proliferation which in turn could reduce the regeneration potential *in vivo*. Therefore, a controlled process of lineage commitment while maintaining proliferation potential is challenging. In the context of diseases affecting the central nervous system (CNS), the exploitation of hADMSCs may aim pre-differentiation into proliferating neural progenitor cells (NPCs) and oligodendrocyte progenitor cells (OPCs). When the derivation of suitable progenitors is achieved, for verification of their translational potential, a suitable disease model may be useful. Most of the commonly occurring CNS diseases cause continuous tissue degeneration; therefore, cell transplantation attempts to face many challenges.

Spinal cord injury (SCI) is a condition resulting from trauma, mainly from accidents or degenerative diseases. According to the world health organization, ~2,50,000 to 5,00,000 young people suffer from SCI every year causing morbidity and mortality. Subsequent to SCI, associated secondary injury cascades lead to the development of astrogliosis and loss of sensory-motor functions. Surgical decompression and methylprednisolone administration immediately after the trauma could reduce inflammation and secondary injuries. Stem cell-based therapies for providing neuroprotection and neuro-regeneration have been found to be promising in pre-

clinical studies. Stem cell-based therapies may help in surpassing the internal hostile environment reducing gliosis at the site of injury and promote regeneration if desirable progenitors are transplanted using an appropriate delivery method. In this background, the use of hADMSC may provide potential benefits in this field.

Pre-differentiation of hADMSCs to NPCs and OPCs may benefit the repair and regeneration of the SCI. A mixed population of hADMSCs, NPCs, and OPCs may provide trophic and neuroprotective effects, whereas astrocytes could lead to progression of astrogliosis in the SCI milieu. Therefore, the primary goal of this study was the exploitation of multipotency of hADMSC for obtaining NPCs and OPCs, in a suitable proliferative stage for transplantation to SCI. Both NPC and OPC differentiation may require related but unique niche conditions. Therefore, standardization of *in vitro* hADMSC induction protocols that have control over both lineage commitment and proliferation is challenging. An *in vitro* induction program which operates via biomimetic signaling cascade seen in neural tissue development and homeostasis would be the best to control cell phenotype in culture.

Previous studies have established the role of the human fibrin-based niche for selective adhesion of NPC from peripheral blood mononuclear cell (PBMNC) fraction. In addition, the signaling capability of the fibrin niche for differentiation of NPC to neurons is also proven. Therefore, cell-specific modifications of fibrin-based niche may be exploited to support the commitment of mesodermal cells to ectodermal neural lineage and further differentiation to NPC and OPCs. With this background, the present study is based on the hypothesis, "Cell-specific niche comprising soluble factors and fibrin may regulate signaling pathways resulting in the *in vitro* lineage commitment of hADMSCs to NPCs and OPCs suitable for transplantation to acute SCI using the injectable biomimetic matrix for better survival".

To verify this hypothesis the following objectives were used.

1. To compare cell culture on bare tissue culture polystyrene surface (TCPS) with those on insoluble fibrin coated surface to distinguish the effect of growth factor included fibrin-based niche on step-wise differentiation of hADMSCs to neurospheres (NS) and NPC and OPC.

2. To establish that the stage-wise differentiation of hADMSCs to NS and OPCs is regulated through receptor-mediated mechanisms, by studying the effect of chemical inhibitors on signaling pathways, triggered via fibrin-based niche components.
3. To study the survival of pre-differentiated progenitors, harvested from *in vitro* niche, upon transfer to spinal cord injury (SCI) model using injectable fibrin as a delivery vehicle.

The goal is to establish the feasibility of autologous hADMSC isolation and multi-lineage commitment for translation into clinics for cell-based therapy to improve the paraplegic conditions of young victims in the long run.

The thesis is divided into six chapters.

Chapter 1 introduces the research problem and its relevance in detail. Based on the defined problem, the study hypothesis and objectives are established. The SCI is a life-threatening condition for which no effective therapy is available. A treatment modality for complete recovery and regaining of neurological functions after SCI is still under investigation. Replacement of neural cells after SCI is one of the various strategies that are widely investigated. Various preclinical studies have indicated that cell replacement therapy has an effect on improving the spinal cord regeneration and BBB scoring in rodents. ADMSC is one of the most studied stem cell types with multipotency and scope for autologous transplantation, unlike embryonic stem cells that cause immune rejection and ethical issues. And for the replacement of specific neural cell populations, there is a pre-requisite for lineage conversion and pre-induction of ADMSC to neural progenitors *in vitro*. Since undesirable changes at the injury site damage the *milieu* required for homing and differentiation of transplanted cells, cells embedded in an injectable form of niche is proposed.

Chapter 2, summarizes the review of current literature in the field of SCI, stem cell transplantation, and regeneration. The currently available strategies for *in vitro* stem cell differentiation have been reviewed and approaches required for turning mesenchymal cells to ectodermal cells are understood by reviewing the field of neural

development biology and stem cell biology. Topics discussed include neurobiology, in brief, embryonic development of CNS, the biology and pathology of SCI, various inflammatory responses in SCI and scar formation, the role of oligodendrocytes in myelination of neurons in the context of SCI, stem cell-based therapies and major hurdles and animal models used for studying the transplantation outcome.

In chapter 3, the experimental design and the various protocols used to achieve the objectives projected in this study to test the hypothesis are explained. The protocols described include the design of biomimetic fibrin niche, both human and rat ADMSC isolation, culture & *in vitro* differentiation of ADMSC to neural cells, co-culture of neural cells, studies to signaling mechanisms involved based on the addition of specific inhibitors, etc. are described. Major techniques used for molecular and cellular studies described include qRT-PCR, immunochemical staining for specific markers for qualitative microscopic and quantitative flow cytometric analysis, fluorochrome based tracking of transplanted cells and histological analysis of explanted SCI tissue to establish survival of transplanted cells.

In chapter 4, the major results of the study are presented with an appropriate illustration. Representative micrographs are presented to demonstrate various stages of differentiation of ADMSCs to NS, NPC, OPC, Neurons, and OLG. Data on quantification of relative gene expressions and protein expressions in cells at various stages of differentiation as compared to that in ADMSC is presented in graphical format. The results of the signaling mechanism studied using chemical inhibitors also are presented in micrographs and in graphs. The results of cell transplantation study are presented mainly as tissue sections stained immunochemically and histochemically.

Chapters 5, results obtained from the various experiments are discussed. Based on previous literature the significance of results obtained in the present study is compared. This study depicts the exploitation of the multipotency of hADMSC to generate NPC and OPCs into a lineage-committed transplantable stage with proliferation potential. The role of fibrin in controlling cell phenotype is the highlight of the study. The simulated biomimetic signaling mechanisms seem elicited by soluble molecules

together with insoluble fibrin-based niche seems to be controlling cell phenotype in culture and in the SCI upon transplantation in the in situ clottable injectable fibrin niche.

In chapter 6, the results of the study are summarized and conclusions are drawn. To summarize, a fibrin-based niche is found efficient for obtaining pre-differentiated neural progenitors with proliferation potential. The biomimetic signaling mechanism is found responsible for the controlled differentiation and maintenance of stemness. Since suitable mixed cell types obtained from ADMSC are able to grow together in co-culture the study indicates autologous therapy for SCI could soon become feasible.

Limitations and future prospects of the study were also listed in chapter 6. The major drawback is only a fixed NPC/OPC dose was studied. Cell numbers required for effective regeneration could be estimated only when different cell numbers are transplanted in separate experiments. In general, homeostasis in neural tissue is a slow process; therefore, 28 d study in an animal model is a very short duration to detect regeneration in terms of BBB scoring and tissue level analysis.

Other supporting documents like acknowledgments, abbreviations, and their expansion, table of contents, list of figures, and declarations are placed at the beginning of the thesis. The citations are listed in the bibliography section. List of courses studied, conferences attended, manuscripts under review and under preparation are also included in the last part of the thesis.

CHAPTER 1

1. INTRODUCTION

Stem cells have the potential to promote the regeneration of tissues either by substitution of the lost cells or by trophic support, homing, and differentiation of progenitors at the injury site. One of the potential issues in using stem cell therapy is immune rejections and ethical issues concerned with embryonic or fetal stem cells. Here comes the advantage of adult stem cell-based autologous therapy as it is associated with minimal threat of immune rejection. Human mesenchymal stem cells (hMSCs) are well studied potent adult stem cells, which have high proliferative capacity and multipotency. In the past decades, the majority of adult stem cell-based studies were focused on bone marrow-derived MSCs. However, the hADMSCs started getting more attention in the current decade due to its ease of isolation and availability. The hADMSCs are multipotent stem cells and hence it can be used for the generation of various cell types of any specific tissue. The only drawback is that these multipotent cells can differentiate into any cell type based on the signals that are present in its environment; therefore, for the desired application the isolated ADMSCs need to be pre-differentiated *in vitro* before transplantation. For regenerative applications in nervous system injuries like spinal cord injury (SCI), both neural and glial cells are required. Through careful *in vitro* manipulation of ADMSCs, using various differentiation inducers such as adhesive proteins and growth factors (GF), progenitors may be derived. The exploitation of single-source ADMSC and similar differentiation niche consisting of GFs can be of greater advantage in the field of central nervous system (CNS) regeneration. Rather than aggressive chemical inducers, natural physiologically relevant inducers may be selected to achieve well-regulated progenitors with regenerative potential. Therefore, the physiology and pathology of CNS need to be well-understood before developing therapeutic regimes and designing strategies for *in vitro* induction of hADMSCs to neurons and glial cells.

1.1 Nervous system

The nervous system is a complex network of certain cells that synchronizes the motor and sensory functions of the body. The nervous system executes its actions by sending electrochemical signals. The nervous system is made up of peripheral nervous systems (PNS) and CNS. The brain and spinal cord constitute the CNS. They are formed of neurons and supporting cells called neuroglia. PNS includes all other parts of the nervous system excluding the brain and spinal cord. The CNS and PNS, act in concert to integrate motor-sensory information and cognitive functions of the body.

The human nervous system development is one of the earliest events that occur in the embryo's development. It is induced from one of the three germ layers called ectoderm, by a process called neurulation (Purves et al. 2001). After patterning of the nervous system, the cellular differentiation occurs. Neurons and glial cells are formed from neuroepithelial cells that give rise to radial glia. Neurogenesis is the formation of neurons from neural stem cells (NSC) and gliogenesis is the formation of glial cells from radial glia or neural stem cells. The final cell divisions and differentiation of the neuronal precursors occur during the early pregnancy period (Kriegstein and Alvarez-Buylla 2009). Neurons are post-mitotic cells that do not enter the cell cycle. The time that a neuronal progenitor cell leaves the cell cycle and gives rise to a post-mitotic neuron is called a Neuron's birthday (Aranda-Anzaldo 2012). Adult neurogenesis occurs mainly in two parts of the body in humans. The subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) the striatum. These two regions are stem cell niches that contain neural stem cells (Ghosh 2019). NSC promotes endogenous regeneration of CNS to a smaller extent.

CNS controls and coordinates the functions of the body with the brain as its main control center. Based on anatomical structure vertebrate brain can be divided into six regions named the telencephalon, diencephalon, mesencephalon, cerebellum, pons, and medulla oblongata. Each of the six areas has a specific internal structure and they are assigned with particular functions (Zhang 2019). The spinal cord extends from the medulla oblongata located in the brainstem to the lowest region of the vertebral column. It conveys signals from the brain to various parts of the body and vice versa. Structures of the brain and spinal cord are arranged in two layers named grey and white

matter. Grey matter is formed of nerve cell bodies and some proximal regions of nerve fibers. White matter is formed of nerve fibers. The brain and the spinal cord are immersed in cerebrospinal fluid, which is a colorless fluid that circulates through these organs. CSF protects & nourishes the CNS.

PNS consist of neurons and processes present in all other regions of the body except in that of the brain and spinal cord. PNS serves as a relay center by connecting the CNS to different parts of the body. It conveys information between the brain and spinal cord and the rest of the body. PNS is divided into the somatic nervous system and the autonomic nervous system. The somatic nervous system is associated with somatic functions such as muscular activities and movements of the body. The autonomous nervous system is concerned with the involuntary function of the body. At the cellular level, the nervous system is made up of two types of cells, neurons, and glial cells.

Neurons are the fundamental cellular unit of the nervous system which receives, processes and transfers information. They are electrically excitable cells that transmit information via electrical and chemical signals. It mainly consists of three parts: soma, dendrites, and axons. The bulbous end of a neuron which contains the cell nucleus and cytoplasm (neuroplasm) is soma or nerve cell body. The neuroplasm contains a large nucleus, nissl bodies, neurofibril, mitochondria, and golgi apparatus. Dendrites are extensions of a nerve cell that receives the electrochemical stimulation from other neural cells. They transfer this information to the soma. Axons are an elongated extension from the soma which transfer information to another neuron via small structures at its end called axonites. Neurofibril is a characteristic feature of neuron which is thread-like structures found in soma and nerve processes. Nerves are made up of bundles or groups of nerve fibers and each bundle is called fasciculus. Each nerve is covered by a membrane called epineurium and each fasciculus is covered by a membrane called perineurium. Nerve fibers have an envelope called endoneurium. In PNS nerve fiber has an outermost layer called neurilemma (Sembulingam and Sembulingam, 2012).

Neuroglia is the supporting cell of the nervous system. They are non-excitable cells that provide support to neuronal cells. The number of glial cells is ten to fifteen times

greater than that of neurons. They are present in both CNS and in PNS: central neuroglial cell and peripheral neuroglial cells. Central neuroglial cells are astrocytes, microglia, and oligodendrocytes. Astrocytes are star-shaped cells that provide form and support in the brain and spinal cord. They form a blood-brain barrier and regulate the transport of substances into the brain. Astrocytes play a vital role in maintaining the levels of neurotransmitters during synaptic transmission. Astrocytes help to maintain the chemical environment of extracellular fluid (ECF) around CNS neurons (Erlichman and Leiter 2010). Oligodendrocytes are glial cells that provide myelin sheath around nerve fibers in CNS. They extend many processes that envelopes a number of axons with multispiral membrane-forming myelin. Myelin insulates axons and makes the synaptic transmission faster. The number of processes of oligodendrocytes can vary in number in different species. Different oligodendrocytes can wrap adjacent regions of a single axon (Simons and Nave 2016). Oligodendrocytes can be classified in relation to the number of their processes, morphology, and thickness of the myelin sheath they form. Myelination of axons is stimulated by signals from axons and astrocytes (Ishibashi et al. 2006). The axons also participate in the regulation of myelin formation as a single oligodendrocyte can myelinate several axons with different diameters. The thickness of the myelin sheath is directly proportional to the diameter of the axon (Baumann and Pham-Dinh 2001). Demyelination due to injury and diseases leads to degeneration and death of axons and neurons.

In PNS there are two types of non- neuronal cells; Schwann cells and satellite cells. Schwann cells are the major glial cells in PNS. They provide myelination around PNS nerve fibers and play an important role in PNS nerve regeneration. They also remove cellular debris during regeneration by phagocytic activity (Jessen, Mirsky, and Lloyd 2015). Satellite cells are glial cells present on the exterior surface of glial cells. They provide physical support and help in regulating the chemical environment of ECF around PNS neurons (Sembulingam and Sembulingam 2012).

1.2 Extracellular matrix of the nervous system

The ECM in the nervous system is synthesized by neuron and glia. The ECM has a major role in the development and maintenance of the nervous system by acting as a

biological scaffold that provides form and structure to the nervous system. They also control the diffusion and availability of various molecules that play a significant role in cell signaling. Moreover, the regenerative capability of the nervous system is dependent on the expression of various ECM molecules (Jessica C. F. Kwok 2016). Proteoglycans, hyaluronan, laminins (La), fibronectin (FN), tenascins, collagens, integrins, and semaphorins are key components of CNS ECM. ECM forms a stable specialized structure called perineuronal net (PNN) composed mainly of HA, CSPGs, and tenascin-R (Tn-R). PNN wraps the surface of the cell body and dendrites of neurons. PNN is formed when the CNS development is completed and it limits the degree of plasticity in adult CNS (Kwok, Foscarin, and Fawcett 2015).

Chondroitin sulfate proteoglycan (CSPG) is the major population of proteoglycan in CNS. Glial cells as well as neurons produce CSPG which aids their arrangement in PNN. CSPGs help in the migration of cells within the developing CNS. In adult CNS to maintain the proper organization of axons, neural connections, and synaptic plasticity a basal level expression of CSPG is needed. High expression of CSPG occurs during injury and certain diseases that further reduces the regeneration potential of CNS (Haylock-Jacobs et al. 2011). Laminins are glycoproteins that are found in the basal membrane and help in axonal guidance during CNS development. They also play an important role in cell morphogenesis, differentiation, proliferation, and apoptosis mediated by integrin, dystroglycan, syndecan receptors. Laminin upregulation is observed after CNS injury (Jessica C. F. Kwok 2016). FN is a glycoprotein in the CNS ECM that facilitates cell adhesion, migration, and differentiation in the CNS (Pires-Neto, Braga-de-Souza, and Lent 1999).

ECM has a significant role as a barrier and as a structural support element. It also provides a signaling environment by providing a substrate for cellular receptors, thus triggering or influencing signaling events across cell membranes. ECM regulates the bioavailability of signals by acting as a niche. Cells contribute to ECM formation and reorganization by determining the composition and quality of ECM and matrix receptors through differential gene expression, glycosylation, and endocytic trafficking and by the release of various extracellular enzymes (Prokop 2011).

1.3 Signaling Mechanisms in development and regeneration

Neurogenesis involves proliferation, specification, differentiation, maturation, migration, and functional integration of newly formed glial and neural cells. During embryonic and adult neurogenesis, signaling via small molecules, neurotrophic factors, growth factors, neurotransmitters, and neurogenic niche determines the fate of every NSC. But same signaling pathways can have similar or dissimilar effects in adult and embryonic neurogenesis. For instance, bone morphogenetic protein (BMP) is a small signaling molecule that regulates NSC fate in adult as well as embryonic neurogenesis. In embryonic development, BMP promotes NSC proliferation and patterning of the nervous system, but in the adult stage, BMP helps in maintain the quiescent stage of NSC (Urbán and Guillemot 2014). In embryonic neurogenesis, Wnt, fibroblast growth factor (FGF), and retinoic acid (RA) signal gradients help to establish the anteroposterior (AP) axis and hedgehog (Hh), Wnt and (BMP) signal gradients establish dorsoventral (DV) axis of CNS. During adult neurogenesis, after CNS injury Wnt, FGF and RA provide signals for axonal guidance and help in regeneration along the AP axis (Cardozo et al. 2017).

Notch signaling acts as a regulator of neurogenesis and gliogenesis. Notch and its ligand delta act through juxtracrine signaling. Notch signaling is required for the proliferation and maintenance of NSC in the developing brain. They also help in regulating neural niche cell identity and plasticity (Faigle and Song 2013). Notch signaling suppresses neuronal as well as oligodendrocyte differentiation from precursors in the adult brain. Notch plays a crucial role in maintaining the stem cell pool in adult CNS (Xiao et al. 2009). Wnt molecules act both in paracrine and an autocrine manner as they are mainly secreted by astrocytes and stem cells. Secreted Wnt molecules induce neurogenic genes such as Neurog2, NeuroD1, and Prox1 directly. They play a vital role in synapse formation and maturation of neurons in embryonic, postnatal, and adult neurogenesis (Urbán and Guillemot 2014).

Neurotrophic factors namely nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) also play a significant role in CNS development. BDNF, NGF and NT-3 promote proliferation and differentiation of NSCs and growth factors like Fibroblast growth factor-2 (FGF-

2), Insulin-like growth factor-1 (IGF-1), and Vascular endothelial growth factor (VEGF) are involved in the regulation of neurogenesis (Oliveira et al. 2013). They act through PI-3 kinase/Akt and the Ras/Raf/MEK/Erk pathway (Faigle and Song 2013). Along with these signals, various neurotransmitters like serotonin and dopamine play a significant role in the proliferation and differentiation of neural progenitors. (Cardozo et al. 2017)

1.4 Injury of the nervous system

Various kinds of damages can occur to the nerves that can be considered as a nerve injury. Based on the first classification of SCI by Seddon, there are three types of nerve injury: neuropraxia, axonotmesis, and neurotmesis. Neuropraxia causes a physiological block in nerve conduction temporarily. Here, the axon remains intact but there is damage in the myelin sheath that causes an interruption in conduction of the impulse. Axonotmesis involves anatomic disruption of axon which is comparatively severe injury than neuropraxia. Connective tissues are not affected by such damages, but Wallerian degeneration occurs. It is the degeneration that takes place in the axon distal to the site of injury and may take time for recovery. Neurotmesis is the most severe injury that results from contusion compression or stretching. This classification was later expanded by Sunderland for including the damages in the connective tissues associated with SCI (Menorca, Fussell, and Elfar 2013). Complete recovery is difficult and may take years to regenerate (Huebner & Strittmatter, 2009). Neuroregeneration differs in PNS and CNS by effectiveness as well as speed.

In PNS, nerve regeneration is relatively faster as compared to CNS. First of all, the Wallerian degeneration occurs, with a thin layer of Schwann cells and connective tissues remain intact. Debris formed is removed by glial cells and macrophages. They eventually build a regeneration tube to which the undamaged proximal end of axon grows. New Schwann cells begin to appear and they myelinate the growing axon finally re-establishing the connection. Macrophages and Schwann cells release neurotrophic factors that enhance re-growth. In PNS, neurons upregulate numerous regeneration-associated genes (RAGs) like Activating transcription factor-3 (ATF-3), SRY-box containing gene 11 (Sox11), Growth-associated protein-43 (GAP-43), CAP-23, small proline-repeat protein 1A (SPRR1A), etc. (Huebner and

Strittmatter 2009). In contrast, CNS does not regenerate spontaneously or up-regulate RAG genes. Genes like GAP-43 is down-regulated adult CNS. The environment of the CNS plays an important role in regeneration. Myelin-associated inhibitors (MAI) and the chondroitin sulfate proteoglycans (CSPG) are two classes of molecules that inhibit axonal regeneration in CNS. Nogo-A, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), ephrin-B3, and Semaphorin 4D (Sema4D) are MAI and are expressed by oligodendrocytes (Huebner and Strittmatter 2009). An astroglial scar is formed after nerve injury in CNS. They help in maintaining the blood-brain barrier (Becerra-Calixto and Cardona-Gómez 2017). It acts as a physical barrier to regeneration. The molecules present in glial scar-like chondroitin sulfate proteoglycans (CSPG) also inhibits regeneration in CNS. Spinal cord injury (SCI) is a serious kind of typical CNS nerve injury. Regeneration is quite difficult in SCI due to all the glial inhibition and neuron's intrinsic inability in axonal regeneration (Moeendarbary et al. 2017). Various studies are ongoing to understand and resolve the issues related to CNS regeneration. SCI is a typical CNS injury model that can be used for experimentation and research for understanding the regeneration hurdles and its solutions.

The spinal cord is a long, tube-like structure that consists of a bundle of nerve fibers that begins from the brain stem and extends to the bottom of the spinal canal through an opening in the base of the skull called the foramen magnum. It is an "information super highway" between the brain and the body through which nerves carry trillions of sensory and motor information. The medical community is still divided on the fact that the spinal cord is an organ or a bundle of nerve tissue. However, it plays an indispensable role as a relay and integration center in the central nervous system (CNS).

1.5 Spinal cord injury

SCI is a condition where damage to the spinal cord results in physical trauma which may be either temporary or permanent. SCI causes loss of sensory-motor functions partly or completely in the parts of the body that lies below the area of injury. SCI affects bowel and bladder control, breathing, heart rate, and blood pressure which have

debilitating effects on patient's health("WHO | Spinal Cord Injury", 2013). The severity of SCI depends on the site and extent of the injury.

According to World Health Organization (WHO) every year around 250,000 to 500,000 people suffer from a SCI and the majority of them are caused by preventable causes such as road accidents, falls, acts of violence, or Sporting accidents ("WHO | Spinal cord injury," 2013). As per WHO Global Burden of Disease Study, by 2020 trauma caused by road traffic injury will be the third-ranked among most disabling conditions. In developed countries, the cost of managing the care of spinal cord injury patients is $3 billion each year which may not be affordable by people in low-income group countries. In developing countries like India, the incidence of SCI is on the rise, which is approximately 20,000 new cases per every year with the majority from regions of poor socio-economic strata. (Singh et al. 2003).People who survive a spinal cord injury usually suffer from complications in bladder & bowel dysfunction, chronic pain, spasticity, respiratory &heart problems. Various secondary conditions associated with SCI like deep vein thrombosis, urinary tract infections, muscle spasms, respiratory complications, etc., are the leading cause of death associated with SCI in low-income group countries ("WHO | Spinal cord injury,2013")

SCI involves a primary injury caused by direct mechanical damage to the spinal cord which leads to secondary injury, a series of cellular and molecular events that result in a neurological deficit. Primary injury is mainly caused by compression, contusion, dislocation, extension, and distraction. Neurons and glial cells in the region get affected along with interruption of blood flow. It will finally lead to hypoxia and ischemia that finally destroys cells and sets off a cascade of biochemical and cellular events called secondary injury. Secondary damage triggers an inflammatory immune response which may take over days to weeks after primary injury. Acute to the chronic phase of SCI involves events like excitotoxicity, oxidative damage, ischemia, and lipid peroxidation which results in glial scar, demyelination, the formation of cavities, necrosis, and infarction (Silva et al. 2014). Excitotoxicity is caused by leakage of excitotoxin, glutamate into the site of injury from damaged neurons. It will cause high calcium ion (Ca++) influx into the cell causing activation of phospholipases,

endonucleases, and proteases. This may lead to increased cell death and damage (Velasco et al. 2017). Glial scar is formed when there is an injury in the nervous system as a result of astrogliosis and various other cellular processes. Glial scar enmeshes the injury site with molecules like chondroitin sulfate proteoglycan results in poor endogenous regeneration. Myelin associated inhibitors like myelin-associated glycoprotein (MAG), Oligodendrocyte myelin glycoprotein (OMgp), the transmembrane semaphorin 4D (Sema4D/CD100)and ephrin B326 released in to the lesion during injury inhibit axonal outgrowth and restoration of neural circuits.(Yiu and He 2006).

Primary damage results in demyelination of axons as well as neuronal necrosis and death. During secondary injury due to the extensive death of oligodendrocytes, more axons will become necrotic and lead to neuronal loss. Myelin can be regenerated as new oligodendrocytes emerge from oligodendrocyte progenitor cells through differentiation. Re-myelination will not be sufficient enough to cover all injured and exposed axons. Also, the rate of de-myelination will be at a higher pace when compared to re-myelination which again results in a large number of exposed axons (Wang et al. 2017). Astrocytes immediately get activated in response to injury and start secreting various cytokines and chemokines which have inhibitory effects axonal regeneration and re-myelination. CSPG is expressed in the extracellular matrix (ECM) of adult CNS and has an inhibitory effect on CNS regeneration. In an uninjured state, they prevent unwanted synaptic plasticity. After the injury, due to astrogliosis CSPG is overexpressed in the ECM. The glial scar ECM contains more inhibitory molecules like Sema 3A and Tenascin-R that can act as a regeneration barrier (Busch and Silver 2007).

Methylprednisolone (MP) is the only approved therapy for SCI. High doses of MP have neuroprotective activity but it has been associated with serious side effects that outweigh the positive outcomes (Varma et al. 2013). This corticosteroid is most effective when administered within 5 hours of injury which is not feasible in many SCI cases. Other treatments to manage SCI include medications to control pain, respiratory problems, blood clots, to improve bladder & bowel control, etc. Surgeries are performed to restore the optimal alignment of the spine by removing bone fragments

or any other particle that compress the spine. Complete restoration of the impairment is challenging because of the poor endogenous regeneration of the CNS and inhibitory environment caused by secondary injury cascade (Silva et al. 2014). To date, there is no established therapeutic intervention that can restore the significant neurological function after SCI.

1.6 Current research approaches in SCI

Various strategies are being studied all over the world to achieve recovery by surpassing the barriers to regeneration such as the cyst formation, glial scarring, and inhibitory environment around the lesion. Neuroprotection, cell replacement, regeneration, restoration of circuits, and plasticity are probably the key principles of the extensively studied field of current and future research(National Institute of Neurological Disorders and Stroke, 2013). Neuroprotection reduces inflammation and associated secondary injury mechanisms which eliminate further damage to the spinal cord. Erythropoietin (EPO), Potassium & calcium channel blockers, Minocycline, GM-1 ganglioside, therapeutic hypothermia are few neuroprotective strategies capable of reducing secondary injury that is being widely studied (Y.-H. Kim, Ha, and Kim 2017). Numerous *in vitro* studies, *in vivo* studies as well as clinical trials, suggest that neuroprotection alone can only reduce secondary cell death and complications related to SCI. Since the cellular and chemical environment around the lesion is constantly varying, combinatorial therapies aiming regeneration and restoration of the injured spine using neuroprotection along with cell replacement may be ideal.

1.7 Advances in Cell Replacement Research

Cell replacement therapy in combination with trophic support may be an ideal combinatorial strategy, as the cell replacement helps in the restoration of the circuit while the neurotrophic molecules help in neuroprotection. Neurotrophic factors or neurotrophins (NT) are growth factors that can promote the growth, proliferation, survival, and regeneration of the neurons. Neurotrophic factors like BDNF, Nerve Growth Factor (NGF), Glial cell-derived neurotrophic factor (GDNF), etc., have a neuroprotective action in the nervous system through influence on Reactive Oxygen Species (ROS) and reducing the oxidative stress associated with various injuries(Altschuler et al. 2014). Stem cells, olfactory ensheathing cells (OEC), fetal

tissue-derived cells, Schwann cells, peripheral nerve tissues are the various cell types researched globally for cell replacement therapy in SCI.

Olfactory ensheathing cells (OEC) are cells that cover the axons of the olfactory receptor neuron. They have properties of both Schwann cells and astrocytes. OECs have limited survival and low proliferation rates in culture over time (Doucette 2002). Fetal derived cells have a high proliferative capability, but they are associated with ethical issues and may have immunogenicity when transplanted (Abdulrazzak et al., 2010). Peripheral nerve tissues on transplantation to CNS fail to re-establish connections. In the case of Schwann cells, one cell can wrap around and myelinate only one neuron. A large number of Schwann cells may be required to promote regeneration at the site of injury. These cells fail to survive on transplantation. Mature cells like neurons or oligodendrocytes are not ideal for transplantation as they are terminally differentiated cells. They cannot proliferate or differentiate at the site of injury. A large number of cells are necessary for overcoming the loss caused by degeneration and injury. Stem cells may be a solution for all these difficulties. Stem cells could undergo various cycles of cell division while maintaining their undifferentiated state. They also have potency, so that they can differentiate into various cell types. Endogenous NSC niches are fewer in CNS and stem cells that migrate from these two regions to injury sites are not enough to supply enough cells for regeneration.

With recent advances in stem cell research, there has been a remarkable hope for the development of new treatment strategies for SCI. Stem cell therapy for spinal cord repair is important for achieving improved regeneration. It offers replacement of damaged neuronal and glial cells, re-myelination of spared axons, restoration of neuronal circuitry, bridging of cavities, the release of various neurotrophic factors & anti-inflammatory molecules and a permissive environment for axonal regeneration (Mothe and Tator 2012). Various stem cells release growth factors as well as neurotrophic factors that can aid neuroprotection together with cell replacement and regeneration.

1.8 Stem Cell Sources

Embryonic stem cells (ESC), Induced pluripotent stem cells (iPSC) and adult stem cells are the three types of stem cells available for therapies. ESCs are pluripotent stem cells that are derived from the inner cell mass of an embryo. ESCs are usually isolated from embryos developed *in vitro* and donated for research purposes along with informed consent. iPSCs are produced *in vitro* from adult stem cells by genetic reprogramming, which attributes them ESC like characters. Quite a few *in vivo* studies have shown that ESCs and iPSCs are associated with increased risk of tumorigenicity when used as cell replacement therapy (Deng et al. 2018). Ethical concerns and immunogenicity are the other issues often associated with ESC research and therapies. Adult stem cells are present throughout the adult human body in an undifferentiated or quiescent form. They are multipotent cells and found in many organs and tissues. Even though potency is higher in ESC and iPSC, adult stem cells may be considered as ideal for autologous cell replacement therapies as they have increased genetic stability and no ethical issues are associated. ADMSCs, bone marrow-derived mesenchymal stem cells (BMSC), and hematopoietic stem cells (HPSC) are the few potent adult stem cell types among the most researched ones.

Mesenchymal stem cells (MSC) are multipotent cells that can differentiate into various cell types like adipocytes, chondrocytes, osteocytes, and neuron-like cells. They are present in the number of tissues like bone marrow (BMSC), adipose (ADMSC), and most connective tissues. MSCs have great homing efficiency and they tend to home in the damaged tissue sites. Exogenously administered MSC tends to migrate specifically to damaged tissue sites with inflammation. The therapeutic benefits of MSC are mainly based on their capacity to release various trophic factors. MSCs home to damaged tissue sites for repair and produce a large number of growth factors that perform multiple functions for tissue regeneration (Wei et al. 2013).

Recently, the stromal cells isolated from adipose tissue, called adipose-derived stem cells have gained increased attention in the field of regenerative medicine. The extraction of adipose tissue is less expensive than bone marrow, with less invasive methods. Greater quantities can be collected through a single procedure. High numbers

of stem cells can be isolated from the adipose tissue since the proliferation rate is higher in ADMSC when compared to BMSC(Zhu et al. 2008a).

ADMSC may be used as a cell source for cell replacement therapy for SCI. Increased proliferative capacity, reduced immunogenicity, affordable expense, availability, and reduced burden to the patient makes ADMSC an ideal stem cell source for transplantation (Patrikoski, Mannerström, and Miettinen 2019). ADMSCs could be used as a source for autologous cell replacement therapy in SCI where the patient's own adipose tissue can be collected by lipoaspiration. It is a less invasive method through which the issue of immune reactions associated with allogeneic cell transplantation may be eliminated. However, since these cells are multipotent, it is a challenge to ensure that these cells undergo the desired differentiation post-transplantation.

Pre-differentiation of stem cells *in vitro* into desired types prior to transplantation could increase the chance for regeneration potential of transplanted cells. For maintenance of spinal cord integrity and function, the key players are neurons, astrocytes, and oligodendrocytes. Out of these 3 cell types, astrocytes contribute to glial scar generation, rather than the regeneration of functional outcome. Neural progenitor cells (NPC) and oligodendrocyte progenitor cells (OPC) are likely to contribute to functional recovery. Both OPC and NPC cell transplantation may foster the endogenous remyelination and regeneration at the injury site.

Cell replacement therapy using oligodendrocyte progenitor cells is based mainly on the fact that they can re-myelinate neurons, re-establish neuronal connections, and thereby reduce the neuronal loss at the injury site. Following injury, remyelination is further challenged by deficiency expression of growth factors like platelet-derived growth factor (PDGF) and neurotrophins like BDNF with supportive roles in myelin maintenance, OPC survival and axonal-oligodendrocytic signaling (Wheeler and Fuss 2016). A large number of oligodendrocytes undergo cell death because of glutamate excitotoxicity and oxidative stress. Demyelination in SCI creates increased energy consumption in neurons for signal transduction. Loss of trophic support from oligodendrocytes and increased energy demands can make demyelinated neurons more

susceptible to oxidative stress and degeneration(Alizadeh and Karimi-Abdolrezaee 2016). Considering all these facts it's apparent that oligodendrocyte cell replacement and remyelination together with neural cell replacement are vital for restoring neurological function following SCI.

1.9 Gap Area

The use of ADMSC for cell replacement therapy in SCI demands pre-differentiation as well as lineage conversion of ADMSC in mesodermal origin to ectodermal origin. The lineage commitment of ADMSC to OPC is a challenge. There are different protocols in the literature which describe methods of differentiation to oligodendrocyte. Differentiated or mature cells may not be suitable for any cell replacement therapy as they may not survive, proliferate, and regenerate injury. Therefore, the stage at which the cultured cells could be harvested for transplantation, and the number of cells needed for effective cell-based therapy has to be optimized. The OPC and NPC phenotype in *in vitro* culture and after transplantation may be controlled by various ECM proteins and growth factors. Therefore, there is a need to identify an *in vitro* niche for transforming mesodermal stem cells to ectodermal progenitor cells. Various signaling mechanisms may operate the control of stem cells in the progenitor stage. How the stem lineage commitment to OPCs is controlled by various signaling mechanisms elicited in the fibrin niche is unknown. Hence *in vitro* signaling studies need to carry out to understand the signaling mechanisms occurring in the niche.

1.10 Definition of the problem

Pathophysiology of SCI is well understood, and it provides great potential for improving therapeutic strategies for spinal cord repair and regeneration. As of now, there is no gold standard for spinal cord injury treatment. Stem cells have been extensively studied as a novel neuroregenerative agents for SCI repair and regeneration. Various animal models of SCI, have demonstrated the therapeutic role of stem cells and stem cell-derived progenitors (Gazdic et al. 2018). Even though, only preconditioned cell transplantation under a controlled environment can ensure successful cell survival and integration.

ESC as well as adult stem cells can differentiate towards neural precursors, motor neurons, and oligodendrocyte progenitor cells. Although *in vitro* induction of stem cells prior to transplantation is crucial to differentiate them into neurons or oligodendrocyte specifically. Stem cells respond to signals in the microenvironment and differentiate accordingly which may be different from its *in vitro* behavior. In a study, proliferating, undifferentiated pluripotent neural stem cells were isolated from the rat embryo, which was able to differentiate *in vitro* into neurons, astrocytes, and oligodendrocytes. When transplanted, the majority of these cells differentiated with astrocytic phenotype rather than becoming oligodendrocytes or remain as nestin-positive undifferentiated cells. (Cao et al. 2001). At SCI site, the astrocyte population is an undesired cell type as it contributes to worsen the glial scar and inhibitory environment. Pre-differentiation of stem cells to the immediate precursor cells of the desired cell type is mandatory to prevent differentiation to such undesired lineages.

In vitro OPC differentiation needs growth factors like EGF and FGF-2. FGF-2 and PDGF-AA contribute to the proliferation and maintenance of OPC. Triiodo-L-thyronine (T3) hormone controls the specification and differentiation of oligodendrocytes. It also promotes myelination of axons (Lourenço et al. 2016). The ECM proteins like FN or vitronectin (VN) are involved in the proliferation of oligodendrocyte progenitors, while La-2 (merosin) promotes their differentiation into mature oligodendrocytes. Chemical inducers like β-mercaptoethanol and Shh agonist Purmorphamine are used widely to differentiate stem cells to OPC spontaneously(Terzic et al. 2016). But these strategies may affect cell stability and health to a greater extend.

A Biomimetic niche that contains these ECM molecules may enable a controlled environment for induction and differentiation of stem cells into other desired cell lineages. There is also a lack of an ideal niche, that can provide a controlled environment for induction and differentiation of progenitor cells to oligodendrocytes at the site of injury. So, the development of a biomimetic niche may help in *in vitro* as well as *in vivo* differentiation of stem cells to OPC. Also, if used as a transplantation vehicle they promote incorporation and integration of grafted cells with host cells at the injury site. Reduced cell survival, proliferation, and migration after cell

replacement therapy can also be addressed by the incorporation of a biomimetic niche containing progenitor cells to the injury site.

1.11 Prerequisite for Niche

A niche can be defined as a microenvironment, which interacts with the stem cells to regulate its cell fate.it can be either *in vivo* or *in vitro*. Stem cell niche helps in maintaining adult stem cells in a quiescent state at the specific anatomical locations of the body. Signaling cues in the microenvironment during injury can promote self-renewal or differentiation of these stem cells. Paracrine interactions between stem cells with differentiated cells, signaling via adhesion molecules, growth factors, and extracellular matrix components play an important role in the proliferation and differentiation of stem cells in a niche.

The biomimetic niche that can replicate *in vivo* stem cell niche characteristics is an inevitable factor for *in vitro* differentiation and transplantation of stem cells. Such a niche can maintain the proliferation and differentiation of stem cells in a controlled manner. Cell transplantation in SCI lesion site demands a niche that can promote cell survival and integration. Transplanted cells may otherwise undergo cell death due to the inhibitory signals and molecules present at the injury site. An ideal biomimetic niche should contain ECM molecules, growth factors, signaling molecules, and cytokines that can help the survival, proliferation, and differentiation in a balanced manner. Various studies are being done in this particular field using different scaffolds and biomaterials and fibrin is one of the potent materials among them.

Fibrin is a natural biopolymer formed as a result of clotting cascade by the interaction of thrombin and fibrinogen. Fibrin is biocompatible, biodegradable, and bioactive, which makes it an ideal biomaterial for differentiation and transplantation studies. Fibrin enables cell attachment, growth, and differentiation. It contains a large number of binding sites for integrin which enables cell attachment and growth. Fibrin can interact with various proteins like FN, vitronectin, and with growth factors such as FGF, insulin-like growth factor-1(IGF-1), VEGF, etc. (Noori et al. 2017). Fibrin based biomimetic niche has been formulated from fibrin and thrombin which direct *in vitro* differentiation of neural progenitors from peripheral blood mononuclear cells

(PBMNC) efficiently. ECM components such as FN and La found in in-house prepared fibrin and they play a crucial role in differentiation to neural lineage (Tara and Krishnan 2015). FN anchors growth factors and provides cell adhesion which helps in the manifestation of various growth factor signaling. Fibrin sealants have been successfully used as a delivery matrix for growth factors in various animal models of wound healing and revascularization. It enables the slow release of growth factors over a long period of time (Arkudas et al. 2007). As a transplantation vehicle, studies show that fibrin improves the survival, migration, and integration of the transplanted cells.

1.12 Current developments

The role of the fibrin-based niche for effective delivery and survival of autologous NPCs isolated from Peripheral Blood Mononuclear Cells (PBMNC) at SCI site in the rat model has been studied in our laboratory. Evaluation of *in vivo* survival of NPC induced from the neural progenitors in PBMNCs in the SCI model was also performed.

1.13 Hypothesis

Cell-specific niche comprising soluble factors and fibrin may regulate signaling pathways resulting in the *in vitro* lineage commitment of hADMSCs to NPCs and OPCs suitable for transplantation to acute SCI using the injectable biomimetic matrix for a better outcome.

1.14 Objectives

1. To compare the effect of soluble factors, for lineage commitment of hADMSCs to neurospheres (NS) and further differentiations to NPC and OPC, upon culture on bare tissue culture polystyrene surface (TCPS) with insoluble fibrin coated surface.
2. To establish that the differentiation of hADMSCs to NS and OPCs is induced by receptor-mediated signaling pathways triggered by fibrin-based niche components.
3. To study the survival of pre-differentiated progenitors, harvested from *in vitro* niche, upon delivery to spinal cord injury (SCI) model using injectable fibrin.

1.15 Rationale of the study

The rationale to develop a "Fibrin based matrix to direct differentiation of adipose-derived mesenchymal stem cell into oligodendrocytes for cell replacement therapy in spinal cord injury" arises from the lack of an ideal niche for the same. An ideal niche like 'fibrin-based niche' for differentiation and transplantation of ADMSC derived OPCs and NPCs may help in overcoming various difficulties such as lack of signaling and adhesion molecules in *in vitro* differentiation and poor incorporation of the cells at the injury sites during transplantation. Efficient and safe therapy for functional recovery in acute SCI may be developed using fibrin niche as a niche for *in vitro* differentiation of ADMSC and transplantation of neural and glial progenitors at SCI lesion sites.

CHAPTER 2

2. LITERATURE REVIEW

This section encompasses the basic concepts and current approaches in stem cell therapy in neurodegenerative conditions from the previously published literature. The overview of SCI, its types, and pathophysiology, the role of glia, and various growth factor signaling involved are reviewed in detail. The role of Oligodendrocytes, its replacement in SCI, Stem cell pre-differentiation strategies, SCI and Stem cell therapy-based clinical trials, and animal models are discussed to justify the current study and its significance.

2.1 Central Nervous System and SCI

The nervous system is a multifaceted, organized network of billions of neurons and glia. They control as well as coordinate all activities of the body. The vertebrate nervous system is basically divided into the central nervous system (CNS) and the Peripheral nervous system (PNS). They are made of neurons and neuroglial cells. CNS includes nerves in the brain and spinal cord while all other nerves present in the body comes under PNS.

The brain is situated in the skull and CNS is continued as the spinal cord in the vertebral canal through foramen magnum present in the skull. The brain and spinal cord consist of three layers called meninges. The outer layer is called dura mater, the middle one is arachnoid mater and the innermost layer is pia mater. CSF is produced from arterial blood by the choroid plexuses and ependymal cells. It provides buoyancy and protects CNS from external impact. The brain plays an important role as the control center of the body and carries out functions such as movements, consciousness, speech, memory, etc. The spinal cord carries signals to and forth between the brain and the peripheral nerves.

The peripheral nervous system consists of spinal nerves, cranial nerves, and peripheral nerves. Cranial nerves emerge from the brain and brainstem. They are mainly associated with the motor-sensory function of the head and neck region of the body. There are twelve pairs of cranial nerves, which are designated as CNI to CNXII. They can be classified as sensory nerves, motor nerves, or mixed. The axons in these nerves

originate from sensory ganglia exterior to the cranium or motor nuclei within the brain stem. Sensory axons forms synapse in the brain while motor axons connect to skeletal muscles of the head or neck. Three cranial nerves are sensory fibers while five are motor and the other four are mixed nerves. Nerves that arise from the spinal cord are spinal nerves. There are 31 pairs of spinal nerves. Based on the level of the spinal cord from where they emerge, they are divided into cervical, thoracic, lumbar, sacral, and coccygeal. There are eight pairs of cervical nerves named C1 to C8, twelve pairs of thoracic nerves; T1 to T12, five pairs of lumbar nerves; L1 to L5, five pairs of sacral nerves; S1 to S5, and one pair of coccygeal nerves. The area of skin connected by a particular spinal nerve is called a dermatome, and muscles connected to a single spinal nerve is called a myotome. When there is a SCI, spinal nerves at that level and below stops functioning. Based on the level and extent of the injury, SCI is classified into different groups.

2.1.1 SCI types

SCI is broadly divided into complete spinal cord injury and incomplete spinal cord injury. Incomplete SCI, the spinal cord gets fully compressed or severed resulting in complete loss of neurological functions below the site of injury. Regeneration of cells as well as the regaining of complete neurological functions after complete SCI is difficult. The partial compression or injury of the spinal cord causes incomplete SCI. The degree of impairment in SCI is based on the extend of the injury. Greater the injury higher will be the neurological deficit.

The extent and severity of SCI can be defined using the American Spinal Injury Association (ASIA) Impairment Scale. A scale of A, B, C, D, E is used in grading the degree of impairment. ‘A’ is a Complete SCI and no sensory or motor functions in the sacral segments. ‘B’ refers to an incomplete SCI with sensory functions preserved below the neurological level. ‘C’ is also an incomplete SCI in which motor functions are preserved below the neurological level and more than half of major muscles below the neurological level cannot be moved against gravity. ‘D’ is an incomplete injury with motor function preserved below the neurological level, at a minimum half of the main muscles below the neurological level are strong and can be moved against

gravity. ‘E’ refers to the normal condition where sensory and motor functions are regained or normal (Maynard et al.,1997).

2.1.2 SCI pathophysiology

The biological response to SCI is divided into three distinct, overlapping phases named acute phase, secondary phase, and chronic phase. Acute phase occurs within seconds to minutes after the injury while, secondary injury cascade occurs over minutes to weeks and chronic injury occurs over months to years after the injury.

The first stage of SCI is the acute phase where the initial mechanical trauma occurs to the cells by compression, shearing, laceration, contusion, or distraction. This causes the disruption of the microvasculature of the spinal cord which may lead to hemorrhage. Eventually, the blood flow in the spinal cord gets disrupted which results in hypoxia and ischemia. This may lead to a local infarction in the central grey matter as the metabolic requirement is higher when compared to the white matter region (Dumont et al. 2001). Acute injury leads to a sub-acute or secondary injury phase.

Primary mechanical injury follows a cascade of secondary injury mechanisms. Secondary injury can be divided into two phases, acute phase, and sub-acute phase. Acute phase occurs within 48 hours of SCI and it is characterized by vascular changes like ischemia, hemorrhage, vasospasm, thrombosis, and edema. Along with vascular changes, disruption of ionic balance occurs which leads to events like increased intracellular calcium, increased extracellular potassium, and increased sodium permeability. It is followed by the accumulation of neurotransmitters mainly, glutamate which leads to glutamate excitotoxicity and causes cell injury and necrotic cell death to a certain extend (Sekhon and Fehlings 2001). Glutamate is an excitatory neurotransmitter and it is released soon after the injury. Rise in the level of glutamate in the injury site stimulate the further release of glutamate from synaptic terminals. This causes calcium influx and excitotoxicity which leads to cell apoptosis. All acute phase events continue in the sub-acute phase along with a cascade of new events like the formation of free radicals, lipid peroxidation, delayed calcium influx, inflammation, and apoptotic cell death (Oyinbo, 2011). ROS and nitrogen species (RNS) formed during SCI causes lipid peroxidation which further causes membrane

damage and cell necrosis. Besides, they cause mitochondrial dysfunction and protease activation which finally leading to apoptosis and necrosis.

As soon as, the acute and sub-acute phases slow down, the repair mechanisms start. This repair phase starts in the intermediate chronic phase and it continues in chronic injury phases. It begins after a few months of injury and continues throughout the lifetime of the injured person. Demyelination of surviving axons, central cavitation, astroglial scar formation, apoptosis, are the major events occurring during the intermediate chronic phase and chronic phase. Maturation of the glial scar and syrinx in the chronic phase is followed by regenerative processes, including sprouting by neurons and alteration of neuro-circuits (Oyinbo, 2011).

After all pathophysiological changes, the final fate of neurons in the area of the lesion is apoptosis or necrosis. Also, degeneration of descending and ascending axons occurs with glial scar formation and loss of myelinating oligodendrocytes (OL). A large number of neuronal cell death occurs because of loss of myelin due to death of OL. Replacement of oligodendrocytes to promote remyelination of neurons to prevent further neuronal loss and damage is inevitable. Any therapy for SCI should focus on re-establishment of neuronal circuits either by replacement therapy or by promoting regeneration (Lowry and Temple 2007).

2.2 Inflammatory responses in SCI

2.2.1 Role of microglia & astroglia

Microglia and astroglia together carry out the innate immune responses during the SCI. (Anwar, Al Shehabi, and Eid 2016). The first line of response comes from the microglia. Various pro-inflammatory cytokines, chemokines, and anti-inflammatory cytokines are released during secondary injury which directly or indirectly controls the events after SCI. Resident microglial cells and bone marrow-derived macrophages are the two groups of macrophages present at the site of injury. The inflammatory responses involve immediate neutrophil invasion and activation of resident microglia. Activated CNS macrophages phagocytize cells and debris. They also help in the recruitment of monocytes from blood to the lesion. Macrophages start to resolve the scar within a week. The release of inflammatory cytokines and chemokines from the

cells in the lesion starts the inflammatory responses which further leads to activation and migration of microglia towards the lesion. It also helps in the recruitment of circulating leukocytes to the injury site (X. Zhou, He, and Ren 2014). Microglia senses the cellular cues and tissue damage in their niche and gets activated. Microglia on activation also release various cytokines which creates an inflammatory microenvironment (Anwar, Al Shehabi, and Eid 2016).

In a way, the inflammatory responses adversely affect the SCI recovery by causing cell damage and extracellular matrix (deterioration. The molecules released by the microglia such as pro-inflammatory cytokines, chemokines, nitric oxide (NO), and superoxide free radicals, that generate reactive oxygen species and reactive nitrogen species (RNS) can cause neuronal dysfunction and cell death in response to injury (Loane and Byrnes 2010). But the inflammation is also associated with potential benefits mediated by activated microglia/macrophages. The activated microglia help in the clearance of damaged and degenerating tissues at the lesion site. Residential microglial cells form a border and block the spread of damage while the bone marrow-derived macrophage phagocytizes apoptotic and necrotic cells and clear tissue debris (X. Zhou, He, and Ren 2014).

The blood-brain barrier (BBB) disrupts as a result of SCI which causes increased permeability and infiltration of cells from the blood that triggers an inflammatory response. The inflammatory response and cell damage activate astrocytes to undergo morphological and molecular changes. The changes involve increased cell proliferation and overexpression of astroglial proteins. This spectrum of changes is called reactive astrogliosis. Reactive astrocytes interact with other cell types and glial cells to form glial scar which re-establishes the BBB. But it acts as a mechanical and chemical barrier to axonal regeneration (Lukovic et al. 2015). The glial Scar prevents the spread of inflammatory cells from the area of injury to other parts of the nervous system. Additionally, Astrocytes play a key role in the regulation of inflammatory responses associated with SCI. The astrocytes interact with microglia and exert both pro- and anti-inflammatory effects on microglia (Sofroniew 2009). Molecules such as cytokines, chemokines, growth factors, and small molecules are released by astrocytes which takes part in various signaling pathways that are involved in the recruitment and

restriction of leukocyte migration, blood-brain barrier repair and resolution of inflammation in the CNS. The anti-inflammatory action of astrocytes is exerted by secretion of various molecules such as retinoic acid (RA) and tumor necrosis factor β (TGFβ). RA and TGFβ help in the restoration of the blood-brain barrier and the mitigation of inflammation (Sofroniew 2015).

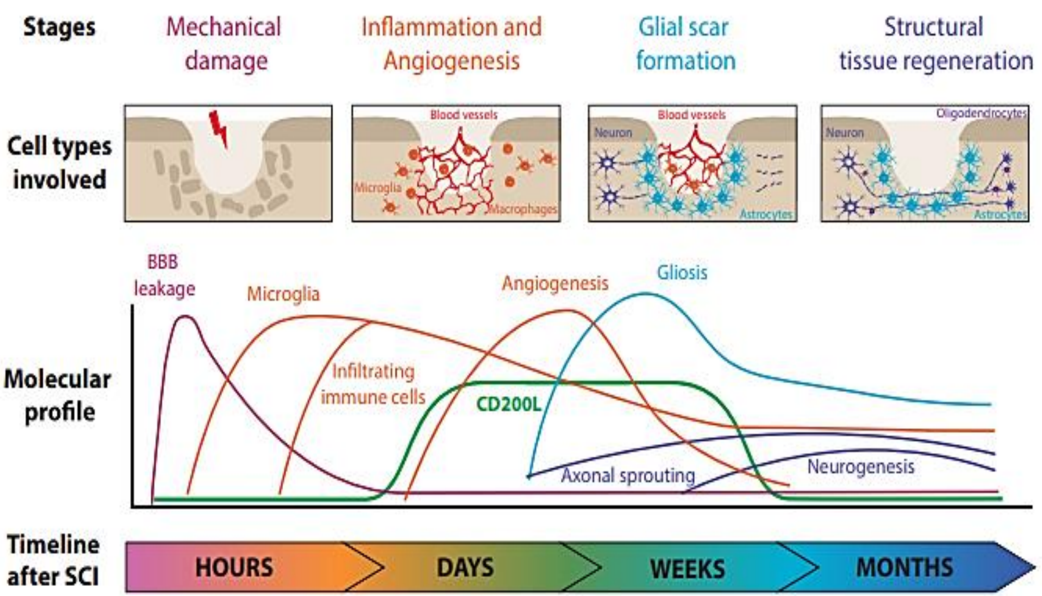
2.2.2 Glial Scar

Glial scar formation occurs when the brain parenchyma becomes permeable to non-CNS molecules due to the disruption of BBB as a result of SCI or brain injury. Glial scarring is maximum in the area where more BBB breakdown has occurred. Breakdown of the BBB causes a neuro-inflammatory response by increasing the recruitment of neurotrophins, macrophages, and lymphocytes which has an adverse effect on CNS health (Hamill et al. 2005). Moreover, macrophages play a crucial role in the upregulation of ECM molecules and glial scar formation. So areas in which high glial scar intensity is found also associated with the presence of a higher number of activated macrophages (Silver and Miller 2004). Transforming growth factor β (TGFβ) signaling plays an important role in triggering the inflammatory response and glial scarring. TGFβ2 considerably increases the production of proteoglycans by astrocytes. Interleukin 1, TGFβ 1 & 2 pathways are involved in the glial scar induction by macrophages (Silver and Miller 2004).

Activated astrocytes show variation in the normal protein expression profile. High expression of the glial fibrillary acidic protein (GFAP) is found in activated astrocytes. Reactive astrocytes also up-regulate epidermal growth factor (EGF) receptors, tyrosine kinase receptors, zinc receptor, and corticotrophin-releasing factor receptors, which are involved in several cell signaling pathways that aid the formation of the glial scar. Up-regulation of molecules like tenascin together with the secretion of Chondroitin sulfate proteoglycan (CSPG) into the ECM by reactive astrocytes is associated with axon growth inhibition (Hamill et al. 2005). CSPG include aggrecan, brevican, neurocan, versican, phosphacan and NG2 are a family of molecules with a protein core attached to highly sulfated glycosaminoglycan (GAG) chains. A gradient of CSPG establishes from the center of the lesion with reducing concentration at the periphery of the lesion. CSPG causes an inhibitory effect on the outgrowth of axons in the injured

area which directly acts as a barrier to regeneration (Shen et al. 2009). The exact mechanism through which CSPG exerts its inhibitory effect is still not completely studied. It is known that a network of reactive astrocytes forms tight gap junctions in the area of injury which acts as a mechanical barrier to axonal regeneration. Along with that astrocytes also secrete molecules like CPSG which act as a chemical barrier that prevents neurite outgrowth from the lesion. The enzymes Chondroitinase ABC (ChABC) removes glycosaminoglycans from the CSPG protein core is effective in the degradation of the glial scar. Various studies show that the disruption of the glial scar is effective in promoting tissue regeneration after SCI (Yiu and He 2006).

2.2.3 SCI: Timeline of injury responses



Four stages in spinal cord injury: During the first few hours, direct mechanical injury causes disruption blood-brain barrier (BBB). Over several days immune cells infiltrate to the injured area and then astrogliosis occurs. Then limited levels of tissue regeneration occur in several weeks to few months following SCI (Rust and Kaiser 2017).

2.3 Oligodendrocytes

In the CNS, glial-subtype responsible for myelin production in oligodendrocytes (OL). Neurons and glial cells of CNS develop from neuroepithelial progenitor cells located

in the ventricular zone (VZ) of the neural tube during embryonic development. The oligodendrocytes are derived from progenitors named as oligodendrocyte precursor or progenitor cells (OPC). OPCs are NG2 positive cells as they express the NG2 proteoglycan on their surface. They are also called as polydendrocytes because of their multiple processes. OPC express molecular markers NG2, platelet-derived growth factor receptor alpha (PDGFα), and transcription factors-Olig2 and Sox10. NG2 and PDGFRα markers are down-regulated as OPCs differentiate into OLs (Bergles and Richardson 2016).

2.3.1 Embryonic development of Oligodendrocytes

Brain and Spinal cord contain distinctive classes of oligodendrocytes that are derived from multiple sources. OPC arises from various regions of the germinal ventricular zone including the telencephalon, diencephalon, mesencephalon, rhombencephalon, and spinal cord in the CNS. Proliferation and migration of OPC occur in developing grey and white matter from these regions and then differentiate into myelin-forming OLs (Bergles and Richardson 2016).

During development, 80 percent of spinal OPCs arise from a particular region of the ventral ventricular zone called the motor neuron progenitor domain (pMN). pMN first gives rise to motor neuron precursors which then undergoes neurogenic or gliogenic lineage switch and to OPCs. After the initial production of OPCs, the second set of OPC production arises in the dorsal cord which is trans-differentiated from radial glia. Radial glia is progenitor cells capable of forming all the cell types in the nervous system. It contributes to the remaining 10–15% of the oligodendrocyte population in the spinal cord. The first wave of OPCs in the forebrain originate in the medial ganglionic eminence and anterior entopeduncular area of the ventral forebrain. These OPCs are found in the whole telencephalon including the cerebral cortex of the embryonic brain. This OPC population gradually declines and get eliminated from the adult forebrain during development. The second wave of OPCs in the brain is derived from the lateral and caudal ganglionic regions and third wave from the postnatal cortex. These distinctive populations of OPCs are practically the same in their functionality. OPCs once formed they migrate to various regions in the CNS controlled

by growth factors such as PDGF, FGF, and ECM proteins and cell surface molecules. (Bradl and Lassmann 2010).

In the adult brain, plenty of OPCs is found. They accelerate the replacement of dying OLs. OPCs migrate and differentiate to various regions in the adult brain based on the need for *de novo* myelin formation to replace dying OLs. The OPC proliferation, migration, and terminal differentiation are dependent on the microenvironment and signals. The CNS triggers OPC cell death when there is a reduced need for myelination. But during CNS injury, the delay between OPC division and OL differentiation is reduced (Michalski and Kothary 2015).

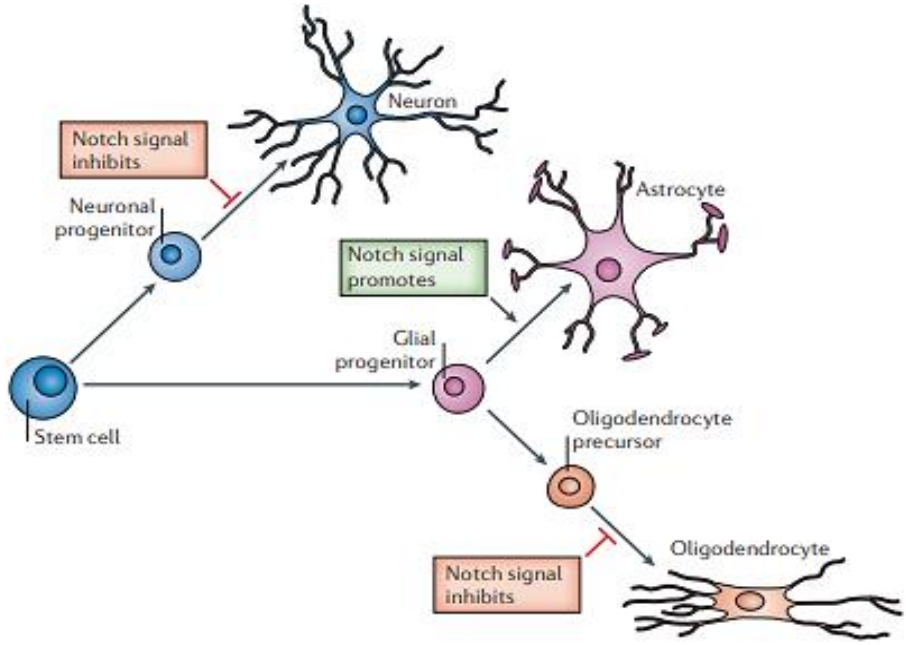
The differentiation of OPCs to oligodendrocytes involves various signaling pathways. The notch signaling pathway involving the notch receptor and jagged ligand located on the axonal surface plays an important role in maturation and myelination. Myelination of multiple axons by an oligodendrocyte is highly coordinated and they have only a small period of time available for this as they are comparatively incapable of myelinating once they are mature. The oligodendrocytes do not ensheath different axons within a brief window of time, 12–18 h (Bradl and Lassmann 2010).

2.3.2 Oligodendrocyte development: Role of the notch and wnt signaling

The canonical Wnt pathway or Wnt/b-catenin pathway is a key regulator of oligodendrocyte development. Canonical Wnt pathway is activated in OPCs simultaneously with the initiation of terminal differentiation. Expression of Tcf4/Tcf7l2, transcription factor which carries out the transcriptional effects of the Wnt/b-Catenin pathway is down-regulated in mature oligodendrocytes. Down-regulation of Wnt signaling may be necessary for the terminal differentiation of OPCs to OL. Elevated levels of Wnt/b-catenin signaling in the OPCs can block the differentiation and myelination of OLs (Emery 2010).

The Notch receptor and its ligands Jagged and Delta, are transmembrane glycoproteins. The notch signaling pathway gets activated when there are close cell-cell interactions between the receptor and its ligands. This signaling pathway plays an important role in axon-glial contact and its regulation. It has been well studied that the notch signaling pathway plays an inhibitory role in OL differentiation. Notch

inhibitory signaling prevents premature differentiation of OPCs and thereby play an instructive role in glial differentiation (Popko 2003). During embryonic development, Notch signaling promotes the formation of OPCs and maintains a population of proliferative precursors of OL in the spinal cord. In pMN, during embryonic development notch signaling inhibits neurogenin expression which is necessary for motor neuron formation and maintains olig 2 expression which is a transcription factor that promotes glial lineage. Terminal differentiation of OPC to OL is regulated by delta-notch signaling and it couples the oligodendrocyte specification and differentiation (Park 2003).



Signaling in OL differentiation: Activation of Notch, Shh, and Wnt signaling inhibits OPC differentiation into oligodendrocytes (Louvi and Artavanis-Tsakonas 2006).

Various intracellular signals also regulate OL development. Transcription factors are among these. They play a role in OL maturation and differentiation. Transcription factors, Hes5, ID2/4, Sox5/Sox6 have a negative effect on OL development whereas, Sox10, Nkx2.2, MRF, Zfp191, Zfp488, and Sip1 promotes OL maturation and development. Another important factor that plays a major role in OL development is the thyroid (T3) hormone. T3 hormone has been widely used to differentiate OPC to

OL *in vitro*. T3 hormone has been implicated in timing control of oligodendrocyte differentiation. It also involves both PDGF signaling and T3 signaling. T3 initiates differentiation of OPC to OL at the appropriate time as an effector (Huang et al. 2013).

2.3.3 Oligodendrocytes: dual role in SCI

Loss of OLs and subsequent demyelination occurs in SCI. OLs play a dual role in SCI as it promotes neuronal loss and enables neuronal survival. Loss of OLs during an injury causes the conduction block and death of neurons. Besides OLs create an inhibitory environment by releasing various inhibitory molecules to the site of injury. Conversely, OLs promote neuronal survival by re-myelination of demyelinated neurons.

In SCI, necrotic cells release proteolytic enzymes which cause damage to OLs and neurons. Ischemia and reperfusion injury cause free radical formation that further causes damage to OLs. Excitotoxicity in secondary injury cascade also contributes to OL death. Besides, neutrophils and activated microglia release various products like inflammatory cytokines, glutamate, and free radicals that promote OL loss. Two or three weeks after SCI, OL apoptosis occurs due to the loss of axons that provide trophic support to OLs (Almad, Sahinkaya, and McTigue 2011). Myelination by OLs is crucial for saltatory conduction of action potential and growth factor support to retain axonal integrity. Acute demyelination during the post-injury period causes a functional deficit in neurons by retracting the conduction of action potential. Demyelinated axons are more susceptible to damage due to injuries, so efficient remyelination is critical for cellular replacement and for neuron-glial function recovery (N. Li and Leung 2015).

2.3.4 Myelin-associated inhibitors

During SCI or CNS injury various molecules in the myelin debris play an inhibitory role in OPC maturation and axon regeneration. Insufficient clearance of myelin debris by microglia and macrophages contributes to inadequate re-myelination. It has been proven that myelin debris is a potent inhibitor of injured spinal cord regeneration and re-myelination. Other OL molecules that inhibit regeneration are netrin, ephrin, and semaphorin 3A (Sema3A). Sema3A inhibits OPC maturation and recruitment in a demyelinating environment during injury. Nogo, Myelin associated glycoprotein

(MAG) and oligodendrocyte myelin glycoprotein (OMag) are the OL proteins that are identified as myelin-associated axon growth inhibitors which are the major inhibitory component in myelin debris (Lee and Zheng 2012).

Nogo protein has 3 isoforms, Nogo A, Nogo B, and NogoC. Among the three isoforms, Nogo A is the most studied one and is implicated with inhibition of neurite outgrowth and growth cone collapse. Nogo-A is found as an intracellular pool as well as cell membrane-bound in OL. However, cell membrane-localized Nogo A is well known for the inhibition of neurite growth and induction of growth cone collapse. NgR receptor along with co-receptors, LINGO-1, and $P75^{NTR}$ or TROY carry out the inhibitory functions of Nogo via various cellular signaling pathways (Schweigreiter and Bandtlow 2006). Other inhibitory proteins MAG and OMag also have a similar role in axon regeneration. These proteins also bind to the same receptor complex (NgR-p75NTR) as that of Nogo (Egawa et al. 2017).

2.3.5 Regeneration and re-myelination: Role OL in SCI

Oligodendrocytes are prone to cell death even after the moderate contusive injury. About 90% of the OLs occur at the injury epicenter in seven days after CNS injury. (McTigue et al., 2001). Axonal degeneration and loss of trophic support from axons lead to further apoptotic loss of OLs. However, OPC in the spinal cord undergoes OL differentiation and carry out remyelination spontaneously after SCI. It replaces the loss of OLs in SCI. Following an injury, some of the NPCs found in the spinal cord get activated, migrate to the site of injury, and differentiate into the glial lineage. But the major population formed from NPC-glial differentiation is astrocytes which contribute to the glial scar (Alizadeh, Dyck, and Karimi-Abdolrezaee 2015).

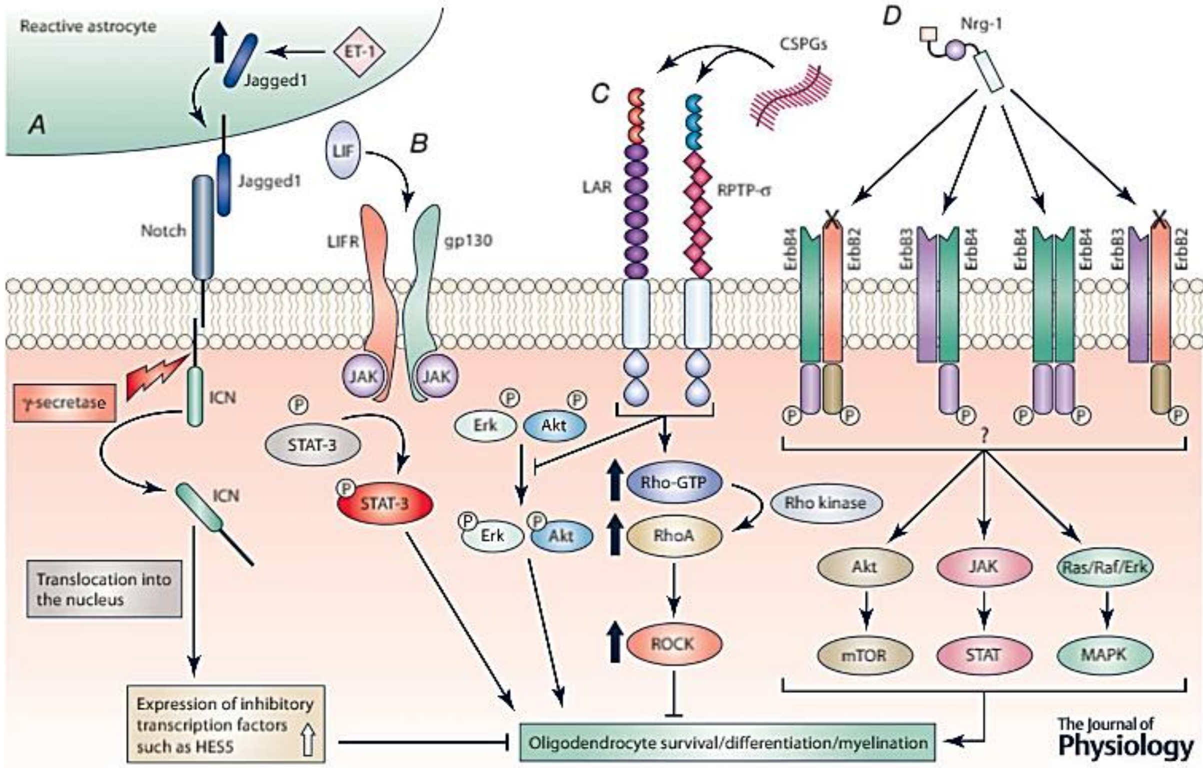
Growth factors like PDGF, bFGF, CNTF, LIF that promote OPCs proliferation and differentiation increase at sites of SCI. Also, the morphogen sonic hedgehog (shh) produced by astrocytes promotes OPC proliferation and survival (Frei et al. 2000). However, glial scar creates a barrier to migrating OPCs along with BMP signaling from astrocytes which inhibit OL formation. So practically in a CNS injury, all the OPCs that are activated may not terminally differentiate to OLs. Even though endogenous regeneration and remyelination are observed following SCI, it is usually

functionally and anatomically incomplete and insufficient to replace the deficits present after SCI (Watson and Yeung 2011).

2.3.6 Oligodendrocyte: Proliferation and survival signaling in SCI

Various extracellular and intracellular signals are involved in the regulation of NPCs and OPCs proliferation, differentiation, and myelination. It has been proven that a repertoire of signaling molecules and pathways initiated by reactive astroglia inhibits oligodendrocytes differentiation and myelination. Signaling pathways like Wnt, notch, and Rho/ROCK pathways are inhibitory to oligodendrocytes differentiation while pathways like PI3K/Akt and MAPK/Erk pathways are involved in the proliferation of neural progenitors, oligodendrocyte differentiation and survival (Alizadeh and Karimi-Abdolrezaee 2016).

Reactive astrocytes release endothelin-1 (ET-1), a soluble factor that increases astrocyte expression of notch signaling ligand Jagged1. It activates Notch signaling in OPCs. Notch signaling inhibits oligodendrocyte differentiation and remyelination. Another signaling pathway, STAT-3 promotes OPC differentiation and remyelination. LIF (Leukemia inhibitory factor) secreted by T-Lymphocytes activates STAT-3 signaling in OPC. CSPG causes activation of the Rho/ROCK pathway in both NPCs and OPCs and inhibit OL growth and myelination. CSPG also activate leukocyte common antigen-related receptor protein tyrosine phosphatases (LAR-RPTPs) pathway. They are cellular receptors of proteoglycans, heparan sulfate, and chondroitin sulfate which is important in axonal growth and regeneration. Neuregulin-1 is a neurotrophic factor involved in OPC differentiation and remyelination. The deficiency of neurotrophic factors arising during SCI is one of the hurdles to optimal re-myelination. Neuregulin-1 signaling activates various signaling pathways like Jak/STAT, Erk/MAPK, and PI3/Akt which plays a vital role in cell proliferation, differentiation, and survival. This results in OL development, survival, maturation, and myelination in the CNS. These molecules also have an additional role in neuronal migration and synapses formation. Level of neuregulin declines after SCI which has a detrimental effect on OL survival (Alizadeh and Karimi-Abdolrezaee 2016).



Various signaling mechanisms in OL maturation and proliferation (Alizadeh and Karimi-Abdolrezaee 2016).

2.3.7 Growth factor Signaling

Platelet-derived growth factor-A (PDGFA) and fibroblast growth factor-2 (FGF-2) are directly involved in the development of OLs. Upregulated expression of PDGFR-α is observed in OPCs in response to the mitogenic effect of PDGF-A. Canonical wnt pathway (Wnt/β-catenin) and phosphatidylinositol 3-kinase pathways are important in the response of PDGF-A signaling. They result in an increased proliferation rate of OPCs. Another signaling pathway, the ERK signaling pathway has a potential role in the recruitment of OPCs after SCI. Evidence suggests that this pathway gets activated through the action of PDGF-A stimuli. FGF-2 is another strong mitogen that enhances the generation of OPCs and promotes the differentiation of OPC to oligodendrocytes. Other growth factors like insulin-like growth factor (IGF) are also involved in OPC development. IGF has synergic actions with PDGF and FGF and they act mainly via PI3K/AKT and ERK pathways that are involved in this (N. Li and Leung 2015).

2.4 ECM: SCI regeneration

The CNS has a poor intrinsic regenerative potential, however, regeneration to some extend does occur. The growth-inhibitory microenvironment is created in CNS injury mainly by the ECM molecules secreted by reactive astroglial and other growth inhibitory molecules associated with the glial scar. Laminin, fibronectin, collagen, HA, tenascins, and CSPGs are the various ECM proteins that play an important role in CNS repair and plasticity.

Laminins are adhesive glycoproteins that are found in the basal laminae. They are growth-promoting molecules that enable neuronal migration and axonal pathfinding in CNS development. It has been demonstrated that the addition of Laminin can reverse the effect of other inhibitory signaling cues to growth cone development. Fibronectin is another protein like laminin that enables the interaction of cell surface receptors and ECM proteins. It has a central role in migration, morphogenesis, and proliferation of CNS cells. Collagen is the most abundant ECM protein present in CNS and the major collagen type present is collagen IV. It acts as a natural scaffold that integrates laminin and fibronectin proteins and forms the basement membrane which is a matrix of glycoproteins and sulfated proteoglycans. Conversely, during CNS injury, type I, III & IV collagen is the major fibrous element of scar tissue. Another major component of ECM is HA and it gives structure to the matrix while taking part in cell proliferation and morphogenesis. It also takes part in evoking the inflammation during CNS injury. CGPG is another CNS component that is upregulated in CNS injury. CSPG creates an inhibitory environment for endogenous regeneration. But during development CSPG is associated will embryonic cytokinesis and it is present in the regions of neural cell proliferation. Lecticans are the most abundant family of CSPGs found in CNS (Burnside and Brandbury, 2014).

ECM plays a vital role in maintaining the neural connections in CNS. The growth inhibitory molecules are present in ECM to restrict the synaptic plasticity so as to maintain the pre-formed synaptic connections. During the injury, re-activation of synaptic plasticity is needed and it is restricted by accumulation of ECM molecules. But, after an injury, there is a drastic change observed in the CNS ECM. Glial scar,

fibrolytic scar, upregulation of CSPGs are the major changes observed during CNS injury (Burnside and Brandbury 2014)

2.4.1 Role of fibrinogen in SCI

Fibrinogen, which is an acute-phase protein has diverse functional activities including its important role in the clotting cascade. Studies show that it is associated with a substantial pro-inflammatory role in SCI secondary injury cascade. Elevation in plasma fibrinogen concentration along with coagulation abnormalities is often observed in SCI. The Fibrinolytic pathway solves the issue of thrombotic activity that occurs soon after the SCI. Fibrinogen activates microglial cells during SCI and activated microglial cells are prominent pro-inflammatory participants in secondary injury. Fibrinogen also triggers astrocyte and its activation results in glial scar formation. The increased concentration of fibrinogen causes red blood cell aggregation which retard the circulatory flow. Moreover, fibrinogen aggregates white blood cells and platelets which causes a threat of coronary heart disease in SCI patients. It is evident that fibrinogen plays a critical role in SCI pathology, causing inflammation, coagulation, hemostasis, and cellular interactions. (Anwar, Al Shehabi, and Eid 2016). Fibrinogen activated inflammation has a positive effect as it helps in the clearance of tissue debris over time. Fibrin is also a potent inhibitor of neurite outgrowth and axonal regeneration in CNS injuries like SCI. Fibrin may trigger an inhibitory signal that controls the differentiation of OPCs to OLs and their myelination potential. *In vitro* studies show that fibrin inhibits the differentiation of OPCs to OLs without affecting the proliferation of OPCs. Fibrin acts via BMP signaling by up-regulating the BMP responsive genes. BMP signaling mediated by fibrin results in differentiation of OPCs to GFAP positive astrocytes (Norris and Strickland 2017)

2.4.2 OPC: replacement therapy

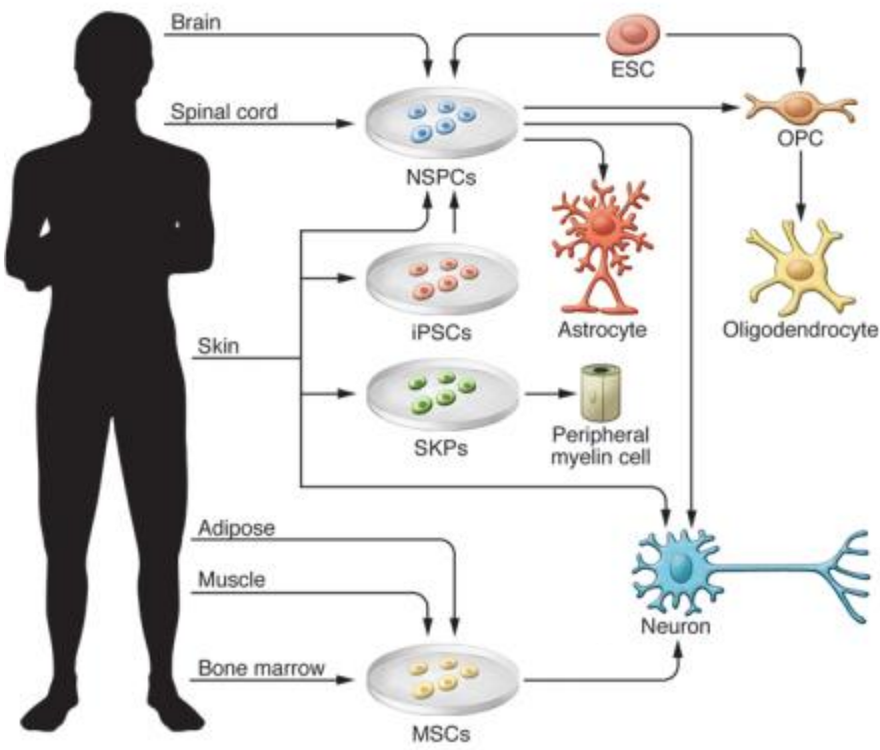
Demyelination in CNS injury contributes to the death of neurons and functional impairment. Replacement of the myelin-forming cells may be a potential strategy to overcome the de-myelination issue. However, oligodendrocytes could not be transplanted as it is a terminally differentiated. Terminally differentiated cells usually undergo cell death without proliferation. Transplantation of Olig2 and PDGFRα positive OPCs may help in re-myelination and restoration of neural circuits. Across

the world, various studies are being done to evaluate the potential of OPCs in resolving the issues related to demyelination in CNS injury. To find a suitable cell source from which OPCs could be generated is also important.

OPC transplantation is the integration of two strategies, to allow remyelination and provide trophic support to create a repair environment that promotes regeneration. The neurotrophic factors secreted by OPCs have the capability to promote neurite outgrowth *in vitro*. Various *in vivo* and *in vitro* study results strongly suggest the application of OPC therapy in human SCI(Watson and Yeung 2011a).

A study using OPC cell transplantation in an SCI rat model found that transplanted cells migrated to the site of injury and 40% of them differentiate into mature myelinating oligodendrocytes. Also, functional recovery was better in OPC treated group. Apart from myelination, OL helps in neuroprotection by releasing trophic factors including IGF-1, GDNF, and BDNF which promote neuronal survival and support synaptic plasticity in axons (Sun et al. 2013). Another group demonstrated improvement of BBB scoring in SCI rats with transplantation of OPCs. It clearly shows that OPC transplantation has the potential to improve motor recovery and relieve mechanical allodynia in rats (J. Yang et al. 2018). OPC transplantation for SCI in humans demands a reliable source of OPC without any potential harm to the patient.

Stem cells have the potential for multi-lineage differentiation and hence they can differentiate to any cell type or cell progenitor. With the use of appropriate trophic factors or cell signaling factors in appropriate concentration the stem cells can be differentiated into the neural lineage, specifically to OPC or NPC lineages.



Sources of stem cells for SCI transplantation (Mothe and Tator, 2012)

The NSPCs, iPSCs, SKPs, MSCs, ESC, and direct conversion methods yield neural cells for transplantation. NSPCs can be isolated from the fetal and adult brain & spinal cord. NPCs can be differentiated into OPCs, OL, astrocytes, or neurons based on the on-culture conditions and growth factor signals. ES cells can be induced to neural cells, and finally to OPC generation by providing appropriate culture conditions. MSCs are from various sources like BM, umbilical cord, adipose tissue, muscle, and dental pulp from deciduous baby teeth. MSCs have shown properties of neural cells *in vitro*. Skin fibroblasts can be reprogrammed into iPSC and are then directed along a neural lineage(Mothe and Tator 2012).

Various issues come as a hurdle to cell transplantation therapies. For transplantation of OPCs, any kind of stem cell has to be pre-differentiated *in vitro* before transplantation. Here comes the question of the purity of OPCs derived by *in vitro* differentiation. ESC and iPSCs are the most studied stem cell types. But the use of ESCs may cause teratoma formation if the whole number of transplanted cells is not differentiated into OPC lineage. The use of ESC has associated with various ethical

concerns also. iPSCs are genetically re-programmed cells and it is not clear whether it causes any potential threats in the long run. Allogenic and xenogenic transplants may also lead to various complications related to immunogenicity and immune rejection. Immunosuppression can be given to overcome immunogenicity related to allogenic graft but it is also associated with various side effects(Watson and Yeung 2011).

Autologous stem cell-derived transplants are best when considering allogenic and xenogenic cell transplantation. Among various stem cells available MSCs are a more reliable source of stem cells for cell transplantation. For autonomous cell transplantation, adipose-derived mesenchymal stem cells may be ideal. They can be isolated from adipose tissue or lipoaspirate of the patient itself. A large number of stem cells can also be isolated and subcultured from a small quantity of adipose tissue. The ADMSCs have been demonstrated to provide neuroprotective and neuroregenerative effects in an animal model of SCI. It also differentiated into neural and glial cells along with functional improvement in hind limb movements when transplanted in to rat SCI. ADMSCs have immunomodulatory potential which can reduce the secondary inflammations (Y.-C. Kim et al. 2016). Pre-differentiation of MSCs *in vitro* to OPCs is essential to ensure that these cells will differentiate to OL lineage. However, a percent of transplanted cells will be MSCs and the may help in regulating the microenvironment of SCI towards a regenerative environment. The MSC secretome contains various trophic factors that promote the proliferation and survival of cells.

2.4.3 Pre-differentiation of stem cells to OPCs

Stem cells can differentiate into cells of multiple lineages because of its potency. This multilineage potential enables any stem cell type to differentiate into a neural lineage or glial lineage. Mesenchymal cells are mesodermal in origin and they have the potential to differentiate into neural cells, which is of epidermal lineage. This mesodermal to epidermal switching is called lineage conversion. Various growth factors and signaling molecules are used for the *in vitro* pre-differentiation of stem cells into OPCs or cells of neural lineage.

Table I: Growth factors and signaling molecules used for pre-differentiation to OL lineage.

Stem cell type	**Differentiation method**
mESC derived embryoid bodies (P. Jiang, Selvaraj, and Deng 2010)	Retinoic acid and the Shh agonist Purmorphamine with medium containing 0.1 mM β-mercaptoethanol
miPSCs (Terzic et al. 2016)	β mercaptoethanol, 3,3',5-triiodo-l-thyronine sodium salt dibutyryl cAMP, GFs (BDNF, PDGF-AA)
Human umbilical cord-derived MSC (Leite et al. 2014)	GFs (EGF, bFGF PDGF-AA) Sonic hedgehog
hBMSC (Abbaszadeh et al. 2014)	GFs (EGF, bFGF, PDGF-AA) T3 Hormone

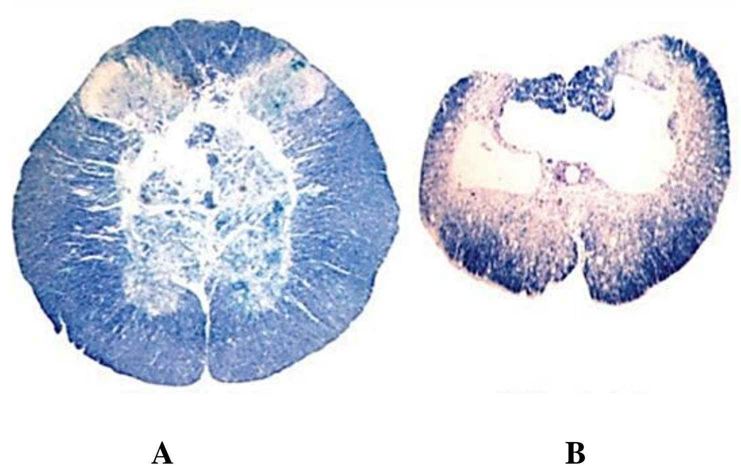
2.5 *In vivo* studies in SCI

For validation of any research for therapeutic purpose demands *in vivo* testing in a suitable animal model. In SCI, animal models are used extensively for studying mechanism underlying pathophysiology, developing effective treatments, and assessing therapeutic safety & efficacy. A variety of animal models have been developed using different animal species with different lesion methods at various locations of the spinal cord, varying the severity. Mice, rats, dogs, cats, and primates are some of the various species that are being used in research as SCI models. A considerable number of studies are conducted using rodent models, of which rat models are most common. Because in mice cells proliferate in the injury area which keeps the ends of the transected spinal cord in contact, with no cyst formation and high

regeneration potential. Completely transected spinal cord gets regenerated after SCI in mice. But in rats and humans, the responses to SCI are similar to a certain extent. They both show cyst formation and complete functional recovery is not possible after complete transection of the spinal cord (Kjell and Olson 2016).

Rats are friendly, inexpensive, and easy to handle and also have reduced surgical complications as well as post-surgery infections. There are well established functional recovery analysis techniques for rat SCI models. Contusion, compression, transection, distension, and chemical methods are the various techniques to induce SCI in animal models. Most popular among all these is the rodent contusion SCI model.

Contusion using a weight drop apparatus or impactor can cause a transient acute spinal cord injury model, which can be used to study various treatment modalities for acute SCI. This kind of injury is most representative of what happens in most human SCI.



A **B**

Rodent SCI contusion model: 1-hour post-injury (A), 60 days post-injury (B) (Ahuja, Martin, and Fehlings 2016)

The contusion model in the rat produces a necrosis pattern in the core region mainly central grey matter, which is surrounded by myelinated fibers and portions of grey matter from dorsal and ventral horns. The pathological and physiological changes are quite similar to human SCI pathophysiology with the cell loss occurs radially in all directions and the lesion grows in the course of time. In figure II only, a thin margin of white matter is visible after 6-0 days of contusion injury. A huge population of cells

in the central core undergoes cell death at the time of injury and further cell death occurs over weeks and months (Ahuja, Martin, and Fehlings 2016).

The contusion rat model at the thoracic level may be used to examine the consequences of the common type of SCI and test various therapies aiming at functional recovery and regeneration of the spinal cord. Functional outcomes of experimentation in these models can be analyzed by methods like behavioral testing, electrophysiology, neuroimaging, and histopathology.

2.5.1 Behavioural testing

Various behavioral tests mainly locomotor tests are conducted to assess the level of recovery in SCI animal models. Open-field behavioral tests are based on the locomotory functions of the proximal forelimb, hindlimb, and tail of the SCI rat. The most commonly used test in the past was Tarlov's open-field test. It is based on the hind limb movements and weight support. This test was sensitive only when the animal is able to show hind limb weight support. So, the test was modified as Tarlov's modified open-field test which is based on a scale of 0 to 6 which represents complete paralysis to normal locomotion. However, these tests were not sensitive enough and it was modified by including all hind limbs motor features. The BBB test developed by Basso, Beatie, and Bresnahan, is the most common test for locomotor function in SCI rats. It is also an open-field test with the grading of hind limb locomotion from 0 with no spontaneous locomotor activity to score 21 representing a normal movement with trunk stability, coordinated gait with parallel paw placement (Sedý et al. 2008).

Behavioral tests that test skeletal muscle functions that are not involved in locomotion are called motor tests. One of the motor tests is inclined plain test where the animal's ability to maintain its position on an inclined plane is assessed. Here strength of the animal is indirectly assessed. Other tests that examine the sensory function recovery in animals are sensory tests. Hot plate-based tests evaluate the thermal pain sensitivity by introducing the animal to a chamber with a hot plate as the floor. Paw licking and jumping are considered as the sensory pain response and the recovery is assessed based on the reaction time. The sensory-motor test is also there which assesses the motor functions along with sensory functions. Electrophysiology and imaging tests are also

available nowadays to assess the recovery and changes that have occurred in SCI animal models in response to injury or any tested treatment (Sedý et al. 2008).

2.6 Clinical trials in SCI

SCI is a devastating condition for which no treatment offers complete cure and functional recovery. There is no gold standard for SCI treatment. Various treatments including immunomodulatory drugs are given for the betterment of the patient's life. Worldwide researchers are developing various stem cell therapies and cell replacement therapies for SCI. All of these therapies are based on various strategies such as neuroprotection, immunomodulation, or cell replacement, or a combination of one or more strategies.

The ideal route of administration of cells, its homing as well as survival, *in vivo* response to transplanted cells in injury microenvironment, the ideal number of cells delivered, and delivery vehicle or matrices should be studied properly before any stem cell or cell replacement therapy.

Table: II: Stem cell therapy in SCI- clinical trials & outcome

Stem cell type	No: of patients	Time/route	Results
ESC derived OPC	5 transplanted	>2 weeks Intra spinal	No safety issue reported but complete results not published
Autologous BM-MNCs	10 (median age 32)	Chronic(3yrs) Intrathecal	No adverse effects, but patients only followed for 12 weeks; efficacy not reported
Autologous BM-MNCs	20 (aged 19–41)	10–30 days intravenous	No complications reported Chronic patients reported improvement.
Autologous MSCs (adipose-derived)	8 (aged 19–60)	>2 months Intravenous	No serious adverse events for 3 months
Autologous BMSCs	12 (aged 20–55)	Acute: >2weeks, Intrathecal	patients showed improvement in upper limb recovery with MRI and electrophysiological changes

2.7 ADMSCs in cell therapy

ADMSCs are highly proliferative adult multipotent stem cells with good self-renewal capability. They can differentiate into different cell types of various lineages, upon induction with inducers like growth factors.

Studies show that ADMSCs have several advantages over BM-MSCs which makes it highly beneficial for regenerative cell therapies. ADMSCs have a high proliferation rate than that of BM-MSCs. In addition, ADMSCs can be sub-cultured for long time periods without a decline in its proliferation when compared to other cells. ADMSCs shows high proliferation, multipotency, and maintains its phenotypic characters even after 25 passages (Zhu et al. 2008). The proliferation potential of ADMSCs is higher

in lower age groups and its found to be declining with age after 60 years(Lei et al. 2007).

2.7.1 In vivo ADMSC niche and their maintenance

Adult Stem cells are present in specialized niches in their specific tissues of origin. MSCs are present in the bone marrow and mesodermal tissue like adipose tissue, tendon, muscle. Recently MSC like colonies were derived from certain vital organs like liver, kidney, brain, etc. It's a question of debate whether all these MSCs have a common niche or they reside independently in the specific tissues.

Recent studies suggest that that the bone marrow-derived and dental pulp derived MSCs reside in perivascular niche throughout the body as they can get access to all tissues for homeostasis. However, any specific ECM components which maintain the stemness in MSCs in the niche are not yet identified. The role of cell adhesion proteins, cadherins, soluble factors in regulating the cell-cell adhesion, migration, and differentiation in the MSC niche is poorly understood (Kolf, Cho, and Tuan 2007). Identification of these factors may help in developing biomimetic *in vitro* niche which can promote the self-renewal or differentiation of MSC as required. The ADMSCs also have a specialized niche which is characterized by its interactions with surrounding cells, ECM, and soluble factors like cytokines in the adipose tissue (Meirelles, Caplan, and Nardi, 2008). It has been reported that the ADMSCs also reside in a perivascular niche similar to that of the BM-MSCs(Zannettino et al. 2008).

Regulating the self-renewal of stem cells is the major feature of its niche. The intrinsic behavior of the stem cell, the niche's microenvironment derived extrinsic cues and the niche architecture induces the stemness of the stem cells by regulating the division and specifying the type of division; asymmetrical or symmetrical. Self-renewal outside the niche is possible only when enough intrinsic factors are available (Fuchs, Tumbar, and Guasch 2004). Therefore, for *in vitro* differentiation or expansion of stem cells, growth factors or inducers need to be supplied exogenously to mimic the microenvironment of the niche.

2.7.2 ADMSC isolation and expansion in culture

The isolation of ADMSCs from adipose tissue was established by Rodbell and colleagues in the 1960s. The primary technique for ADMSC isolation was developed for the adipose tissue from the rat fat pad. The tissue was minced and then washed to remove the hematopoietic cells. Digestion of the tissue was done using collagenase enzyme. The digested tissue was centrifuged to get the stromal vascular fraction (SVF) as a pellet. The SVF is a heterogeneous cell population, consisting of blood cells like RBCs, fibroblasts, pericytes, and endothelial cells and adipocyte progenitors. Finally, after the seeding of SVF, the plastic adherent ADMSCs were selected during the subculture. This procedure has been modified for the isolation of human ADMSCs from adipose tissue specimens. The advances in the field of liposuction surgery made these steps simpler and now usually people rely on lipoaspirates for hADMSC based studies (Bunnell et al. 2008).

During the expansion of ADMSCs, increasing serum (fetal bovine serum, FBS) concentration may cause adipogenesis. The subculture of 80% confluent ADMSCs is usually done using the trypsin-EDTA method. The ADMSC proliferation is influenced by basal medium, glucose concentration, FBS quality, and percentage and cell seeding density (Ghiasi et al. 2016). The inadequate medium, contamination, or senescence of the primary cells induces granularity around the nucleus, cytoplasmic vacuolations, or detachment of the cells from the plastic surface.

ADMSCs can be stored for the long term without affecting its potency and proliferation. Slow freezing in 80% FBS,10% DMEM media and 10% cryoprotectant (DMSO) is an ideal way of storing MSCs (Bunnell et al. 2008).

2.7.3 ADMSC characterization

During the emergence of MSC based cell therapy research, various study groups put forward certain methodologies for the isolation and expansion of MSCs. There was a lack of universal criteria for comparing the MSCs and the results. As a solution to this issue, Mesenchymal and Tissue Stem Cell Committee of the ISCT proposed a set of standards for defining the human MSC for both *in vitro* and for pre-clinical studies. Those were adherence to plastic, specific surface antigen (Ag) expression, and

multipotent differentiation potential. Regarding the specific antigen expression, more than 95% of the MSCs should express CD105, CD73, and CD90 and these cells must lack the expression of CD45, CD34, CD14/ CD 11 b, CD79a/CD 19 and HLA class II. This analysis for cell surface markers is done using flow cytometry. CD45 is a leukocyte marker, CD34 is a hematopoietic marker, CD14 and CD11b are monocytes and macrophages marker, and CD79a and CD19 are B cells marker. The HLA-DR molecules are not expressed by MSCs without stimulation (Dominici et al. 2006). These markers are collectively called negative and positive MSC markers and various commercially available kits are there to analyze and sort the MSCs. Based on the cell surface antigens, the MSCs can be sorted to get 100% pure MSC culture using Fluorescent activated cell sorting (FACS) and magnetic-activated cell sorting (MACS). The final criterion for being an MSC is the test for multipotency. The cells must differentiate to adipocytes osteoblasts and chondroblasts in standard *in vitro* conditions. Commercially available media are available for the trilineage differentiation and specific stains are used to confirm the differentiation. The cell culture parameters like the glucose content, calcium concentration, media composition, and the presence of growth factor supplements have a profound effect on the maintenance of stemness in ADMSC (Irioda et al. 2016). The environmental parameters and the growth factor signaling in the milieu cause the differentiation or lineage commitment of ADMSCs.

2.7.4 Multipotency of ADMSCs

The ADMSCs are plastic, which means MSCs have the ability to cross the lineage barriers and adopt the phenotypical, biochemical, and functional properties of cells of other tissues. The *in vitro* differentiation of ADMSCs can be induced by the addition of specific growth factor cocktails or chemical inducers. Differentiation efficiency is age, sex, and patient dependent (Bunnell et al. 2008).

The MSCs tend to differentiate to cells of various lineages in the different germ layers in response to the signals they receive. The ADMSCs have the potential to differentiate to cells of mesenchymal lineages like bone, cartilage, fat, etc. Apart from this, various *in vitro* studies have shown the differentiation of ADMSCs to non-myogenic cell types like skeletal muscles, cardiomyocytes, and neural cells (Strem et al. 2005).

Currently, the differentiation of ADMSC using chemical inducers or biomolecule inducers to almost all lineages is known. However, the exact mechanisms involved in the differentiation are not completely delineated. Certain inducers like butylated hydroxyanisole, valproic acid, and insulin promote the neuronal differentiation of ADMSCs *in vitro*. Similarly, transferrin, IL-3, IL-6, VEGF promote cardiomyocyte differentiation and ascorbate, epidermal growth factor, basic fibroblast growth factor, hydrocortisone induce differentiation to endothelial lineage(Gimble Jeffrey M., Katz Adam J., and Bunnell Bruce A. 2007). The stability of the chemical-based inducers and the reversibility of lineage-committed cells need to be understood before adopting this for regenerative purposes. The EGF, FGF, and PDGF based neural differentiation was reported to be more reliable for providing long term effects up to 3 months, while the chemical inducers like DMSO and BME provided only transient effect (Tao, Rao, and Ma 2005). The growth factor-based differentiation protocol seems more attractive because they mainly rely on the signal mechanisms similar to that of embryonic development.

2.7.5 Mechanisms of ADMSC maintenance in culture

The molecular mechanisms playing behind the stemness of ADMSCs is poorly understood. No particular transcription factor is associated with MSC stemness, while a number of markers like NRF2, CD49f, BCL-6 co-repressor are found to be associated with the stemness and differentiation of MSCs (T. M. Liu 2017). However, Long term handling may cause a reduction in stemness and differentiation potencies of MSCs. To maintain the stemness various growth factors like FGF and PDGF are used *in vitro.* The FGF signaling pathway together with the ERK pathway plan an important role in maintaining MSC stemness. Canonical wnt signaling pathways and its cross-talk with TGF-β has also been shown to persuade MSC self-renewal (Arezoumand et al. 2017).

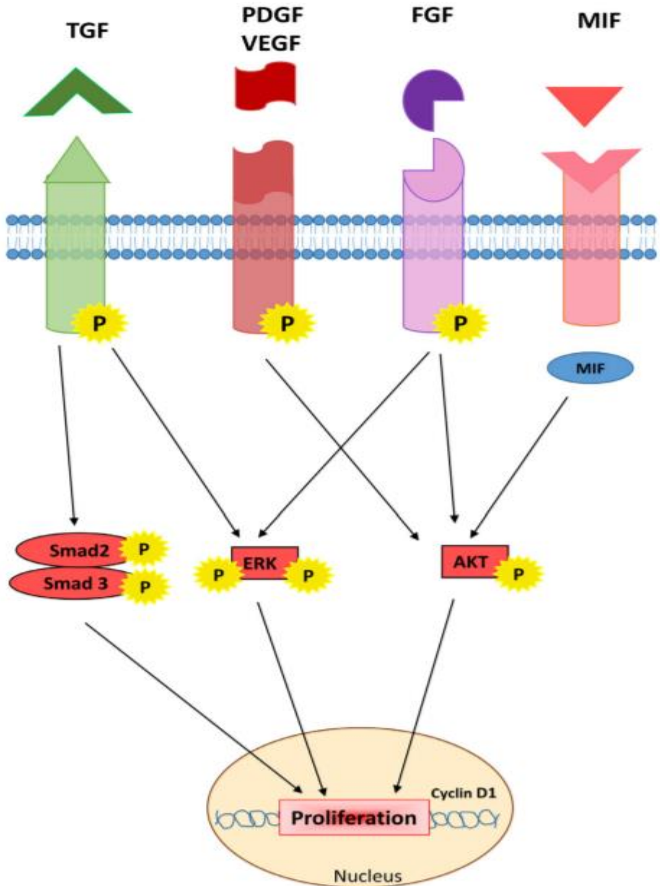
The hADMSCs in long term culture change its cell morphology, in tandem to a decrease in proliferation, and differentiation potential. The cultured ADMSCs has a doubling time of 2 days in 15th passage while the doubling time reduces to 4 days by passage 15. The autocrine FGF-2 signaling allows the maintenance of ADMSC self-renewal *in vitro*. The decrease in the FGF-2 release in cells of higher passage number causes an increase in doubling time. The FGF signaling has a crucial role in long term

maintenance of ADMSC *in vitro.* Exogenous FGF-2 can reverse the progressive reduction in the ADMSC proliferation (Zaragosi, Ailhaud, and Dani 2006).

Another key player in the maintenance of ADMSC in culture is Activin A. It is secreted by human undifferentiated ADMSCs which plays a vital role in maintaining the proliferation of cells *in vitro*. Autocrine and paracrine signaling of Activin A via C/EBPβ and Smad2 pathways promote the proliferation and hinders the differentiation of ADMSCs. Apart from Activin A another TGF-β family protein BMP4 regulates hADMSCs proliferation, and maintains its potency by autocrine signaling in a dose-dependent manner (Villageois et al. 2011). However, extensive studies need to be done to draw a complete picture of the mechanisms of ADMSC maintenance *in vitro.*

2.7.6. Signaling Mechanisms in ADMSC Proliferation in vitro

The TGF-β, PDGF, and bFGF signaling are key pathways that regulate MSC growth and proliferation *in vitro*. Inhibition of any of these pathways causes reduced proliferation of MSCs (Ng et al. 2008). The hedgehog protein also plays a key role in maintaining the proliferation of ADMSCs. The Mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase PI3K/ AKT, and Smad2/3 are the main signaling pathways are the downstream pathways that directly causes the proliferation of ADMSCs. These pathways are activated by the binding of the cytokines; VEGF, TGF, PDGF, FGF, and Macrophage migration inhibitory factor to their receptors (Fatemeh Atashi 2015).



Signaling pathways that regulate the ADMSC proliferation (Fatemeh Atashi 2015): the effect of the cytokines in the proliferation of patient-derived ADMSCs was established by culturing the cells with the patient's PRP.

2.7.7 *In vitro* differentiation of ADMSCs to neural lineage

ADMSCs are capable of trans-differentiation and they can differentiate into cells of ectodermal lineage upon induction. Evaluation of the neural cell fates of MSCs is difficult as the undifferentiated cells constitutively express certain neural specific markers. However significant upregulation in genes, as well as functionality, can be observed after stable neuronal differentiation (Phinney and Prockop 2009).

ADMSCs cultured at higher cell densities spontaneously form a clump of cells called neurospheres. NS is a heterogeneous population of cells of different sizes, granularity, metabolism, cytoplasmic content, and are in different phases of the cell cycle. They

also contain NSC (Bez et al. 2003). FGF-2, EGF, retinoic acid, and supplements such as B27 and N2 are used by almost all researchers to induce NS from stem cells. FGF-2 is the most significant component in neural differentiation and it promotes the proliferation of neural progenitor cells (Maio et al. 2011). The NS derived cells cultured in neurobasal medium supplemented with B27, 20 ng/ml bFGF and 20 ng/ml EGF for a week give rise to neural cells (Bunnell et al. 2008). Neuronal induction of NS derived cells using BDNF and RA induced 50% MSCs to neurons in another study (Anghileri et al. 2008). Growth factor induction of NS derived cells can give rise to oligodendrocytes and neurons which may be used for regenerative purposes in neural tissue.

2.7.8 ADMSC based therapies in SCI

Various *in vivo* studies have demonstrated the ability of ADMSC to promote the regeneration and repair in SCI due to its capacity to differentiate into neural cells as well as with its neuroprotective effects.

ADMSC is an ideal source for stem cell therapy for neuro-regeneration as it has been demonstrated to improve functional recovery in the number of SCI related studies. Transplantation of three-dimensional (3D) cell mass of ADMSCs and transplantation of neurogenin 2 overexpressed ADMSCs have shown to improve the functional recovery in SCI (Shi et al. 2017). It has also been reported that MSCs express neuronal and oligodendrocyte protein markers post-transplantation (Qu & Zhang, 2017). The transplantation of human ADMSCs in rat cervical spinal cord injury modified the structure of the glial scar and stimulated axonal sprouting (Kolar et al. 2014). The intravenous infusion of OPCs derived from rat ADMSCs provided a betterment in the motor function in spinal cord injury (SCI) rat models. These transplanted OPC cells survived, migrated, and differentiated at the injury site efficiently as well. The study reports a 30-35% efficiency in 4-5 weeks post-transplantation (S.-K. Kang et al. 2006). ADMSCs also have a protective effect on the induced neuronal cells when co-transplanted to the SCI site, thereby enabling a better survival of the transplanted cells (Oh et al. 2011).

MSCs when transplanted to the injury site produce various growth factors, neuroprotective factors and they reduce the inflammatory reaction by suppressing lymphocyte effects, by modulating the glial scar formation and by downregulating the Caspase-3 mechanisms(Qu and Zhang 2017). More insights into the mechanisms of ADMSC mediated neuroprotection, immune suppression and neuro-regeneration will be promising for developing an ADMSC based cell therapy for SCI.

CHAPTER 3

3. MATERIALS AND METHODS

This chapter focuses on the details of protocols used for the preparation of fibrin-based biomimetic niche, isolation of human/rat ADMSCs, and its differentiation to NPCs, OPCs, neurons & OLs. The methodologies used for the analysis of differentiation and for studying the role of the notch and wnt signaling in *in vitro* differentiation of ADMSCs to NS and OPCs are also included in this section. This section comprises the detailed protocols used for the creation and maintenance of SCI rat models. The dosage, administration, and tracking of ADMSC-derived progenitors used in this study are explained in detail.

3.1 Human ADMSC Isolation from lipoaspirate samples

Human lipoaspirate samples were collected from patients undergoing liposuctions approved by the Institutional Ethics Committee of KIMS hospital, Trivandrum, India. From each participant before adipose tissue collection informed consent was obtained (SCT/IC-SCR/44/ March 2017; IEC NO: SCT/IEC/1231/June 2018; IEC No: KIMS-IEC/18012018 dated 08-05-2018).

Lipoaspirate sample was washed with Hanks' Balanced Salt Solution (HBSS) to remove the blood content by centrifugation. Collagenase enzyme type 1 (Millipore, SCR 103, 4mg/ml working solution) was added to lipoaspirate in a ratio of 1: 4. Enzymatical digestion was achieved at 37° C for 10 minutes in a shaking incubator. The enzymatic action of collagenase was stopped by the addition of FBS containing media. The digested lipoaspirate was then diluted with HBSS and centrifuged at 500g for 5 minutes to obtain the stromal vascular fraction (SVF). The supernatant was discarded and SVF was seeded DMEM- Low Glucose (Gibco) with 10% Fetal Bovine Serum (FBS) (Gibco), and 1% Antibiotic antimycotic (AB/AM) solution (Invitrogen) on TCPS (Nunc). Cells were incubated in 5% CO2 at 37^0C. The cells were washed with AB/AM containing HBSS for the next two consecutive days to remove the tissue debris, fat molecules, RBCs, and dead cells. At 80%-90% confluence, the cells were passaged using standard trypsinization protocol.

3.2 ADMSC Characterization

3.2.1 Flowcytometric analysis

Miltenyi Biotech MSC phenotyping kit based on ISCT guidelines was used for analysis. Cocktails of fluorochrome-labeled antibodies were used for staining MSCs. One cocktail with MSC positive markers, CD73 (APC), CD90 (FITC), CD105 (PE)) and another with MSC negative markers, CD45, CD34, CD14, CD 20 (PerCP) was used to stain passage 3 ADMSCs. 10 minutes incubation at 4ºC was given. Fluorochrome labeled antibody in another cocktail was used as isotype control (PerCP, PE, APC, FITC).

The analysis was done using flow cytometer (Beckman Coulter, Germany). CytExpert software was used for data analysis.

3.2.2 Trilineage Differentiation

The StemPro Adipogenesis differentiation kit (Catalog no: A1007001, StemPro™ Adipogenesis Differentiation Kit, Life Technologies, USA), StemPro Chondrogenesis differentiation kit (Catalog no: A1007101, StemPro™ Chondrogenesis Differentiation Kit, Life Technologies, USA) and the StemPro Osteogenesis differentiation kit (catalog no: A1007201, StemPro™ Osteogenesis Differentiation Kit, Life Technologies, USA) were used to induce differentiation of MSCs into adipogenic, chondrogenic and osteogenic tissues, respectively according to the manufacturer's instructions.

Adipogenic Differentiation

Passage 3 (P3) hADMSCs were seeded at a density of 10,000 cells/cm^2 and cultured in low glucose DMEM, 10% FBS, and 1% AB/AM solution for 24 hours. The medium was then replaced with adipogenesis differentiation medium and the cells were incubated at 37°C with 5% CO_2. The differentiation medium was replenished every third day for an induction period of 21 days. After induction, the cells were fixed with 3.7% formaldehyde, rinsed with PBS, and then stained with specific stain Oil Red O staining to confirm differentiation.

Osteogenic Differentiation

P3 hADMSCswere seeded at a density of 10,000 cells/cm^2 in DMEM low glucose medium with, 10% FBS and 1% AB/AM. The medium was replaced with osteogenesis differentiation medium after 24 hrs. and was incubated at 37°C with 5% CO_2. Every third day the differentiation medium was replenished till an induction period of 21 days. Post induction, the cells were fixed with 3.7% formaldehyde, rinsed with PBS, and stained with specific stain Alizarin Red to confirm the differentiation.

Chondrogenic Differentiation

P3 hADMSCs were seeded at a density of 5,000 cells/cm^2 in basal growth medium for 24 hours. Chondrogenic differentiation medium was supplemented thereafter and the cells were incubated at 37°C with 5% CO_2. The medium change was done every third day for an induction period of 21 days. After induction, the cells were fixed with 3.7% formaldehyde, rinsed with PBS, and stained with specific stain Toluidine blue to confirm the differentiation.

3.3 Preparation of fibrin matrix coated TCPS for induction studies

Fibrin matrix-based biomimetic niche used for differentiation and signaling studies was prepared using a modified, well-established protocol (Sreerekha, Divya, and Krishnan 2006). Pharmacopoeia grade fibrin sealant (Drug controller approved) was used for coating tissue culture polystyrene (TCPS, NUNC, Roskilder, Denmark).

Fibrinogen and thrombin were reconstituted using sterile distilled water. Thrombin at a concentration of 5IU was poured on to TCPS. It was incubated for 30 minutes. Thrombin was completely poured off after the incubation period. The thrombin adsorbed surface was layered with a thin layer of diluted fibrinogen (2 mg/ml). Then the clot formed was stabilized by incubating the coated TCPS at 37°C for 30 minutes. The plates were frozen overnight at -80°C. Plates were lyophilized (Modulyo 4K, Edwards, UK) and stored at 4°C in a sterile environment.

3.4. Induction of OL from ADMSC

As a part of objective 1 of the study, the differentiation of ADMSC to Neurospheres was performed. This NS derived cells were induced to form NPCs and OPCs. NPCs

were further differentiated into neurons and OPCs into OLs. A comparative study was done between the bare TCPS and fibrin niche to evaluate the effect of fibrin in the induction of NS, OPCs, and OLGs. OPCs and neurons were cocultured to study the effect of neurons on the terminal differentiation of OPCs in the niche.

3.4.1 Neurosphere induction

The ADMSCs were plated on Fibrin coated TCPS and bare TCPS at a density of 10,000 cells/cm2 in (DMEM/F12 medium, with 10% fbs and 1% AB/AM). After 24 hours the basal media (with 1% FBS) supplemented with AB/AM, 20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF) was added to the cells induced on bare (INB) & induced on a niche (INF) (Abbaszadeh et al., 2014). 10% FBS and AB/AM supplemented basal media were added to the control group (un-induced ADMSC on fibrin). The medium was changed every 4th day. Cells were maintained for 7 days and culture was terminated for further analysis using immunocytochemistry (ICC), real-time PCR (qRT-PCR), and flow cytometry.

3.4.2 Reseeding of NS and Secondary NS formation assay

Reseeding after manual picking of NS: The well-developed NS in fibrin niche were manually picked using a pipette tip (10µl) and reseeded into bare TCPS and fibrin coated TCPS. The basal media, DMEM F12 supplemented with 1% FBS and AB/AM was provided for 7 days. The culture was terminated for further analysis of molecular markers.

Reseeding after enzymatic digestion of NS: The NS induced in fibrin niche were dissociated through mild trypsin treatment (4 min, 0.25% trypsin-EDTA, Invitrogen) by day 7. These NS derived cells were seeded on to bare TCPS and fibrin matrix supplemented with the growth factors (10ng/ml of EGF and bFGF). The basal media, DMEM F12 supplemented with 1% FBS and AB/AM was provided for 5 -7 days. Another set of NS derived cells were seeded on to bare TCPS and Fibrin matrix without GF and was provided basal media, DMEM F12 supplemented with 1% FBS and AB/AM for 7 days.

3.4.3 Neural progenitor and Glial progenitor induction

Adherent NS formed from ADMSCs, cultured in NS induction media till 7th day were harvested and plated on fibrin coated and bare TCPS in DMEM/F12 basal medium

supplemented with AB/AM. The seeding density was 5000 cells/cm^2. The next day the medium was supplemented with 10 ng/ml EGF and 10 ng/ml bFGF for NPC proliferation. The cells were maintained for another 4 days for the proliferation of NPCs

The cells were further supplemented with 10ng/ml PDGF-AA and bFGF in basal medium (1% FBS) for OPC induction. (Modified protocol published by Abbaszadeh et al., 2014) . The culture was terminated in another 4 days to evaluate the differentiation using ICC, qRT-PCR, and flow cytometry.

3.4.4 Neuronal induction

For neuronal induction, induced NPC culture was provided with the addition of 20ng of bFGF alone for 4 days and 25mM final concentration, KCL for 2 days (Modified protocol Tara and Krishnan, 2015) in DMEM F12 basic media with 1% FBS. Control group, un-induced ADMSC on bare TCPS was maintained in basal media with 10% FBS & AB/AM. Cells were maintained for 4 days and culture was terminated for further analysis using RT-PCR and ICC.

3.4.5 Terminal differentiation of OPC

Induced OPCs were further induced to OL lineage with tri-iodothyronine (T3) hormone (20ng/ml) with growth factor removal for 72 hours on bare TCPS and fibrin coated TCPS. The control group used was, ADMSC grown on bare TCPS using basal DMEM f12 media with 10% FBS and AB/AM. The culture was terminated for further analysis after 48 hours using ICC, qRT-PCR, and flow cytometry.

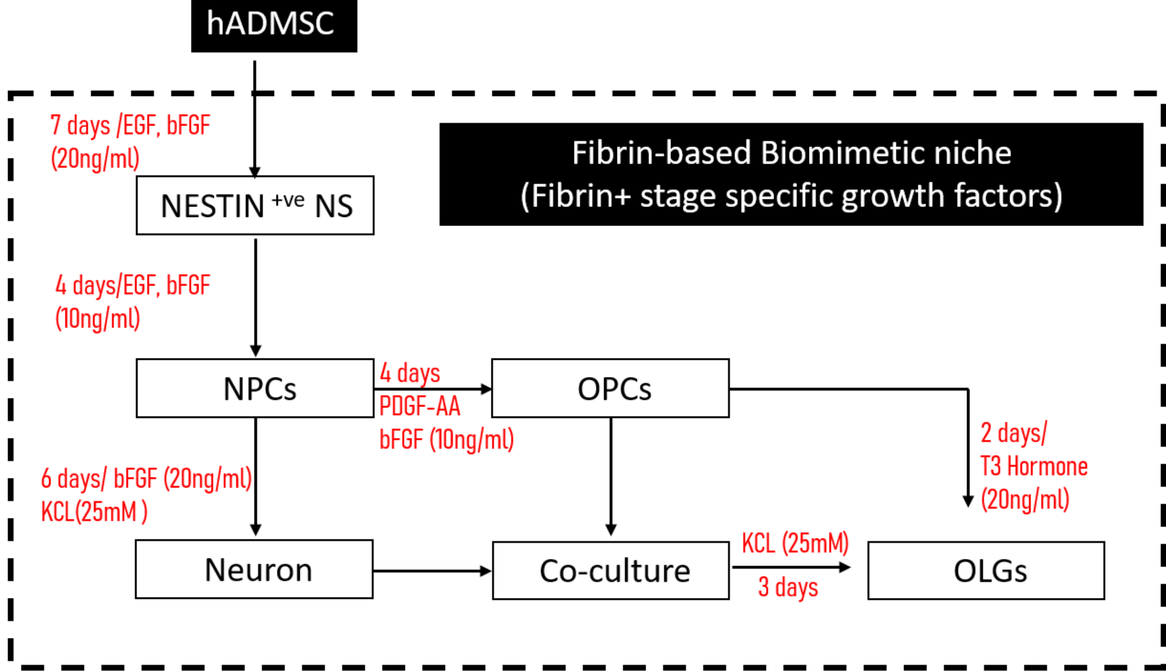
3.4.6 Co-culture of induced neurons and OPCs

The NPCs were induced to OPCs and neurons separately in fibrin niche with seeding density 5000 cells/cm^2 using previously described protocol (Section 3.4.3 and 3.4.4) The NPCs induced to neural cells in fibrin niche were maintained in KCl medium. The Induced oligodendrocyte progenitors were trypsinized and seeded onto the induced neural cells at a density of 5000 cells/cm^2. The cells were maintained for 48 h in KCl (25mM)- DMEM F12 medium. ADMSC grown in basal DMEM F12 medium with 10% FBS on bare TCPS was used as control.

In vitro differentiation of ADMSC into NPCs, OPCs, and OLGs on a fibrin-based bio-mimetic niche was confirmed by analyzing the expression of various lineage-specific

markers at genetic and protein levels using molecular methods real-time RT-PCR and ICC. During different levels of differentiation, the cell morphology was periodically analyzed using phase-contrast microscopy (DMIRB, Leica Microsystems, Wetzlar, Germany).

Flow chart depicting the *in vitro* differentiation protocols for transforming hADMSCs to different neural lineage cells.



3.5 Signalling study

3.5.1 MTT Assay

The human ADMSCs grown on fibrin niche at a seeding density of 5000 cells /cm ^{2} were used for MTT assay. The inhibitors were added to the ADMSCs for 48 hrs. The Wnt inhibitor and notch inhibitor was added at a concentration of 40µg/ml and 20 µg/ml respectively. The cells were washed with sterile HBSS and the MTT reagent (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma) was added to the culture and was incubated for 2 h. MTT lysis buffer, DMSO (100 µL) was added and the incubation continued for 4 h more. The suspension was transferred to a 95 well plate and the absorbance was measured at 570 nm. The cytotoxicity or cell proliferation rate

was calculated using the formulae: Absorbance of Test X Absorbance of Control/100 (Aravind and Krishnan 2016).

3.5.2 Effect of notch & wnt inhibitor on Neurosphere induction

The ADMSCs were plated on Fibrin coated TCPS and bare TCPS at a density of 10,000 cells/cm² in (DMEM/F12 medium, with 10% FBS and 1% AB/AM). After 24 hours the basal media (with 1% FBS, AB/AM) supplemented with 20 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, and either one of the inhibitors. Two concentration of inhibitors were used, 20 µM/ml (LC); 40 µM/ml (HC) wnt inhibitor (PNU 74654, Tocris) and 10 µM/ml (LC); 20 µM/ml (HC) notch inhibitor (DAPT, Abcam). Induction media (20 ng/ml bFGF & EGF, 1% FBS, AB/AM) was added to cells in fibrin coated TCPS (niche only). In cells seeded on bare TCPS, 10% FBS supplemented DMEM f12 medium was added (Control group). The medium was changed every 4th day.

3.5.3 Effect of notch & wnt inhibitor on OPC induction

The ADMSCs were plated on Fibrin coated TCPS and bare TCPS at a density of 10,000 cells/cm² in basal media (DMEM/F12 medium, with 10%fbs and 1% AB/AM). These cells seeded on fibrin coated TCPS were induced to form adherent NS using the previously described protocol (#3.4.1). The cells seeded on bare TCPS were the control groups used in the study. After the 7th day, the induced NS were trypsinized, dissociated, and seeded on to fibrin coated TCPS at a seeding density of 5000 cells/cm² in basal media. After 24 hours the basal media (with 1% FBS, AB/AM) supplemented with 10 ng/ml bFGF, 10 ng/ml EGF was added in the fibrin coated TCPS. Control group cells were reseeded on bare TCPS and supplemented with 10% FBS, AB/AM basal media.

After 3 days induced cells on fibrin coated TCPS were supplemented with 10 ng/ml bFGF, 20 ng/ml PDGF-AA, and either one the inhibitors. Two concentration of inhibitors were used, 20 µM/ml (LC) and 40 µM/ml wnt inhibitor (HC) or 10 µM/ml (LC) and 20 µM/ml (HC) notch inhibitor (DAPT). In cells seeded on bare TCPS, 10% FBS, AB/AM supplemented DMEM f12 medium was added (Control group). The

medium was changed every 4th day. The culture was terminated for further analysis on day 4.

3.6 Analysis of differentiation

In vitro differentiation of ADMSC into OPCs and OLs on the fibrin-based bio-mimetic niche was confirmed by analyzing the expression of various lineage-specific markers at genetic and protein levels using various molecular methods.

During different levels of differentiation, the cell morphology was periodically analyzed using phase-contrast microscopy (DMIRB, Leica Microsystems, Wetzlar, Germany). After confirming the lineage conversion and differentiation of induced cells by morphological analysis further characterizations were performed.

3.6.1 RNA Isolation and cDNA preparation

Following NS induction, OPC induction, and OL induction, gene expression of stage-specific molecular markers was estimated.

The total RNA from cells were collected using TRIZOL reagent (Invitrogen, USA) based on the manufacturer's protocol. RNA quantification and purity analysis were done using spectrophotometry in a Nanodrop equipment (ND 2000; Thermo Scientific, USA). After quantification, 200ng of total RNA was converted to cDNA using the OrionX cDNA kit (Origin, India) in a thermal cycler (Master cycler; Eppendorf). Variation in gene expression was analyzed in fold change using stage-specific primers by qRT-PCR.

3.6.2 Real-time PCR (qPCR)

Stage-specific gene expression of the induced cells after the induction time period was analyzed using real-time PCR.

Real-time PCR was carried out in forty cycles of reaction using the Bio-Rad iQ5 (Reaction mix: 20ng cDNA, 100 pmol each of specific forward and reverse primers,7.5 µl of OrionX 2X Real-time PCR master mix (Origin, India)). GAPDH was used as a house-keeping gene.

Reaction Steps: Enzyme activation: 95°C for 15 minutes; Denaturation: 95°C for 30 seconds; Annealing: 50°C for 20 seconds; Extension: 72°C for 20 seconds.

The melt curve was analyzed for single peaks to confirm the specificity of the polymerase reaction. Product size and specificity were analyzed by agarose gel electrophoresis. The variation in gene expression was calculated as fold change after normalization with GAPDH expression using the formula $2^{-\Delta\Delta Ct}$

Table: III Primer sequences for human genes

Sequence	**Sense Primer**	**Anti-sense Primer**
GAPDH	GCTTGTCATCAATGGAAATCCC	TCCACACCCATGACGAACATG
GFAP	ACATCGAGATCGCCACCTACA	GTCTGCACGGGAATGGTGAT
β-111 TUB	GCTCAGGGGCCTTTGGACATCTCTT	TTTTCACACTCCTTCCGCACCACATC
Olig 2	CCCTGTCTCTCGTTGATTT	GTGGTTCTACTCTGAATGTCT
PDGFRα	AGGTTGAGAGGAGGACTT	CCACTGAGATGCTACTGAG
OSP	ACTGCTGCTGACTGTTCTTC	GTAGAACGGTTTTCACCAA
MBP	GATGGCGTCACAGAAGAG	CCGATGGAGTCAAGGATG
Ng2	AAGAGATGATGGTGGCATATC	CAAGTCCTTCGGCGTTAA
Axin 2	AGTCAGCAGAGGGACAGGAA	AGCTCTGAGCCTTCAGCATC
LEF	GACGAGATGATCCCCTTCAA	AGGGCTCCTGAGAGGTTTGT
Cyc D1	ACCTGGATGCTGGAGGTCT	GCTCCATTTGCAGCAGCTC
PCNA	GGCCGAAGATAACGCGGATAC	GGCATATACGTGCAAATTCACCA
Hes 1	CGTCTACACCAGCAACAG	TCCTCTTCTCTCCCAGTATTC
Hes 5	ATCCTGGAGATGGCTGTC	CTTCGCTGTAGTCCTGGT
Mash 1	AAGATGAGTAAGGTGGAGACA	GTTCAAGTCGTTGGAGTAGTT
TCF 7	TGATGCTAGGTTCTGGTGTA	CTTCTTGATGGTTGGCTTCT
TCF7L2	CCAGACTACTCCGTTCCT	AACACCAACAACAACAAGAAG

Table: IV Primer sequences for rat genes

Sequence	Sense Primer	Anti-sense Primer
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
OLIG 2	GGTGGCTTCAAGTCATCT	TCAGTCATCTGCTTCTTATCTT
PDGFRα	GCCTTACGACTCCAGATG	CTTCACAGCCACCTTCAT
OSP	GCGTTCCATTGTTGTTGAT	TCTCTACGAGGCTTCCAT
MBP	GCATCTTGTTAATCCGTTCTAAT	TCTGGAGGGTTTGTTTCTG

3.6.3 Immunocytochemistry (ICC)

The ICC was done to evaluate the stage-specific marker expression in the induced cells. Specific markers for each type of induced cell is selected and assessed for their expression at specific cell compartments.

The cells were fixed using 3.7% formaldehyde for 20 minutes. Then washed with PBS and permeated using 0.1 % Triton-X for 5 minutes for all nuclear and cytoplasmic proteins. For reducing non-specific binding, 3% BSA in PBST for 30 minutes was used for blocking. Then cells were incubated overnight at 4ºC with the standardized dilution of primary antibodies (Table V). Following the incubation, cells were washed with PBS and further stained with specific secondary antibodies for 1-hour (Table VI).

Then the cells were washed thrice with PBS for 5 minutes and were counterstained with DAPI followed by 3 washes, 5 minutes each using PBS.

The nestin antibody used was primarily conjugated to FITC. For immunostaining, conjugated primary antibody, overnight incubation at 4ºC was given followed by DAPI staining and washing.

For colocalization studies, both the primary antibodies (PCNA and TUJ 1) were added together and incubated overnight at 4ºC. This was followed by secondary antibody incubation for 1h.

For all other markers, primary antibody incubation was given for overnight at 4ºC and Secondary antibody incubation was given for 1 h at room temperature.

Reagents used:

Antibodies against Nestin (1:100), synaptophysin (1:100) & O4 (1:200) : R& D systems, Minnepolis, USA; GFAP: BD Biosciences; Olig 2 (1:1000), PDGFRα (1:1000), OSP (1:1000), MBP (1:500),PCNA (1:500), Beta catenin (1:1000), Anti-notch 1 (1:500) , Anti- Hes 1 (1:1000) , Anti-wnt 3a (1:500), TUJ 1(1:500) **:** Abcam, UK; Nuclear stain: 4,6-diamidino-2-phenylindole (DAPI; 1:5000), Invitrogen USA.

Table V: **List of Primary Antibodies used for ICC and FACs**

Antibody	**Isotype**	**Details**
Nestin (1:100; (FITC conjugated)	Mouse IgG	R&D systems, Minnepolis, USA
PCNA (1:500)	Rabbit IgG	Abcam, UK
TUJ 1(1:500)	Rabbit IgG	Abcam, UK
Beta-catenin (1:1000)	Rabbit IgG	Abcam, UK
Anti- notch 1 (1:500)	Rabbit IgG	Abcam, UK
Anti-wnt 3a (1:500)	Rabbit IgG	Abcam, UK
Hes 1 (1:1000)	Rabbit IgG	Abcam, UK
Olig 2 (1:1000)	Rabbit IgG	Abcam, UK
PDGFRα (1:1000)	Rabbit IgG	Abcam, UK
O4 (1:200)	Mouse IgM	R& D systems, Minnepolis, USA
Synaptophysin (1:100)	Mouse IgG	R&D systems, Minnepolis, USA
TH (1:500)	Rabbit IgG	Abcam, UK
NGFR (1:500)	Rabbit IgG	Abcam, UK
MBP (1:500)	Mouse IgG	Abcam, UK
OSP (1:1000)	Mouse IgG	Abcam, UK
GFAP (1:500)	Mouse IgG	BD Biosciences, India
MAP-2 (1:100)	Mouse IgG	Santa Cruz, Heidelberg, Germany
Nuclear stain: 4,6-diamidino-2-phenylindole (DAPI; 1:5000)		Invitrogen, USA.

Table VI: List of Secondary antibodies used for ICC and Flow cytometry

Antibody	Isotype	Catalog Number
Anti-mouse Alexa Fluor 488 (1:1000)	Goat IgG	Abcam ab150113
Anti-rabbit Alexa Fluor 488 (1:1000)	Goat IgG	Abcam ab150077
Anti-mouse Alexa Fluor 488 (1:1000)	Goat IgM	Abcam ab150121
Anti-mouse Texas Read (1:2000)	Goat IgG	Abcam ab6787

The fluorescence was observed using a fluorescent microscope (Leica microsystems, DMIRB, Germany). Sequential separate images were taken and the overlay was created (LAS image overlay system, Leica, DMIRB). Leica microsystems Blue filter (Band pass 450-490) was used for Alexa fluor 488 and Green filter (Band pass BP 545/30) was used for Texas red conjugated antibodies. For DAPI, UV filter (Band pass340-380) was used.

The image J software (NIH) was used to measure the area, integrated density and mean gray values of selected cells in the ICC images (40X magnification; (MAP 2, Syn & NGFR of induced neurons; TH & OSP in co-culture). The cells of interest were marked using the drawing tool and the area, integrated density, and mean gray value was measured. Corresponding background values were also measured. The fluorescence measure of cells was calculated as corrected total cell fluorescence (CTCF) using the equation, CTCF = Integrated density – (Area of the selected cell X Mean fluorescence of background readings) and represented as histograms. Average CTCF of 15 cells from 5 or more fields were used for accuracy.

3.6.4 Flow cytometry

The cells positive for the stage-specific marker was evaluated using flow cytometry. The cells were fixed using 3.7% formaldehyde for 20 minutes. The fixed cells were washed with PBS. For all the nuclear and cytoplasmic proteins, the cells were

permeated using 0.1 % Triton-X for 5 minutes. The cells were incubated with 3% BSA in PBS for 30 minutes was used for blocking the non-specific binding. The cells were then incubated with the particular dilution of primary antibodies overnight at 4^0C. Following the incubation, cells were washed with PBS and further stained with specific secondary antibodies for 1-hour incubation at room temperature. The cells were then washed with PBS by centrifugation at 300g for 6 minutes. The cells were resuspended with the 500 µl of PBS.

Unstained cells were used for gating the cells in the flow cytometer (Cytoflex, Beckman Coulter, Germany). For primary antibodies conjugated with fluorochrome, unstained was used as the control. And for the secondary antibodies conjugated, cells incubated with secondary antibody alone used as the control. The data was analyzed using FlowJo and CytExpert software.

3.7 *In vivo* cell transplantation studies in SCI rat model

One of the major objectives of the experiment was to develop NPCs and OPCs from adipose-derived mesenchymal stem cells for transplantation in rat SCI. The major problem associated with cell transplantation is that they do not survive due to a lack of appropriate niche at the injured spinal cord. Therefore, based on a preliminary transplantation study with the circulating blood-derived NPC that has been already conducted in the laboratory, which yielded very promising results, biological matrix, fibrin was used as a niche in *in vivo* experiments. The cell survival at the transplanted site was found to be significantly higher in tests, cells implanted with fibrin as compared to control, cells implanted in the medium in the previous study (patent filed:201641015636, Tara and Krishnan, 2016.). However, the previous study was terminated in 1 week and the current objective is to test the long-term efficacy (28 days) of transplantation of NPCs and OPCs in a standardized niche for better survival and proliferation at the transplantation site.

In this study, ADMSCs were isolated from adipose tissue of inbred rats and were expanded. These cells were transplanted to the SCI model which has already been standardized in-house. Being inbred, immune reactions may be eliminated even

though allogenous transplantation was performed. Spinal cord contusion injuries were made using an in-house built impactor and transplantation was carried out.

3.7.1 Rat ADMSC isolation

Rat ADMSCs were isolated, sub-cultured, and differentiated into OPCs on fibrin matrix for transplantation in SCI rat models. *In vivo* study was aimed to verify the effect of OPCs, NPCs, and fibrinogen in SCI.

Rat adipose tissues (Approximately 5gm) were collected from Wistar rats approved by the Institutional Animal Ethics Committee (IAEC) of Sree Chitra Tirunal Institute for Medical Sciences & Technology (SCT/IAEC-220/MARCH/2017/9). The isolation of mesenchymal stem cells from rat Adipose tissue was performed using a modified protocol published by Yanxia Zhu et al (2008).

The adipose tissue was washed 2 to 3 times with HBSS to remove the blood content and was enzymatically digested at 37° C for 20 minutes in the shaking incubator using Collagenase NB 4. The enzymatic action of collagenase was stopped by the addition of 10% FBS containing medium. The suspension was filtered through a 70 µm cell strainer and the filtrate was centrifuged at 300g for 10 minutes.

The stromal vascular fraction (SVF) obtained was resuspended in medium consisting DMEM- Low Glucose with 10% FBS and 1% AB/AM. The cells were seeded into TCPS and incubated at 37° C with 5% CO_2. The cells were washed with 1% AB/AM added HBSS for the next two to three consecutive days to remove the tissue debris, fat molecules, RBCs, and dead cells. At 80%-90% confluence, the cells were passaged using standard trypsinization protocol.

3.7.2 Rat ADMSC induction to neural progenitors

The protocol used in human NS induction, OPC induction, and OL induction protocols were used for rat ADMSC induction to NS and its further induction to OPCs and OLs (#3.4).

Neurosphere Colony count

Rat ADMSCs were seeded on Four well plates (TCPS; NUNC, Roskilder, Denmark) at a seeding density of 10,000 cells/cm². Cells were induced to form adherent and non-

adherent NS using NS induction protocol (EGF and bFGF: 20ng/ml in basal media with 1% FBS, supplemented with AB/AM)

Phase-contrast/Fluorescent microscope (LAS, Leica, Germany) was used for NS imaging and counting. Low power lens (4X) was used to manually count the total number of NS formed per well.

Analysis of differentiation was done using ICC, Flow cytometry, and qRT-PCR as per the protocols #3.6

3.7.3 Allocation of animals

All experiments were conducted as per the guidelines issued by the CPCSEA committee and approved by the Institutional Animal Ethics Committee (IAEC). Twenty-six adult female inbred strains of Wistar rats of body weight: 150-180g each were used for the experiments. The animals were obtained from the Division of Laboratory Animal Sciences, SCTIMST, India. The animals were kept under standardized animal care conditions. The experimental animals were provided with enough food and water throughout the study. The animals were assigned randomly to one of the following eight groups before surgery with a minimum of 3 animals in each group.

Table VII : Allocation of animals for *in vivo* studies

Group I	SCI Control	Untreated (saline injection)
Group II	SCI Control	fibrin matrix only
Group III	SCI Control	Media only
Group IV	Test 1	OPCs transplanted in the fibrin matrix
Group V	Test 2	OPCs transplanted in media.
Group VI	Test 3	NPCs transplanted in the fibrin matrix
Group VII	Test 4	NPCs transplanted in media.
Group VIII	Test 5	OPCs+ NPCs in fibrin matrix

3.7.4 Labelling of cells for transplantation and tracking

Passage 3, rat ADMSCs were induced to NPCs and OPCs using *in vitro* differentiation protocols in fibrin coated TCPS. Induced NPCs and OPCs were harvested on 4th day after induction. Cells were labeled with fluorescent dye PKH26 (Sigma, USA) as per manufacturer's instructions. The PKH26 is a membrane intercalating cell tracker dye with red fluorescence.

The induced cells were trypsinized and washed in sterile HBSS. The cells were then mixed with 0.5 ml of the diluent supplied with the kit (diluent). The dye solution was also added to 0.5 ml of the diluent. Both were mixed and incubated for 2.5min at room temperature in dark with periodic mixing. An equal volume of FBS was added to the cell suspension mixed with dye for 1min, to bind the excess dye. The cell suspension was centrifuged at 400g for 10min. Then the cells were washed with DMEM F12 containing 10% serum-containing medium to remove the unbound dye.

3.7.5 SCI: Surgery, Transplantation, and Post-operative care

The animals were prepared by the removal of fur and disinfection done by wiping the Povidone-iodine solution. Under deep surgical plane of anesthesia, with Xylazine (5mg/kg body weight) and Ketamine (50-70 mg/kg body weight), dorsal hemi-laminectomy of thoracic vertebrae was performed to expose the spinal cord. Extra care was taken to maintain an aseptic condition. A contusion injury of (140kdyne) was made on the thoracic region (T10) using an in-house-built Impactor. Cells were transplanted soon after the injury, to the injury site using a microliter syringe. 10^6 cells in 25 microliter cell delivery vehicle (media or fibrin) was injected to the site of injury.

SCI animals developed paraplegia and soft bedding material (paper pulp based) was supplied to avoid laceration and abrasions of ventral skin. Preventive analgesic was given once daily to manage the pain for five days post-surgery. Antibiotic injections were also given for five post-operative days once daily. The wound site was smeared with Povidone-iodine ointment (Betadine) daily until the sutures are removed on the 7th post-operative day. Manual evacuation of urinary bladder was done in animals with

retention of urine in initial post-operative phases. Animals were fed with ad libitum feed and water. The animals were maintained for a period of 28 days.

The animal weight was noted and the BBB score was taken every 7th day for 28 days. The animal was allowed to move freely on a flat surface to evaluate the hind limb movements and the BBB score.

3.7.6 Tracking of transplanted cells

The animals were sacrificed and the injured spinal cord was collected 28 days, post-surgery. The tissue sections were washed with PBS and cell tracking was done using IVIS Spectrum Preclinical *in vivo* imaging system, Perkin Elmer, USA.

The excitation wavelength used for the imaging was 535nm and the emissions were measured at 580nm. The cell retention at the transplanted site was assessed by the fluorescence intensity at the SCI explant from the IVIS image captured.

3.7.7 Analysis of the SCI site using Immunohistochemistry

To evaluate the presence of PKH26 cells in the tissue sections and to identify the transplanted cells differentiated at the SCI site to neuronal or glial cells the explant cryosections were used.

For cryosectioning, the SCI explant was initially dipped into iso-Pentane and was cooled using liquid nitrogen. The cryopreserved tissue was then covered with tissue freezing media (OCT, Jung, Lecia Microsystems, Germany). Using a cryostat (Leica, Germany) the sections were cut into 12 µm thick sections. The sections were fixed using 3.7% formaldehyde for 20 minutes. The tissue was then permeated with Triton-X 100 (0.2%) for 5min. The sections were blocked with 3% bovine serum albumin (BSA; Sigma, USA) for 30 minutes and incubated with neural or glial specific primary antibodies overnight at 4°C. The corresponding secondary antibody was added after washing the tissue with PBS thrice and incubated for one hour at room temperature.

Nuclear staining was done using 4,6, diamidino-2-phenylindole (DAPI, Invitrogen, USA). DAPI staining alone after fixing the tissue with 3.7 % formaldehyde was used to track the PKH26 stained cells in the tissue sections.

The paraffin-embedded sections were used for H &E staining, GFAP, and CD 68 immunohistochemistry. For this, the explant was collected and fixed using

paraformaldehyde. After fixation, decalcification was done to eliminate the vertebral column. The paraffin embedding of the spinal cord was done using standard histology procedures.

The sections were then deparaffinized and were used for standard H & E staining. For GFAP and CD 68, protein retrieval was done using incubation in high pH for 20 minutes. Ready to use IHC kits were used for staining of CD 68 and GFAP (DAKO, India).

3.8 Statistical analysis

Statistical significance was calculated by ANOVA (single factor) for all quantitative data having more than two groups. Students t-test was used for comparing two groups. Mean values, standard deviation (SD), and standard errors were calculated for all parameters and are represented in graphical form. Significance is labeled in the graphs with '***' ($P<0.001$); '**' ($P<0.01$); and '*' ($P<0.05$).

CHAPTER 4

4. RESULTS

The results of the study are presented in this chapter with appropriate illustrations comprising representative micrographs and compiled quantitative data in graphical format. The results are separated into 3 parts; the first part (#4.1) comprises the results of *in vitro* experiments carried out with human ADMSCs demonstrating their differentiation to glial and neural progenitors and their further differentiation into mature cells. The second section (#4.2) delineates the signaling mechanisms responsible for lineage commitment and differentiation of hADMSC to neural progenitor cells; pointing to the involvement of both wnt and notch signaling. The third part (#4.3) demonstrates that the rADMSC derived neural and glial progenitors are suitable for transplantation and that they survive in the spinal cord injury in rats when the cells are delivered using injectable fibrin matrix as cell delivery vehicle. Results relate to the materials and methods described in chapter 3.

4.1 ADMSC isolation and characterization

ADMSCs were isolated from human adipose tissue and lipoaspirates, approved by the Institutional Ethics Committee of Sree Chitra Tirunal Institute for Medical Sciences & Technology (SCTIMST IEC No: SCT/IEC/1231/June 2018; SCT/IC-SCR/44/ March 2017) and KIMS Hospital, Trivandrum (IEC NO: SCT/IEC/1231/June 2018)

4.1.1 ADMSC Isolation

The Stromal vascular fraction (SVF) obtained from human adipose tissue after mincing and collagenase digestion resulted in a good yield of ADMSCs (Fig.1 A). Contaminating cells were successfully removed by repeated medium changes in 1st few hours of seeding. The removal of unattached cells resulted in the isolation of plastic adherent hADMSCs with spindle-shaped morphology towards the end of the confluent stage of passage 0, which was attained in 5-7 days. The cells also showed contact inhibition. Upon trypsinization, passage 1 cells were showing elongated spindle-shaped morphology till 10-12th passage. (fig.1.B) The cells after the first passage became confluent in 5-6 days after trypsinization (Fig.1.C)

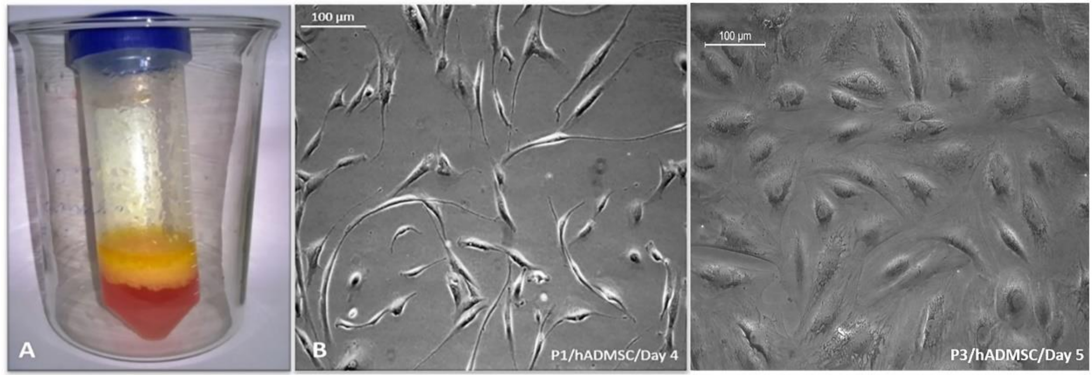


Fig. 1 ADMSC Isolation: (A) Tissue sample minced for collagenase digestion and hADMSC isolation (B) Micrograph of hADMSCs, passage 1, showing typical spindle shape morphology on day 4 (C) P3 hADMSC confluent by day 5.

4.1.2 ADMSC Characterization

Passage 3, hADMSCs showed the potential to differentiate into adipogenic, osteogenic, and chondrogenic lineages. The multipotency of ADMSC was confirmed using special stains. Oil Red O staining revealed the presence of lipoid substances confirming adipogenic differentiation of hADMSC (Fig. 2 C), Alizarin Red Staining detected the presence of calcium precipitate formed in osteo lineage-committed cells (Fig. 2 B) and Toluidine blue staining identified the presence of acidic proteoglycan present in cells committed to chondrogenic differentiation (Fig. 2 A)

Flow cytometric analysis of stem cell markers CD105, CD73 & CD90 showed ~95% of the cells in culture to be positive. The markers of hematopoietic stem cells (HSCs) CD14 & CD45 showed < 5% positivity for the same population (Fig.3).

The results of trilineage differentiation and flowcytometric analysis of surface markers are in accordance with the standards recommended by ISCT. Thus, the isolation protocol was suitable for getting pure hADMSCs with good proliferation potential and multipotency to exploit their application in pre-differentiation to neural lineage cells.

Fig. 2 Micrograph depicting multipotency of hADMSCs (A) Toluidine blue staining of acidic proteoglycan in induced chondrocytes (Magnification-10x) (B) Alizarin Red Staining of the calcium precipitate in induced osteocytes (Magnification-10x) (C) Oil red-O staining of lipoid droplets in induced adipocytes (Magnification-40x)

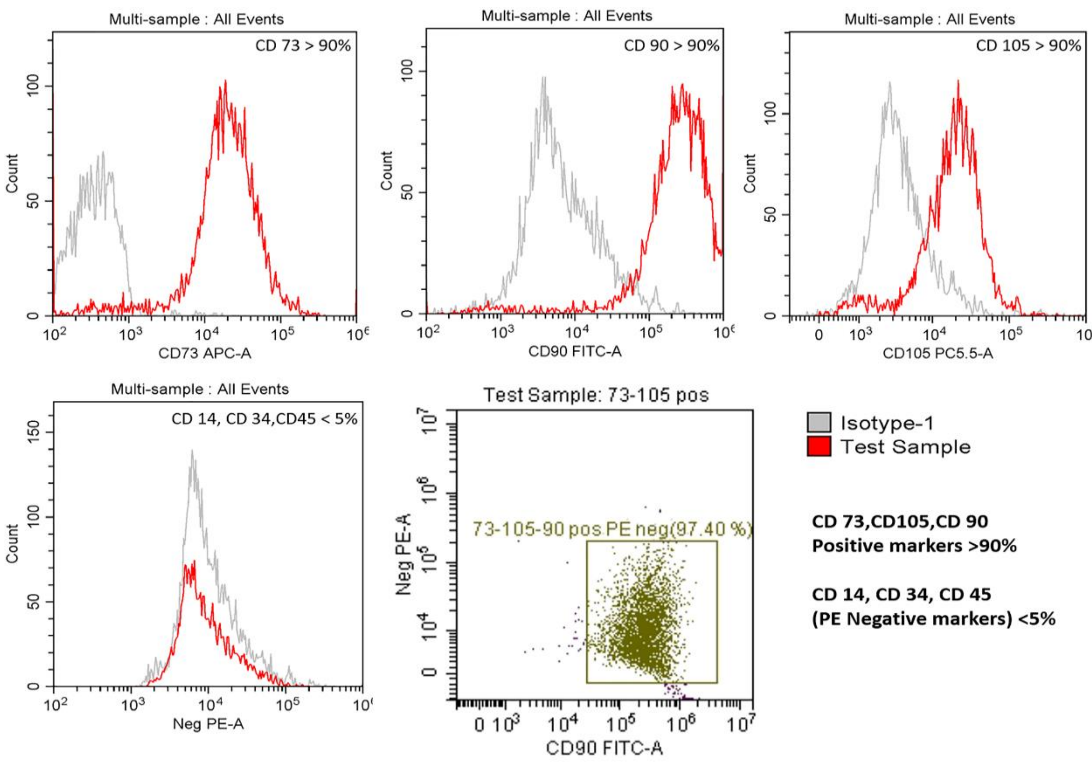


Fig. 3 Histograms showing flow cytometric analysis of hADMSCs for a panel of positive and negative CD markers. Histograms showing positive MSC markers: (A) CD 73 (B) CD 90 (C) CD 105 (E) Histogram : negative cocktail of markers CD 14, CD 34 & CD 45 (F) Dot blot showing 97% MSC population which is CD 73,34 and 105 positive and negative for CD 14, CD 34 and CD 45.

4.1.3 Effect of fibrin matrix on hADMSC to NS conversion

The hADMSCs induced in fibrin niche and bare TCPS started grouping by day 4 (Fig. 4 A). The morphology of induced cells became thin and elongated. The grouped cells transformed into distinct spheres or clusters like neurospheres by day 7 in both fibrin coated niche, INF and bare TCPS conditions, INB (Fig.4 B) & Fig.4 C). In both INB and INF, the adherent neurospheres (NS) appeared similar at the early stage of induction, however, the attachment was better on the fibrin niche. NS developed on bare TCPS detached from the dish in the time period between 7 to 10 days and appeared dark & dead, while the NS on fibrin niche was stable and alive at least till 10 days.

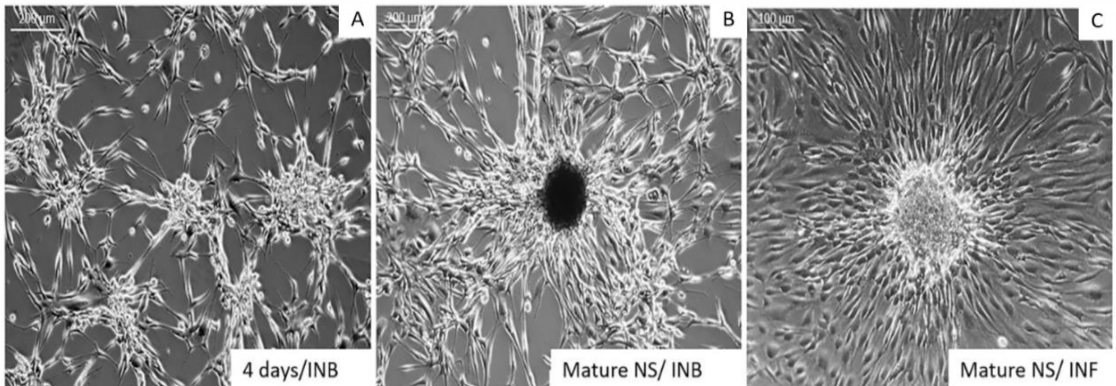


Fig. 4 Phase-contrast Micrographs of neurospheres derived from hADMSC (A) Neurospheres formation and grouping by day 5(B) Mature NS by day 7 in bare TCPS (INB) (C) Mature NS by day 7 in fibrin niche (INF).

The ESEM analysis showed the fiber morphology and porous nature of the fibrin mesh formed from the particular concentration of fibrin (2 mg) and thrombin (5 IU) used in this study (Fig 5 A & B). The morphology of the NS grown on fibrin matrix and the cells growing out from the NS to the porous matrix is visible in the ESEM analysis (Fig 5 C & D).

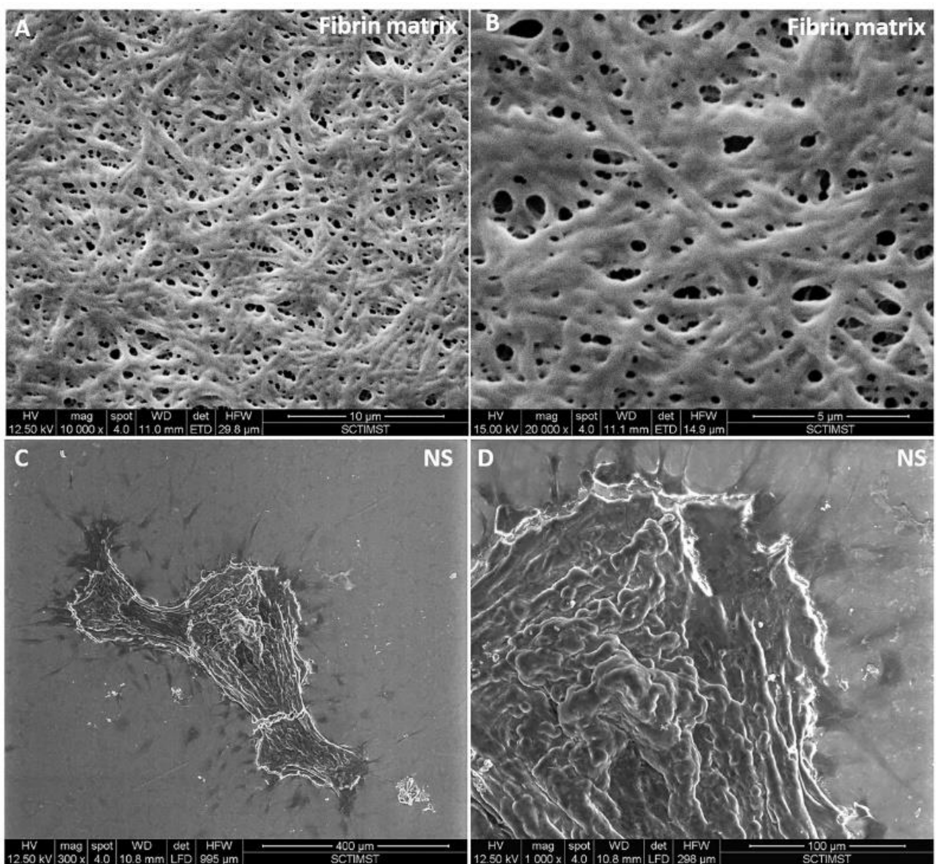


Fig. 5 Environmental Scanning Electron Micrograph of Fibrin niche and NS: (A) Fibrin niche fiber morphology and porosity at (10,000x magnification) (B) Higher magnification of fibrin matrix (20,000x) (C) eSEM image of NS (300x) (D) NS at higher magnification (1000X).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis done using culture terminated on the 7th day of NS induction showed a significant upregulation of neural stem cell marker, Nestin (NES). The cells induced on fibrin niche, INF had better NES gene expression, when compared to those induced on bare TCPS, INB (Fig.6).

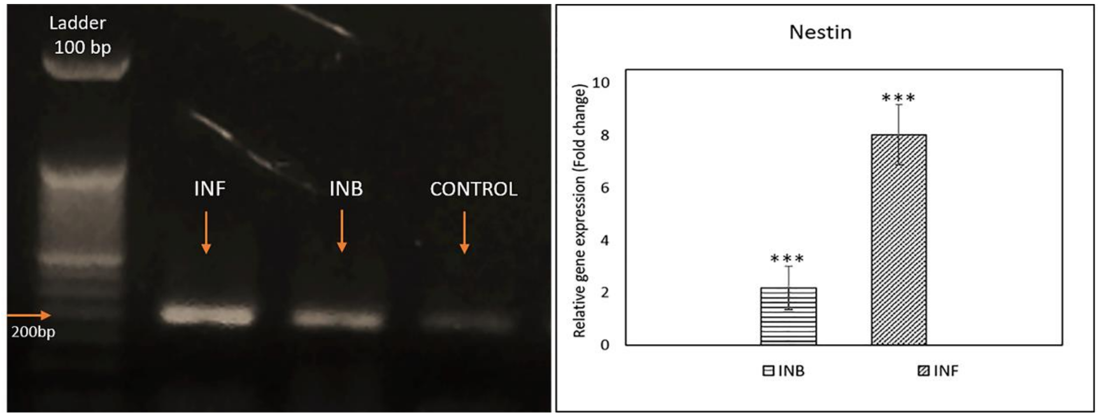


Fig.6 Graphical representation showing PCR analysis of NES gene expression: (A) Agarose gel image showing standard PCR analysis of NES gene. DNA ladder loaded to lane 1, cells induced to NS on fibrin (INF) in lane 2, cells induced to NS in bare TCPS (INB) in lane 3 and control, ADMSC in bare TCPS in lane 4 (B)Nestin gene expression in hADMSC induced to NS after 7 days; hADMSC in bare TCPS in DMEM F12 media for 7 days used as the experimental control; GAPDH used as the House keeping gene; ANOVA: Control, INB & INF; P= 0.001(n= 3) ('***' ($P \leq 0.001$),'**' ($P \leq 0.01$),'*' $P \leq 0.05$)); Error bars represent mean ± SEM

The qualitative analysis of immuno-stained cells using fluorescence microscopy and quantitative analysis using flow cytometry confirmed the presence of Nestin^{+ve} population in neurosphere derived cells. Flow cytometric analysis showed that ~ 45-50% cells were Nestin^{+ve} (Fig.7 B) and this result was in correlation with fluorescence microscopic analysis (Fig.7 A).

The results of the experiments demonstrate the role of fibrin in the survival of NS; NS formed on TCPS did not show good attachment and survival.

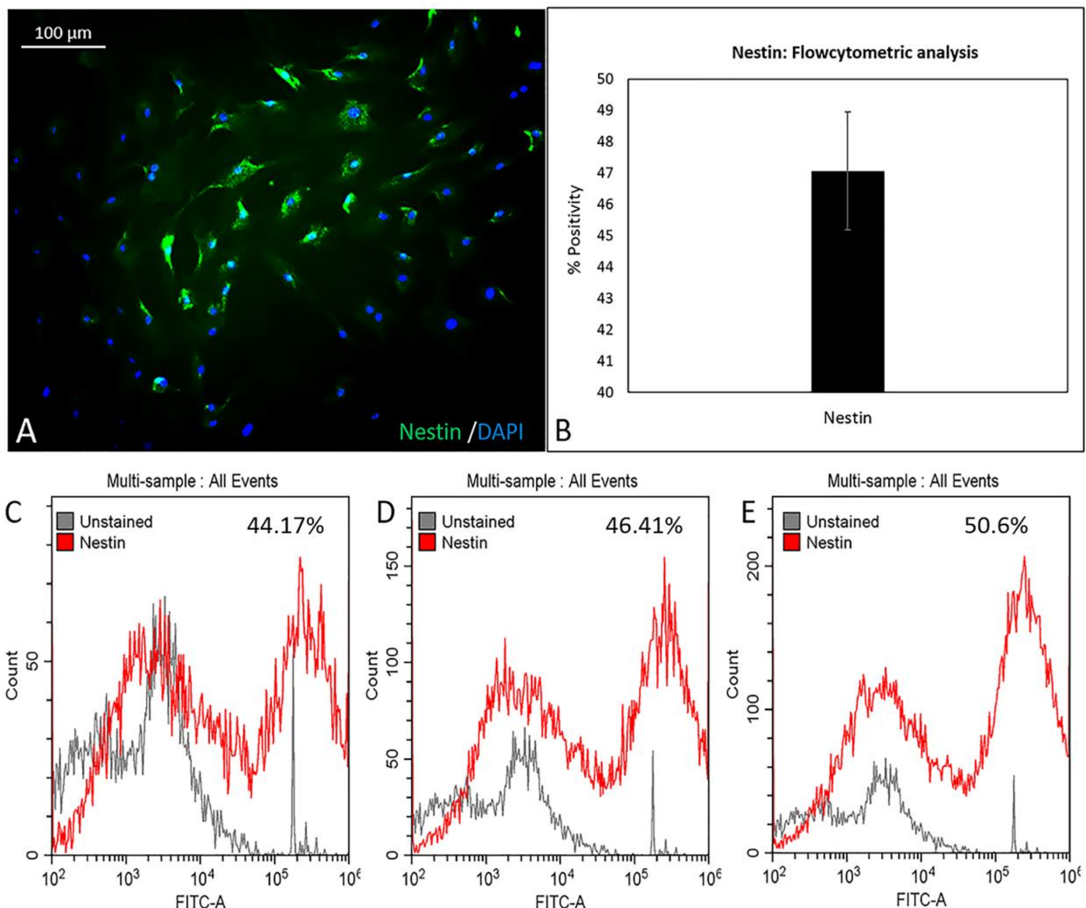


Fig.7 Immunofluorescence analysis of NS using Immunocytochemistry and Flow cytometry: (A)Fluorescent micrograph of Nestin stained NS cells by day 7 (Cells in the periphery of NS; 20x magnification) (B) The graphical representation of Flow cytometric analysis data of Nestin from 3 donors. Error bar represents SEM. Histogram representing the flow cytometric analysis of nestin in NS using CytExpert software in different donors (n=3) (C) Donor 1 (D) Donor 2 (E) Donor 3. Primary conjugated antibody used for analysis. Unstained NS induced cells in fibrin used as control.

4.1.4 Effect of Fibrin in the formation of secondary NS

Manually picked NS, reseeded in bare TCPS, and fibrin niche attached well with good proliferation in 24h (Fig.8 A). In 5 days, the reseeded NS became confluent in both fibrin and bare culture dish (1.75 $cm^{2)}$ (Fig.8 B). A similar effect was observed in both culture conditions.

Nuclear staining done using DAPI, the reseeded NS showed the presence of numerous nucleus in blue color (Fig. 8 C). The maintenance of Nestin positivity and survival of the neural stem cell population in the reseeded NS was confirmed using nestin immunostaining. The Nestin positive cell population in reseeded NS, after antibody staining was observed in green. (Fig.8 D).

The secondary sphere formation potential of the primary NS was confirmed by NS formation assay. The sphere formation was observed in the culture of enzymatically dissociated NS. Secondary sphere formation by neural stem cells was evident in 5 days without growth factor induction or fibrin niche (Fig.8 E). The dissociated primary NS in bare TCPS with NS induction media containing EGF and bFGF induced formation of denser secondary spheres (Fig.8 F).

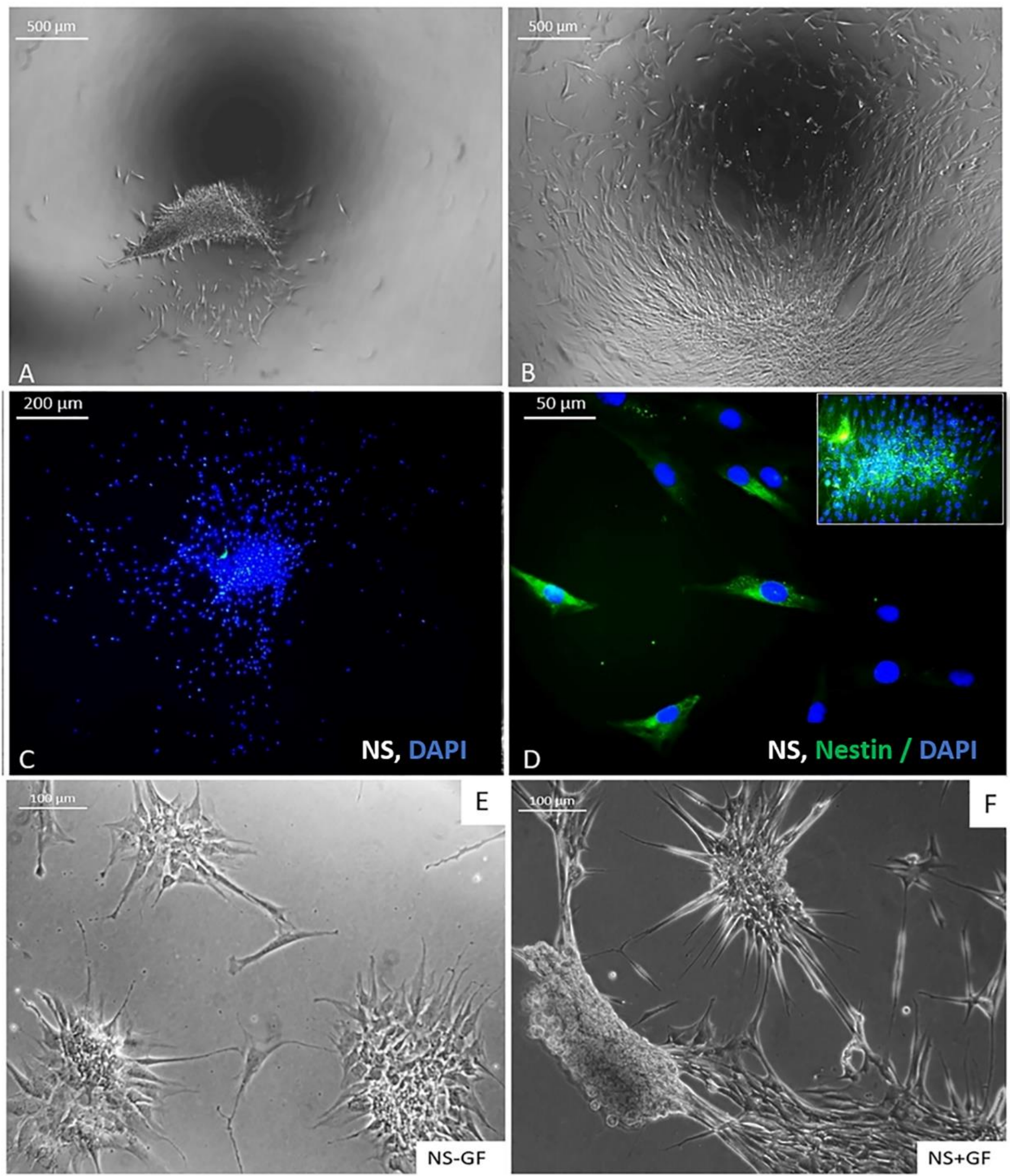


Fig. 8 Secondary sphere formation potential of induced NS and maintenance of Nestin positivity after reseeding (A) Phase-contrast micrograph showing the morphology of NS reseeded by manual picking without trypsinization by day 1 on bare TCPS (B) Reseeded Adherent NS in another 5 days in bare TCPS (C) DAPI staining of the cell nucleus in reseeded NS in fibrin niche (D) Fluorescent micrograph showing immunostaining of reseeded NS in the niche for NES marker counterstained with DAPI in 20x magnification;10x magnification shown as inset. (E) Secondary NS formation by day 5 without niche or growth factor induction (F) Morphology of secondary NS by day 5 with growth factor induction on bare TCPS.

4.1.5 Induction of NS to proliferating NPCs

NS derived cells induced with NPC induction medium showed NPC like thin elongated morphology in 4 days of induction (Fig.9 A). qRT-PCR analysis of the NPC like cells using neural progenitor markers, NES, and TUJ 1 /Beta-3-tubulin showed significant upregulation in the induced NPCs (fig.9 B). Fold change (relative gene expression) >70 was observed for the Nestin marker while fold change >30 was obtained for TUJ-1.

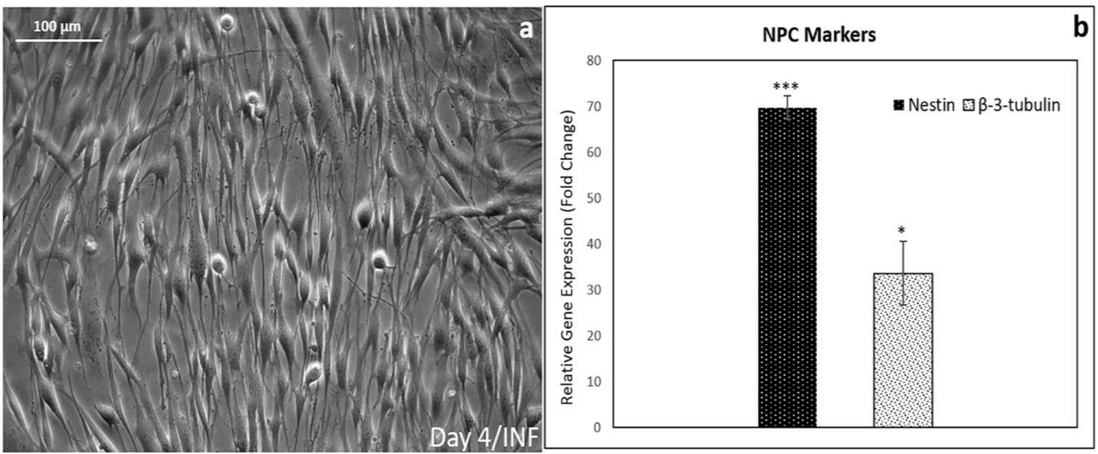


Fig.9 NPC morphology and gene expression after induction on fibrin niche: (A) Phase-contrast micrograph of induced NPCs in the culture after 4 days of NPC induction (B) **Graphical representation of qRT-PCR analysis of NPC markers:** NS derived cells induced to NPC after 4 days of induction.NES and TUJ-1 (β-3-tubulin) gene expression represented as individual bars; hADMSC on bare TCPS in DMEM F12 media for 7 days was used as the experimental control; GAPDH was used as the Housekeeping gene; Student's t-test: Control & NPC in niche; Nestin: P=0.001 (n=3); β-3-tubulin : P=0.05 (n= 3) ('***' ($P \leq 0.001$) ,'**' ($P \leq 0.01$),'*' $P \leq 0.05$)); Error bars represent mean ± SEM

Maintenance of the nestin-positive neural stem cell population or the formation of NPCs in the culture was confirmed by Nestin immunostaining (Fig.10 A). The proliferation of NPCs in the culture was established by PCNA-TUJ-1 co-immunostaining. The PCNA antibody specifically stained the nucleus of proliferative cells and the TUJ 1 antibody stained the cytoplasm associated specific tubulin proteins. Secondary antibodies used were of different origins and with different specificities but

the fluorochrome-conjugated was Alexa Fluor in both cases. After co-immunostaining both immuno-markers were evidently localized to specific cell compartments in green color (Fig.10 C). The PCNA marker colocalized with DAPI, nuclear marker appeared in Cyan color in the nucleus of proliferating cells with green cytoplasm of TUJ-1 stain. The cells stained with green cytoplasm and cyan nucleus (Orange arrows) indicate proliferating NPCs (Fig.10 D).

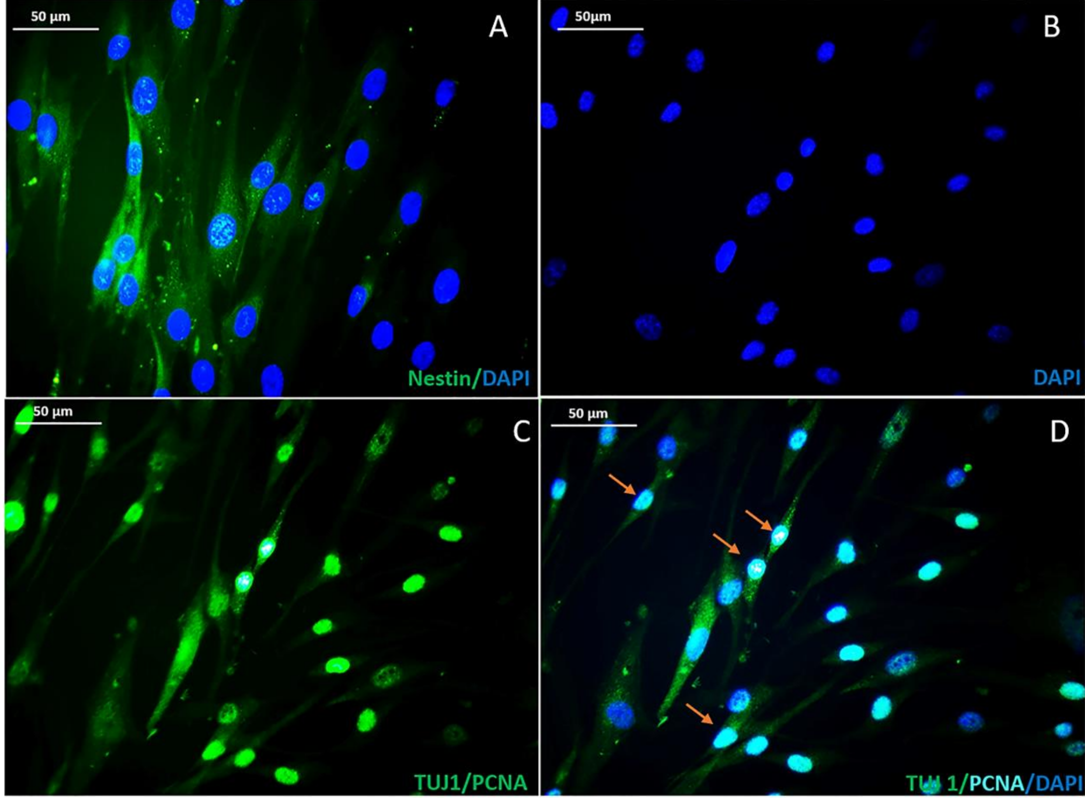


Fig.10 Immunostaining of induced NPCs with NES, TUJ, and PCNA antibodies: Fluorescence micrographs showing DAPI/PCNA/TUJ 1 co-immunostaining (Magnification 40X) (A) Immunostaining of NES marker on day 4, DAPI represents nucleus and NES represents cells induced to neural stem cells (B) DAPI stained NPC nucleus (C) Fluorescence micrograph of NPC stained with PCNA & TUJ -1 antibodies, PCNA in the nucleus of the proliferating cell and TUJ-1, tubulin protein in the cytoplasm. (D) PCNA and DAPI co-localized in nucleus as cyan color and TUJ-1 seen as green. Proliferating NPCs indicated by Orange arrows.

The flow cytometric analysis of hADMSC derived NPCs stained with TUJ 1 antibody (n=1 donor) showed ~30% TUJ 1 positivity. Donor to donor variability was observed in percentage of positivity obtained in different experiments (Fig. 11).

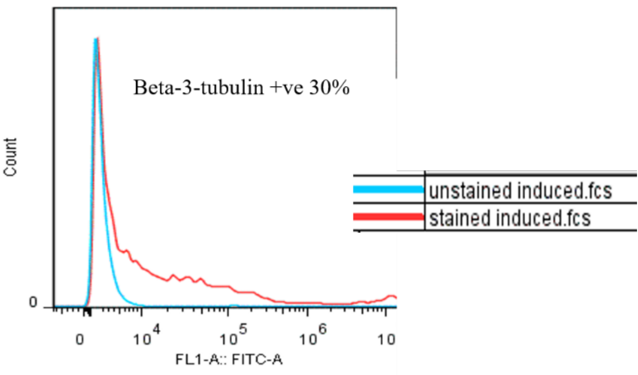


Fig 11: Flowcytometric analysis of NPCs: Histogram (Flow Jo software) showing 30% TUJ 1 positivity in NPCs on day 5

4.1.6 Effect of Fibrin matrix on NPCs to OPCs conversion

The NPCs induced to OPCs showed a change in morphology similar to OPCs on both INF and INB conditions (Fig 12 A). The effect was more prominent in fibrin-based niche, as more OPC like phase bright cells were observed in the niche (Fig.12 B).

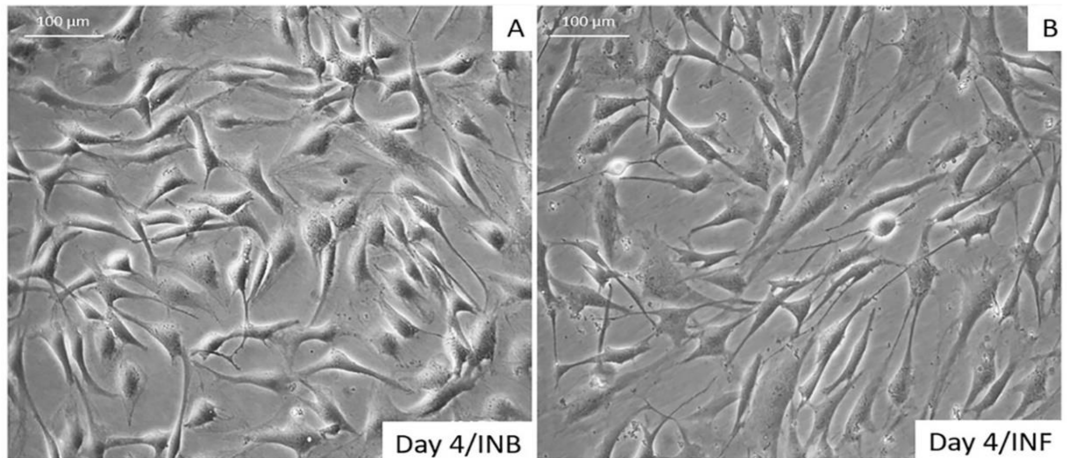


Fig. 12 Phase-contrast micrograph of OPC induced from NPCs: (A) Morphology of NPCs induced to OPCs on bare TCPS (INB) by day 4 (B) Morphology of NPCs induced to OPCs on fibrin niche (INF) by day 4.

Transcriptional analysis of the induced OPCs using qRT-PCR confirmed the upregulation of OPC markers, Platelet-derived growth factor receptor-Alpha (PDGFRα) and OLIG 2 (Fig.13 A). The proliferation potential of these progenitors was confirmed by PCNA gene up-regulation. Both PDGFRα and PCNA gene upregulation was found to be significantly higher on fibrin niche compared to bare TCPS. The Olig2 was upregulated in both INF compared to the control ADMSC.

OPC markers, O4 antigen, OLIG 2, and PDGFRα was prominently expressed in induced cell culture as confirmed by immunostaining. O4 and PDGFRα molecules were observed in the cytoplasm and cell membrane respectively (Fig 13 B and C). The staining of induced OPCs using OLIG 2 antibody confirmed the presence of OLIG 2 transcription factor in the nucleus (Fig 13 D).

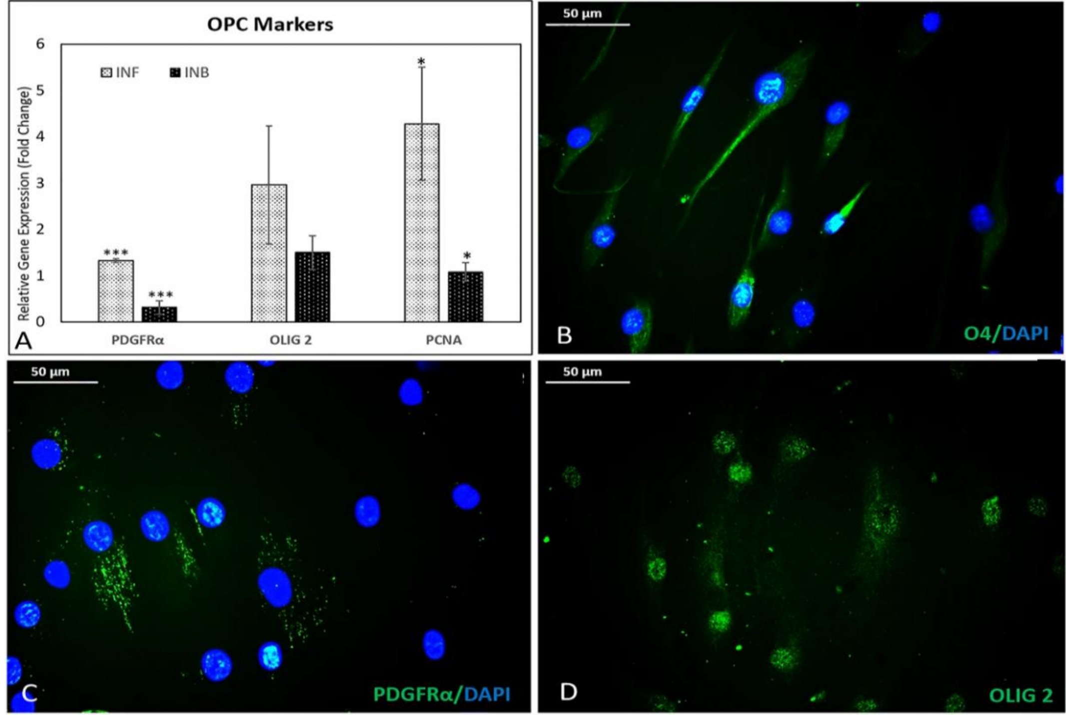


Fig. 13 OPC marker expression in induced cultures: (A) Gene expression of NPCs induced to OPCs after 5 days of induction; PDGFR-α, P = 0.0004 (n = 3), PCNA, P = 0.02 (n = 3) ; hADMSC on bare TCPS in DMEM F12 media for 7 days used as the experimental control; GAPDH used as the House keeping gene; ANOVA: Control, INB & INF; ('***' (P ≤ 0.001) ,'**' (P ≤ 0.01),'*' P ≤ 0.05)); Error bars represent mean ± SEM . (B) Fluorescence micrograph showing O4 antigen stained induced cells counter stained with DAPI (C) OLIG 2 nuclear specific marker stained positive in induced OPCs, Day 4. (F) Immunostaining of OPC cell surface marker, PDGFRα & DAPI.

Quantitative estimation of induced OPCs in fibrin niche by day 5 showed that ~30% cells were positive for the Oligodendrocyte progenitor marker, OLIG 2 (Fig.14). Flow cytometric analysis data from replicate experiments (n=3) has been compiled and presented as individual histograms and bar diagram.

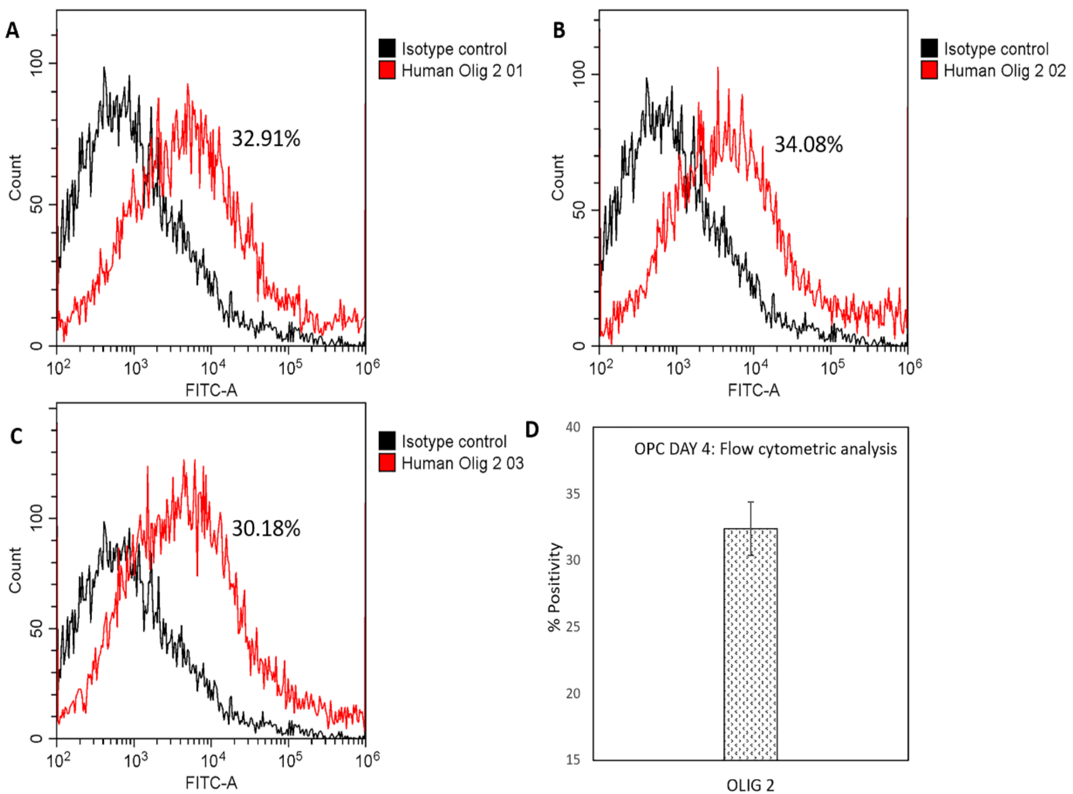


Fig.14 Flow cytometric analysis of induced OPCs using OLIG 2 marker: The histograms (CytExpert software) representing OLIG 2 positivity in induced OPCs in fibrin (A) Donor 1 (B) Donor 2 (C) Donor 3 (D) Graphical representation of the flow cytometry data(n=3). Secondary antibody alone stained induced cells used as control

4.1.7 Comparison of fibrin & bare TCPS on OPC maturation

The OPCs showed morphological changes similar to OLGs after induction of terminal differentiation for 48h. in both niche and bare TCPS (Fig.15 A and B). Cells started to detach from the cell culture dishes in INB and INF after 2 days of induction.

Gene expression analysis of the cells induced to OLGs using qRT-PCR showed upregulated expression of mature OLG markers, Oligodendrocyte specific protein

(OSP),& Myelin basic protein (MBP) in both INB & INF. Significant upregulation of OSP & MBP expression was observed as compared to control ADMSCs (Fig. 15 C)

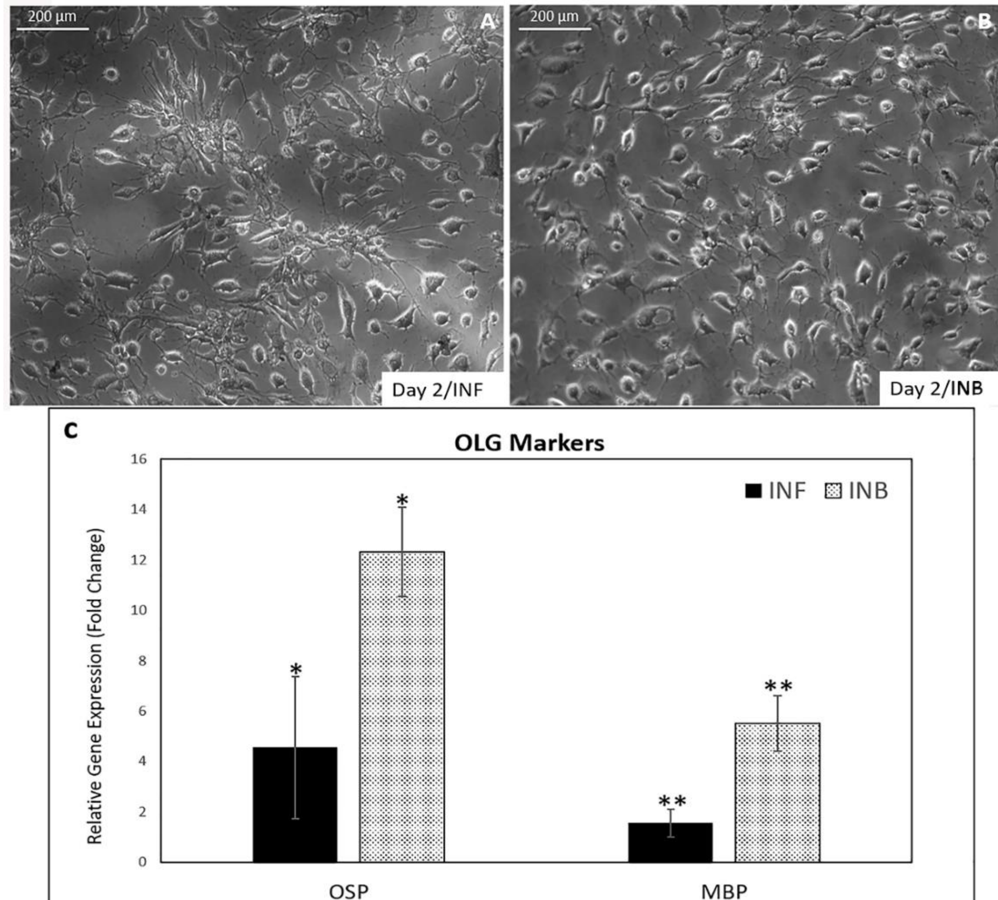


Fig.15 Morphology and gene expression analysis of OPCs induced to OLGs: (A) Phase contrast micrograph showing morphology of induced OLGs on fibrin niche (INF) by day 4(B) Morphology of induced OLGs on bare TCPS (INB) by day 2 (C) Graphical representation of qRT-PCR analysis of OLG markers: OSP: P = 0.015 (n = 3) MBP: P = 0.008 (n = 3);hADMSC on bare TCPS in DMEM F12 media for 7 days used as the experimental control; GAPDH used as the House keeping gene; ANOVA: Control, INB & INF; ('***' ($P \leq 0.001$),'**' ($P \leq 0.01$),'*' $P \leq 0.05$)); Error bars represent mean ± SEM.

The induced OLGs stained with MBP and OSP marker antibody showed prominent expression in both INB and INF conditions (Fig.16). The protein expression of OLG markers on bare TCPS and fibrin niche was similar when stained with specific antibodies.

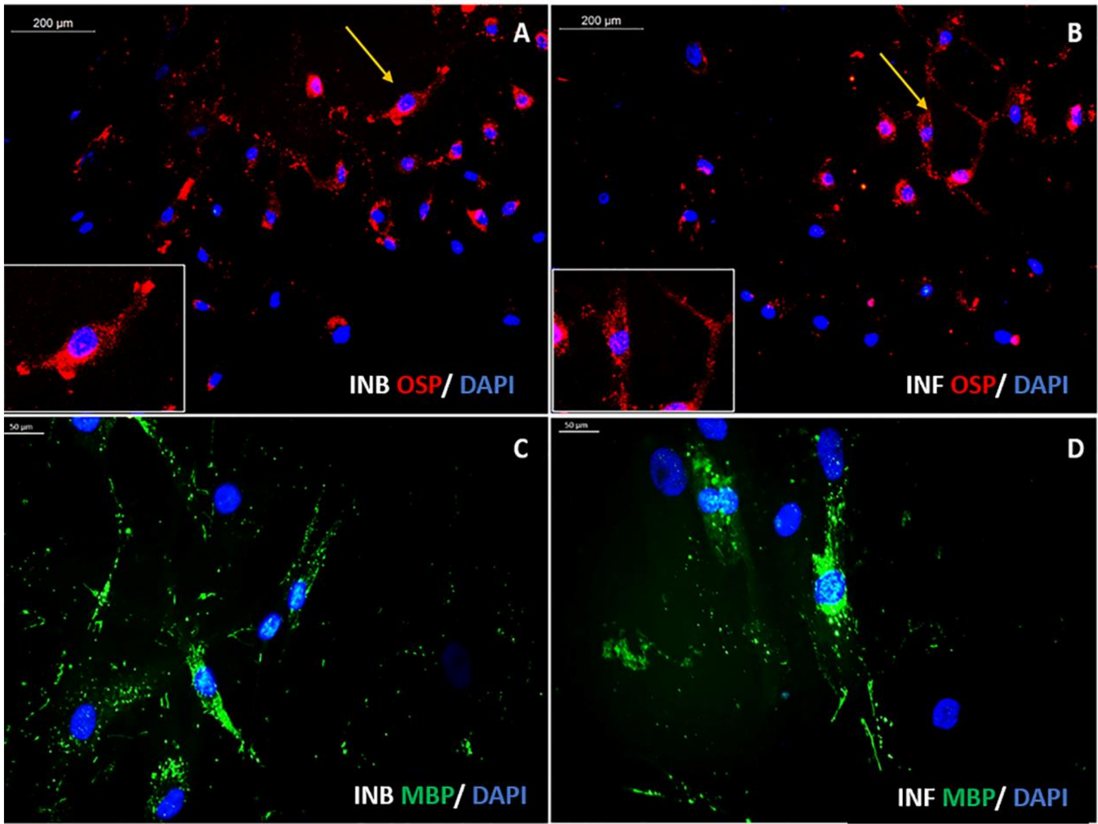


Fig. 16 Immunostaining of OLG markers: Fluorescence micrograph showing induced OLGs in niche and TCPS on day 2 (A) OSP stained induced OLGs on bare TCPS (B) OSP stained induced OLGs on Fibrin niche; Higher magnification of the region of interest in fluorescence micrograph shown as inset (Region of interest indicated in yellow arrow) (C) MBP stained induced OLGs on bare TCPS (D) MBP stained induced OLGs on bare TCPS. DAPI used as nuclear stain.

4.1.8 Fibrin niche induced differentiation of NPCs to Neurons

NPCs derived from NS showed neuron-like elongated cell morphology upon induction to NPCs. The neural extensions were more prominently observed after the addition of KCL to the culture (Fig.17 A). The upregulated expression of the mature neuronal marker at mRNA level was observed in the neuronal culture using qRT-PCR analysis on day 6 (Fig.17 B). Microtubule-associated protein -2 (MAP-2), the neuron-specific cytoskeletal protein was found to be upregulated at the mRNA level in the induced neurons. mRNA level of Neuron-specific enolase (NSE) was also expressed significantly higher in the induced culture compared to control ADMSCs. The tyrosine

hydroxylase (TH), dopaminergic neuron marker was also significantly upregulated in the induced cells. This indicates that the terminal differentiation of NPCs to neurons also includes a dopaminergic neuronal population.

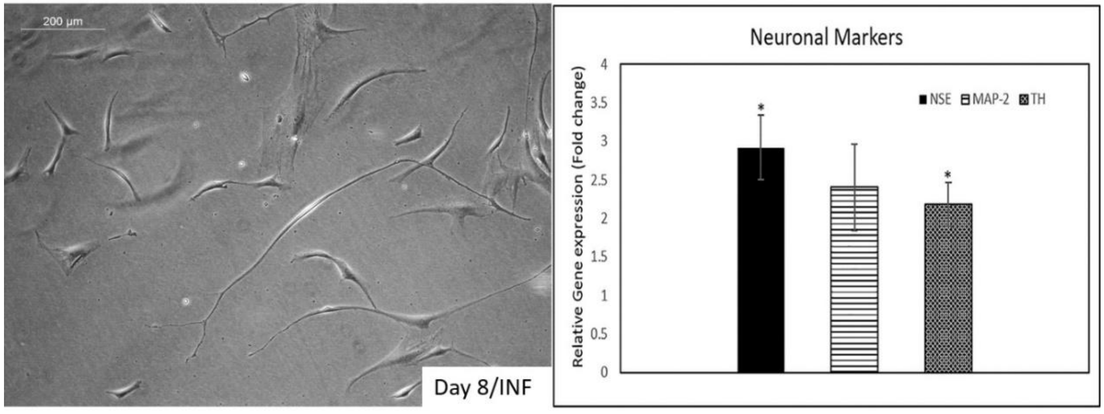


Fig.17 Morphology and specific marker expression of the induced neurons: (A) The phase-contrast micrograph showing elongated neural morphology of cells induced for 6 days (B) **Graphical representation of qRT-PCR analysis of neural markers**: NSE, MAP-2 and TH gene expression compared to control ADMSCs. Mature neuronal markers NSE (P=0.05) and TH (P=0.04) showed significant upregulation of gene expression when compared to control cells. hADMSC on bare TCPS in DMEM F12 media for 7 days was used as the experimental control; GAPDH was used as the House keeping gene; Student's t-test: Control, INB & INF; ('***' ($P \leq 0.001$), '**' ($P \leq 0.01$), '*' $P \leq 0.05$)); Error bars represent mean ± SEM.

The findings from the qRT-PCR analysis were further established at protein level by immunostaining of induced neuronal cells against MAP 2 antibody (Fig. 18 A), Synaptophysin antibody (Fig. 18 B), TH antibody (Fig.18 C) and Nerve growth factor Receptor (NGFR) antibody (Fig.18 D). Neuronal marker immunoreactive cells were present in the induced neuronal cells by day 6. TH expressing cells could be dopaminergic neurons. About 40.0 % of cells were found to be TH positive upon manual counting of cells in >10 fields.

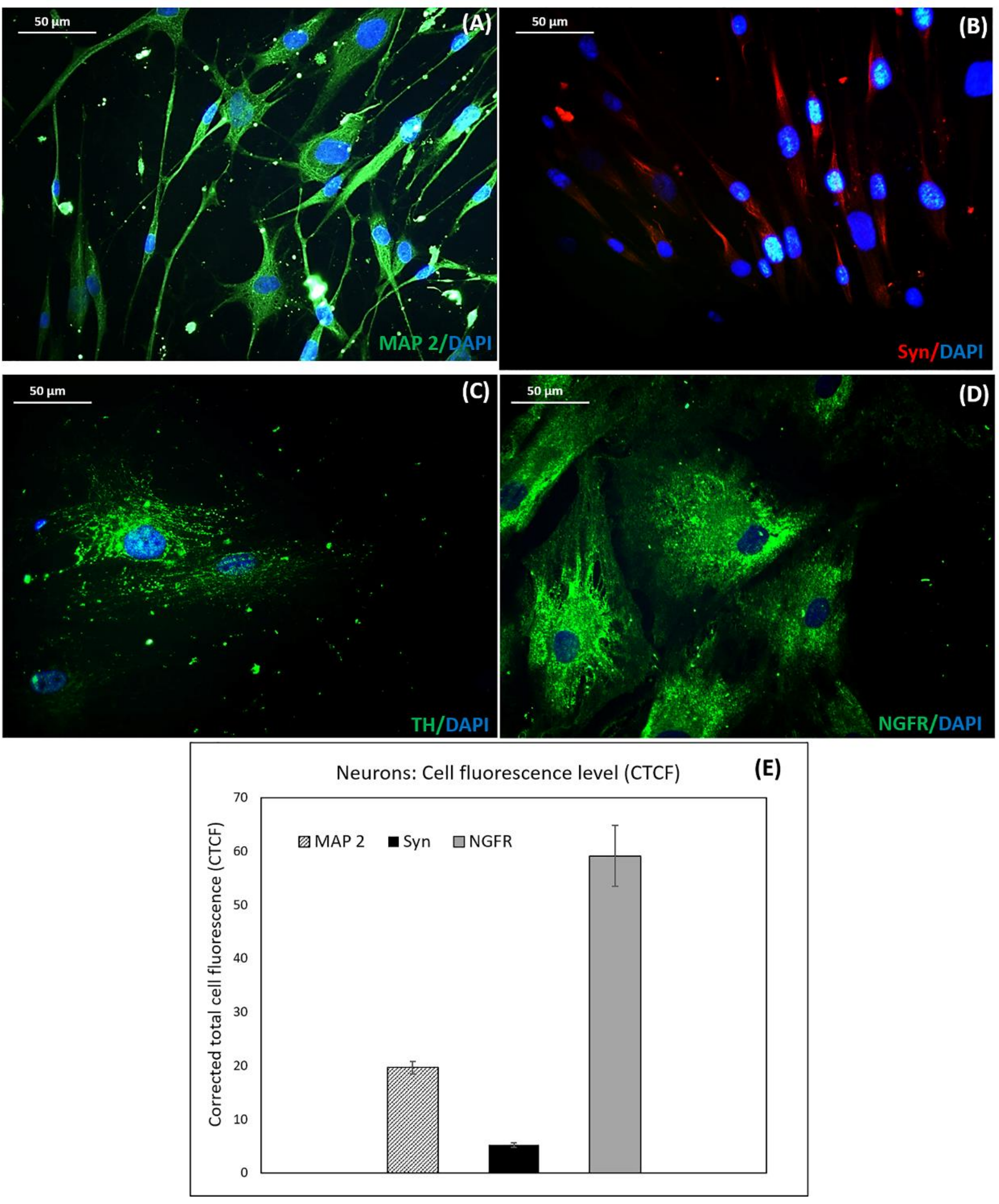


Fig. 18 The fluorescence micrograph showing mature neuronal markers in induced cells: (A) MAP 2 (B) Synaptophysin (C) TH (Dopaminergic neuron marker) (D) and NGF positive cells observed in induced neuronal culture (Magnification 40X). DAPI used as nuclear stain. (E) Quantitative fluorescence image analysis: Corrected total cell fluorescence (CTCF) calculated from ICC images of MAP 2, Syn and NGFR (Average of CTCF of 15 cells from 5 fields). Error bars represent SEM.

4.1.9 Co-culture of Neurons and OPCs on Fibrin

The cells with OLG morphology appeared after 3 days of co-culture of neurons and OPCs in KCL added basal DMEM F12 media (FIG.19 A).

The mRNA level gene expression profile of OLG and Neuronal markers was done using qRT-PCR (Fig.19 B). The cultured cells showed significantly upregulated immature neuronal marker, TUJ-1, and the mature neuronal marker, MAP-2 expression. OLG marker OSP was also found to be upregulated at the mRNA level. Glial fibrillary protein (GFAP), specific astrocyte marker was significantly upregulated in the mRNA level.

Immunostaining of the coculture population with neuronal and OLG specific markers confirmed the presence of terminally differentiated neuronal and glial cell populations in the culture. MAP-2 was prominently seen in few thin elongated neuron-like cells (Fig.19 C). Dopaminergic neuronal marker, TH was also prominently expressed in the cocultured cells (Fig. 19 D). MBP immunopositivity was also present in the coculture population suggesting the differentiation of OPC to OLGs (Fig.19 E). The MBP and TH immunofluorescence intensity is expressed as corrected total cell fluorescence (Fig 19F) However, GFAP protein expression could not be confirmed at the protein level using immunocytochemistry.

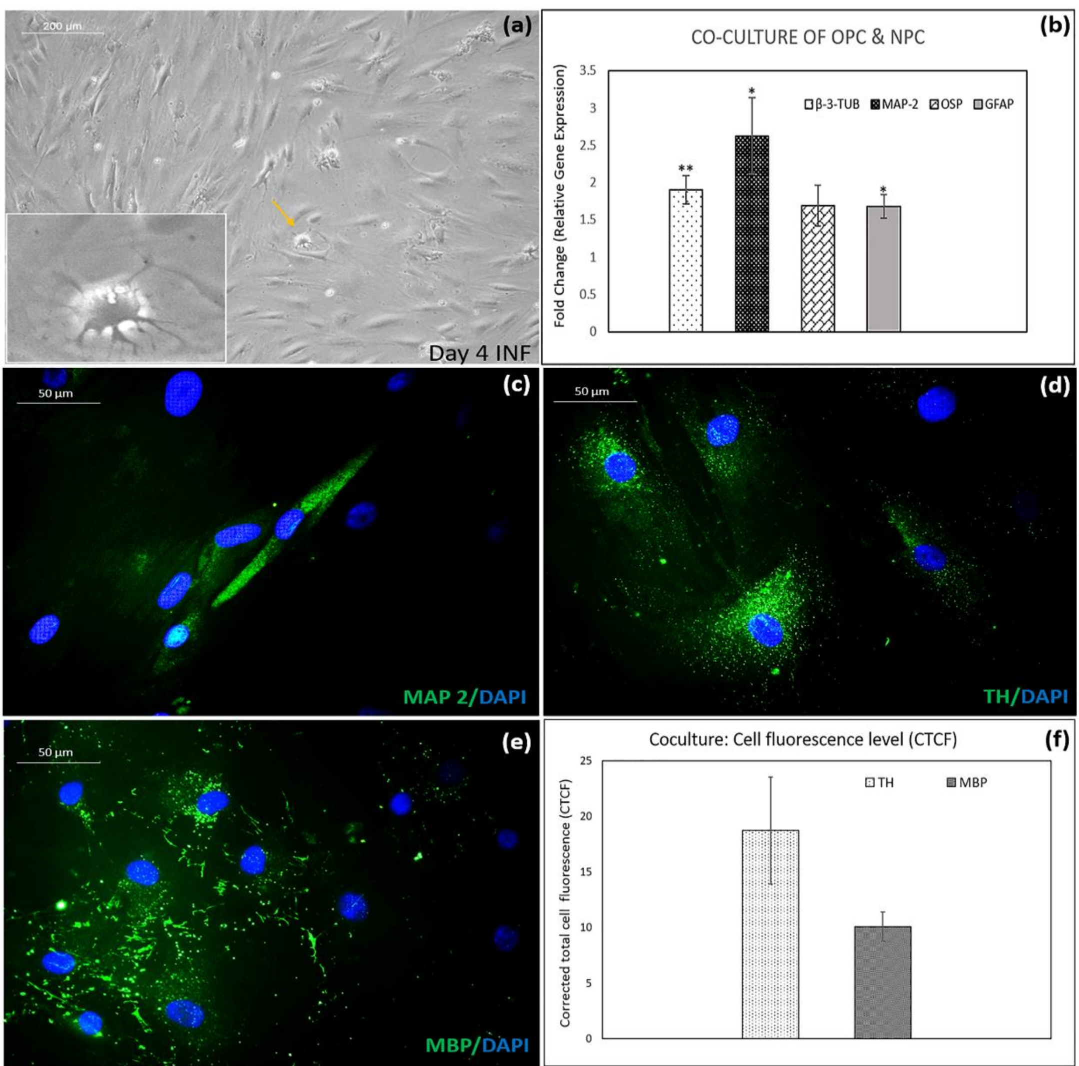


Fig.19 Coculture of OPCs and neurons: (A) Phase-contrast micrograph showing the morphology of co-cultured neurons and OPCs. Higher magnification of a cell with OLG morphology showed as inset (Region of interest in black arrow) (B) **Graphical representation of qRT-PCR analysis of neuronal and glial markers in the co-culture**: TUJ 1, MAP-2, OSP, and GFAP gene expression compared to control expressed as individual bars. TUJ I (P=0.009), MAP-2 (P=0.05) and GFAP (P=0.04) were significantly upregulated. hADMSC on bare TCPS in DMEM F12 media was used as the experimental control; GAPDH was used as the House keeping gene; Student's t-test: Control & INF; ('***' ($P \leq 0.001$), '**' ($P \leq 0.01$), '*' $P \leq 0.05$)); Error bars represent mean ± SEM. Fluorescence micrograph showing (C) MAP 2 (D) TH (E) MBP immunopositive cells in the coculture. DAPI used as nuclear stain. (F) Quantitative fluorescence image analysis: Corrected total cell fluorescence (CTCF) calculated from ICC images of TH and MBP (Average of CTCF of 15 cells from 5 fields). Error bars represent SEM.

4.2 Signaling cascades elicited by fibrin-based niche

Upon comparison of fibrin-matrix based niche and bare TCPS, the role of fibrin in supporting *in vitro* hADMSC growth and differentiation to various stages of neural progenitor cells was evident from results presented in #4.1. Both wnt and notch signaling pathways were explored and found to be involved for *in vitro* signaling in matrix directed differentiation of ADMSCs to NS and OPCs; both test and control represent cells cultured on fibrin

4.2.1 MTT assay to evaluate the cytotoxicity of the inhibitors

The results suggested that the percentage of cell proliferation or the cell viability in the culture added with inhibitors of the notch and the wnt signaling pathways (20 μM/ml of notch inhibitor and 40 μM/ml of wnt inhibitor) were ~ 80% and ~100% respectively (Fig. 20). Minimal cytotoxicity was observed in notch inhibitor added cultures. The wnt inhibitor added culture showed cell viability comparable to that of the cells in non-inhibited fibrin niche.

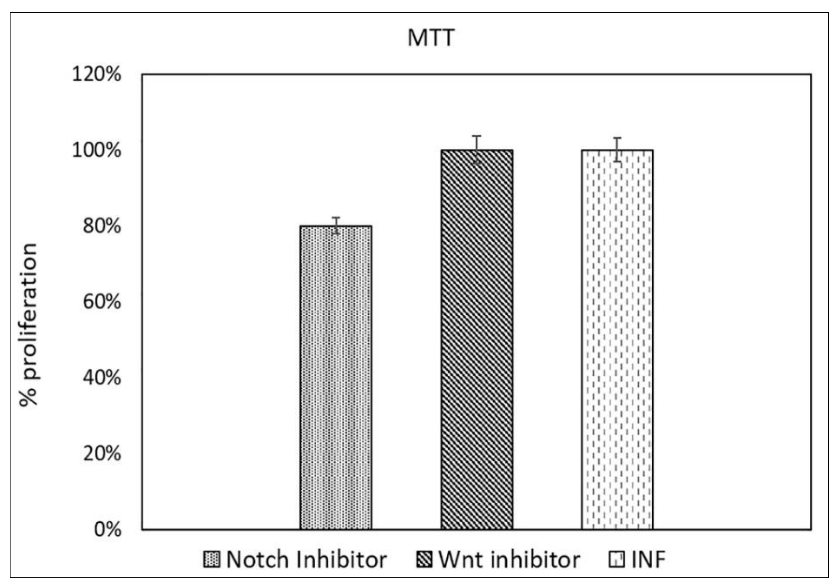


Fig. 20 Graphical representation MTT assay: Cell viability, 48 h. after addition of inhibitor to the ADMSC culture in neural induction niche. Error bar represents SEM. ADMSC in the neural induction niche without the addition of inhibitor is used as control.

4.2.2 Action of inhibitors of Notch and Wnt on NS formation

The control cells showed grouping after 5 days of NS induction (Fig.21 A). The inhibitor added test cultures did not form cell groups and the cell to cell contact was sparingly seen. The cell density in wnt inhibited culture was comparatively lower as compared to notch inhibited cultures (Fig.21 D). The morphology of notch and wnt signal inhibited cultures were also not comparable to that of ADMSCs induced to NS on fibrin niche. Notch inhibitor added cultures showed thin elongated neural cell-like morphology (Fig.21 B). The basic level of signaling responsible for ADMSC proliferation, the cells grown on bare TCPS without any induction was used as the experimental control (Fig 21 C).

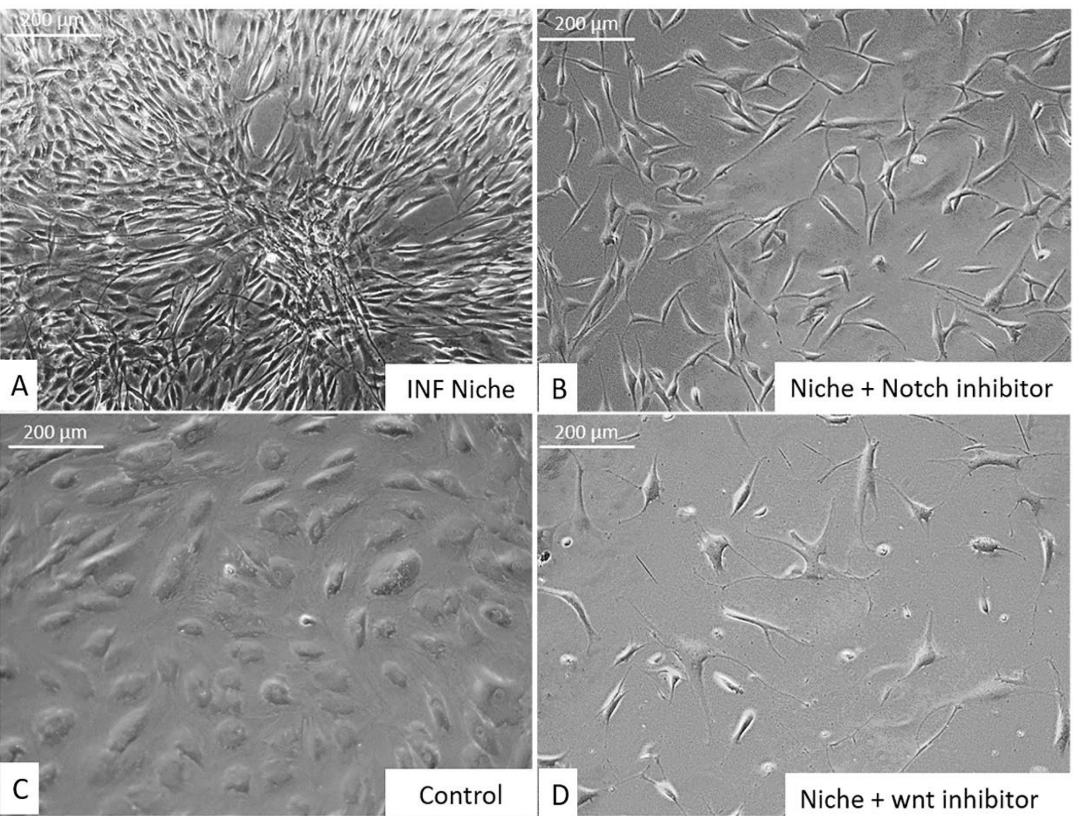


Fig.21 Phase-contrast micrograph of morphological effect of signal inhibitors in NS induction: (A) INF, hADMSCs induced to NS in fibrin niche showing grouping by day 5 (B) Morphology of notch inhibitor added cultures by day5 with no group formation (C) Control hADMSCs grown on bare TCPS (D) Morphology of cells induced on fibrin niche added with wnt inhibitor.

4.2.3 Effect of Notch and Wnt inhibitors on Nestin gene expression.

The qPCR data analyzed against normal ADMSC grown for 5 days indicated the upregulation of nestin at mRNA level in the standard niche (Fig. 22). Significant downregulation of NES gene expression was observed in both wnt inhibitor & notch inhibitor treated cells. However, the inhibition was more prominent upon treatment with wnt inhibitor.

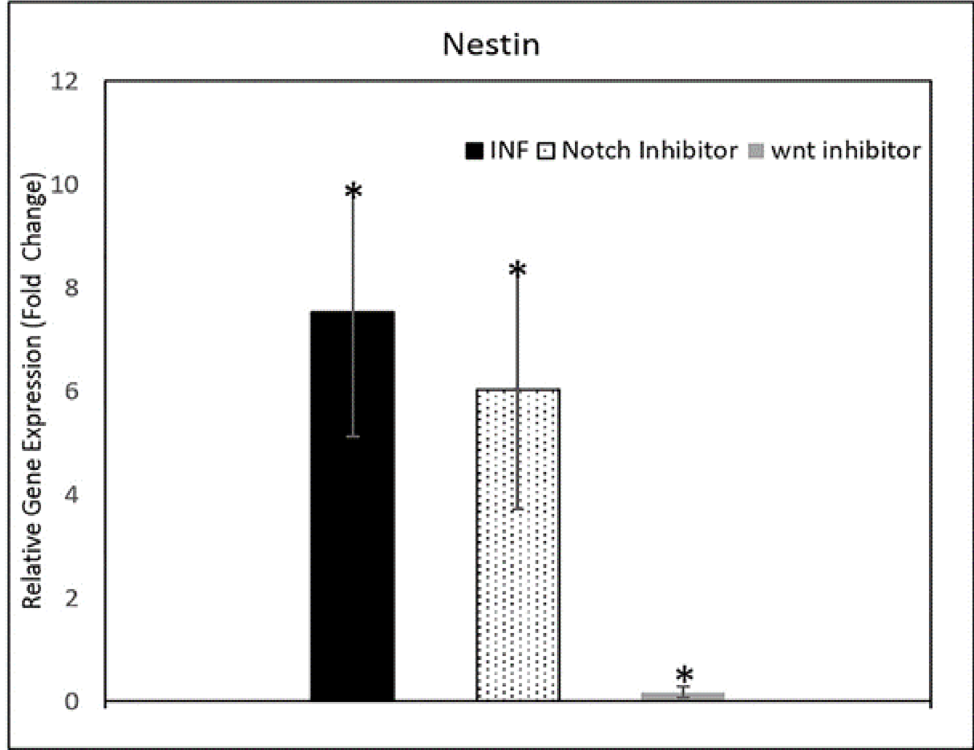


Fig. 22 Graphical representation of qRT-PCR analysis of nestin marker: The gene expression analysis of NS induced in the niche in the presence and absence signal inhibitors. ADMSC grown on bare TCPS used as experimental control and GAPDH used as Housekeeping gene; ANOVA: Control, INF, & Inhibitor (Wnt & notch) +INF; P= 0.03(n= 3); '*' (P<0.05) '**' (P<0.01) '***' (P<0.001)

4.2.4 Effect of inhibitor on gene expression of Notch downstream molecules.

The qRT-PCR analysis of the cells after 5 days of induction and inhibition of signals using 20 µM/ml of notch inhibitor and 40 µM/ml wnt inhibitor demonstrated changes in the level of expression of various notch markers at mRNA level (Fig 23). Expression of TCF 4 was reduced in the cells induced in fibrin niche Hes 1, gene expression remains quiescent or unchanged in all conditions. Neurogenin 2 (NGN 2) is upregulated in inhibitor added culture compared to induced inhibitor-free controls. Another downstream molecule Hes 5 is significantly upregulated in INF compared to notch signal inhibited culture.

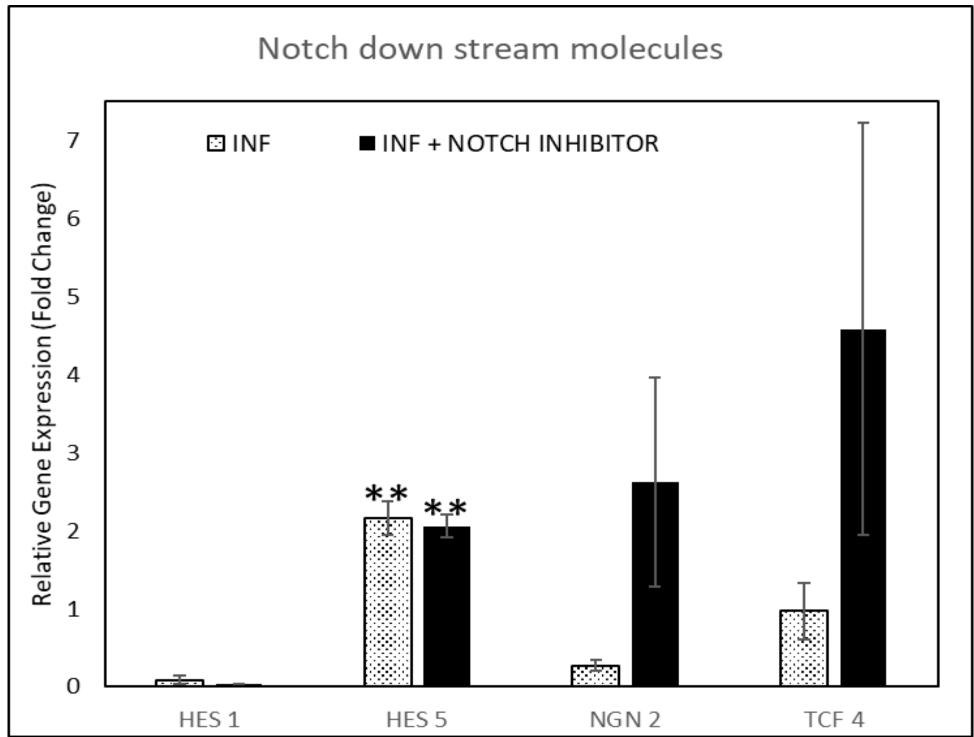


Fig. 23 Graphical representation of qRT-PCR analysis of notch downstream markers: TCF 4, NGN 2, HES 1, and HES 5 gene expression in induced cells. ADMSC on bare TCPS used as experimental control and GAPDH used as Housekeeping gene; ANOVA: Control, INF, Notch inhibited INF; Hes 5, $P = 0.002$; $n = 3$; ('*' ($P < 0.05$) '**' ($P < 0.01$) '***' ($P < 0.001$)).

4.2.5 Identification of notch and wnt downstream molecules

The notch is prominent in induced cells in fibrin with a large number of notch molecules in the nucleus in cyan color indicating an active notch signaling from the niche (Fig. 24 A). Control ADMSC grown in bare TCPS did not show much active notch in the nucleus (Fig. 24 B). The presence of active notch molecules was barely present in inhibitor added cultures; however, few cells were positive for Notch even with a higher concentration of inhibitors (20 µM/ml ; LC and 40 µM/ml ; HC) ((Fig 24 C & D). The inhibitor did not eliminate the notch signaling completely.

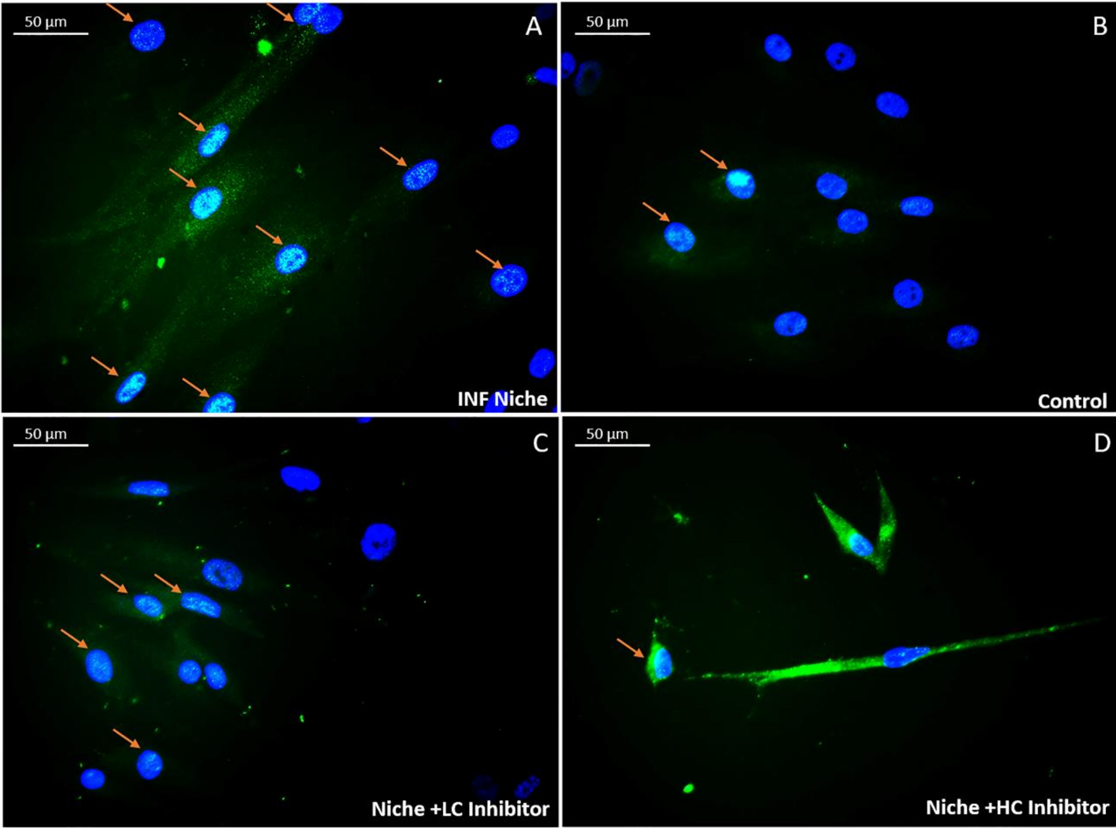


Fig.24 Fluorescence micrograph of cells immunostained with Notch antibody: (A) Cells induced to NS in fibrin niche, INF (B) hADMSC control on bare TCPS (C)Niche added with low concentration of inhibitor (LC; 10 µM/ml) (D) Niche added with a higher concentration of inhibitor (HC; 20 µM/ml). DAPI used as nuclear stain. Orange arrows indicate notch molecules in the nucleus.

HES 1 protein was observed in control ADMSC cells (Fig.25 B) and induced INF cells (Fig. 25 A) after immunostaining. The induced cells added with notch inhibitor showed a lesser number of DAPI stained nucleus in a concentration-dependent manner (Fig.25 C, D). HES 1 was prominently seen in the nucleus of cells in all cultures immunostained with HES 1 antibody.

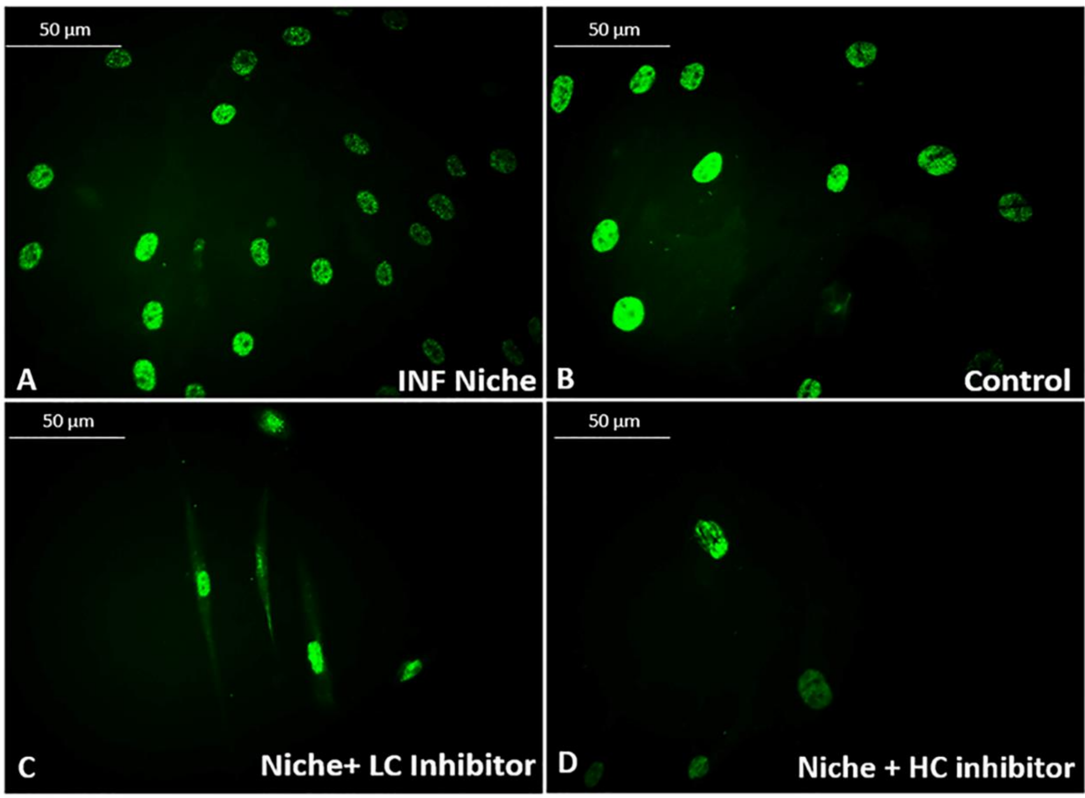


Fig.25 Fluorescence micrograph of cells immunostained with HES 1 antibody: Cells induced to NS showing HES 1 protein expression in nucleus of (A) cells induced in INF (B) hADMSC control on bare TCPS (C) Niche added with low concentration of inhibitor (LC; 10 µM/ml) (D) Niche added with a higher concentration of inhibitor (HC; 20 µM/ml).

Antibody staining of the induced cells and wnt inhibitor added cells showed expression of wnt 3a molecules in cytoplasm and extracellular matrix. Secreted Wnt 3a is expressed prominently in ECM of INF cells (Fig.26 A) which were not seen in ADMSCs grown on bare TCPS (Fig.26 B). The addition of inhibitor showed dose-dependent disappearance of wnt molecule from the extracellular matrix of the cells (20 μM/ml; LC and 40 μM/ml; HC) (Fig.26 C, D). Active paracrine wnt signaling occurring in the fibrin matrix is noticeable in the induced cells which was not much observed in inhibitor added cultures (10 μM/ml; LC and 20 μM/ml; HC).

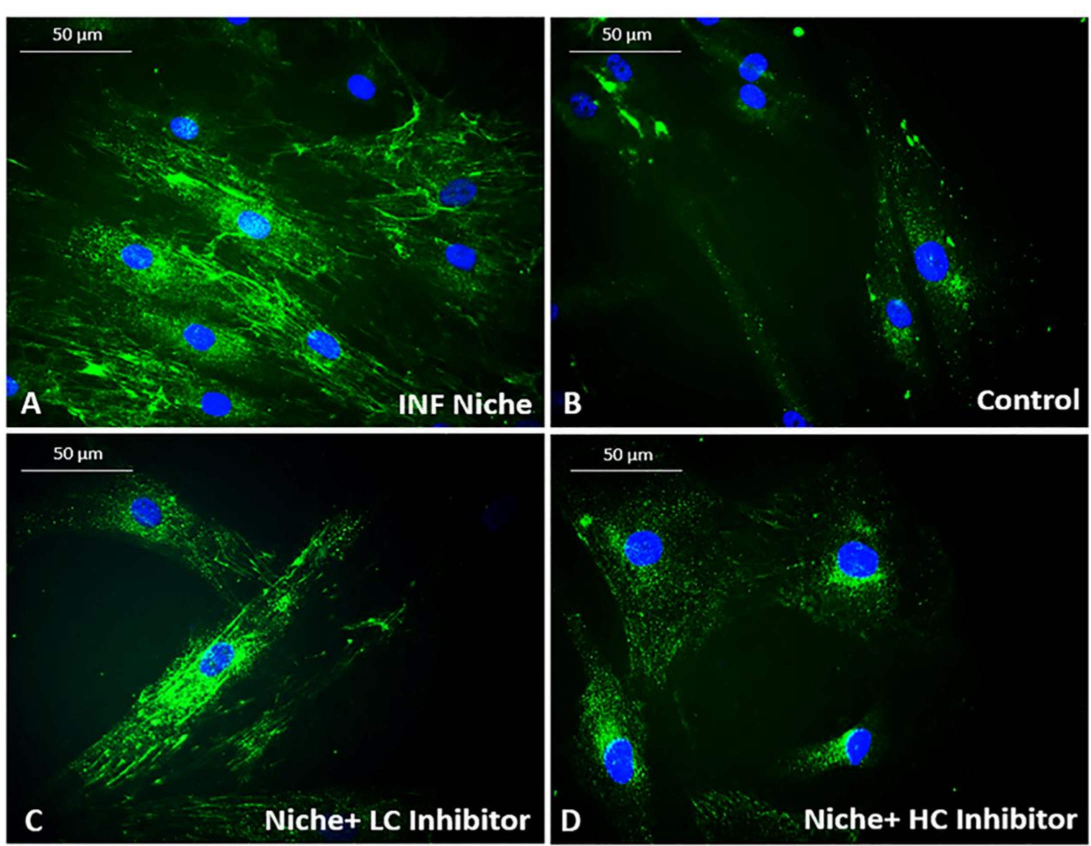


Fig.26 Fluorescence micrograph of cells immunostained with wnt 3a antibody: Cells induced to NS expressing wnt 3a molecules in cytoplasm and ECM (A) niche, INF (B) hADMSC control on bare TCPS (C) Niche added with low concentration of inhibitor (LC; 20 μM/ml) (D) Niche added with a higher concentration of inhibitor (HC; 40 μM/ml). DAPI used as nuclear stain.

Immunostaining of cells using the β-catenin antibody showed a prominent expression of active β-catenin in the nucleus of INF cells. Active β-catenin colocalized with DAPI stain in the nucleus and observed in cyan color indicating active wnt signaling (Fig.27 A). Few control ADMSCs also showed active notch signaling with few β catenin molecules in the nucleus (Fig. 27 B). Inhibitor treated niche lack active β-catenin in the nucleus (Fig. 27 C, D). Red arrows indicate cyan-colored β-catenin molecules in the nucleus.

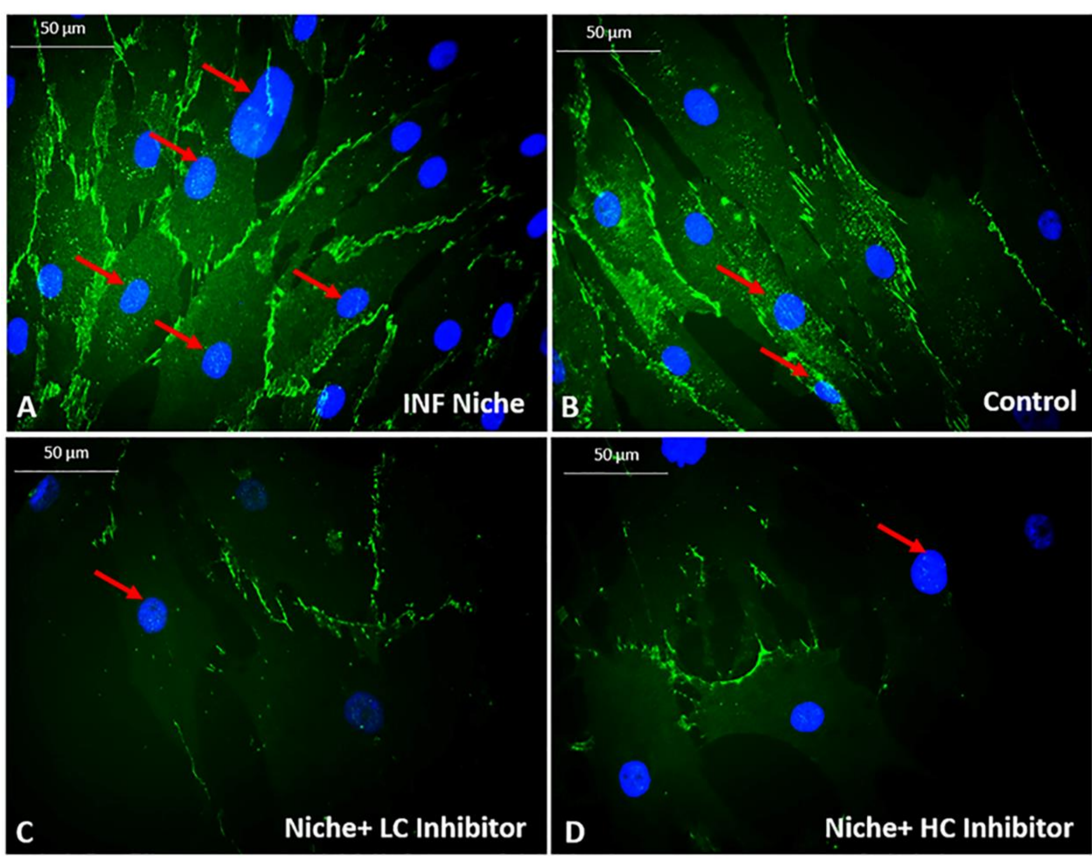


Fig.27 Fluorescence micrograph of cells immunostained with Beta catenin antibody: Cells induced to NS expressing beta-catenin in cytoplasm and nucleus in (A) INF culture (B) hADMSC control on bare TCPS (C)Niche added with 20 µM/ml of inhibitor (D) 40 µM/ml of inhibitor. DAPI used as nuclear stain. Active β-catenin in the nucleus seen in cyan color (Red arrows)

4.2.6 Action of inhibitors of Notch and Wnt on NPC to OPC differentiation

Induced cells started showing OPC like morphology in the INF condition after 4 days of induction (Fig. 28 A). Both notch and wnt inhibitor added cultures did not show any OPC morphology. The cell density in the wnt inhibited culture was comparatively low (Fig.28 D). Also, the morphology of notch and wnt signal inhibited cultures were not comparable to that of induced OPC in fibrin niche. Wnt inhibitor added cultures showed thin elongated neural cell-like morphology (Fig.28 B). ADMSC grown in bare TCPS without any induction was used as the control for the experiments (Fig 28 C).

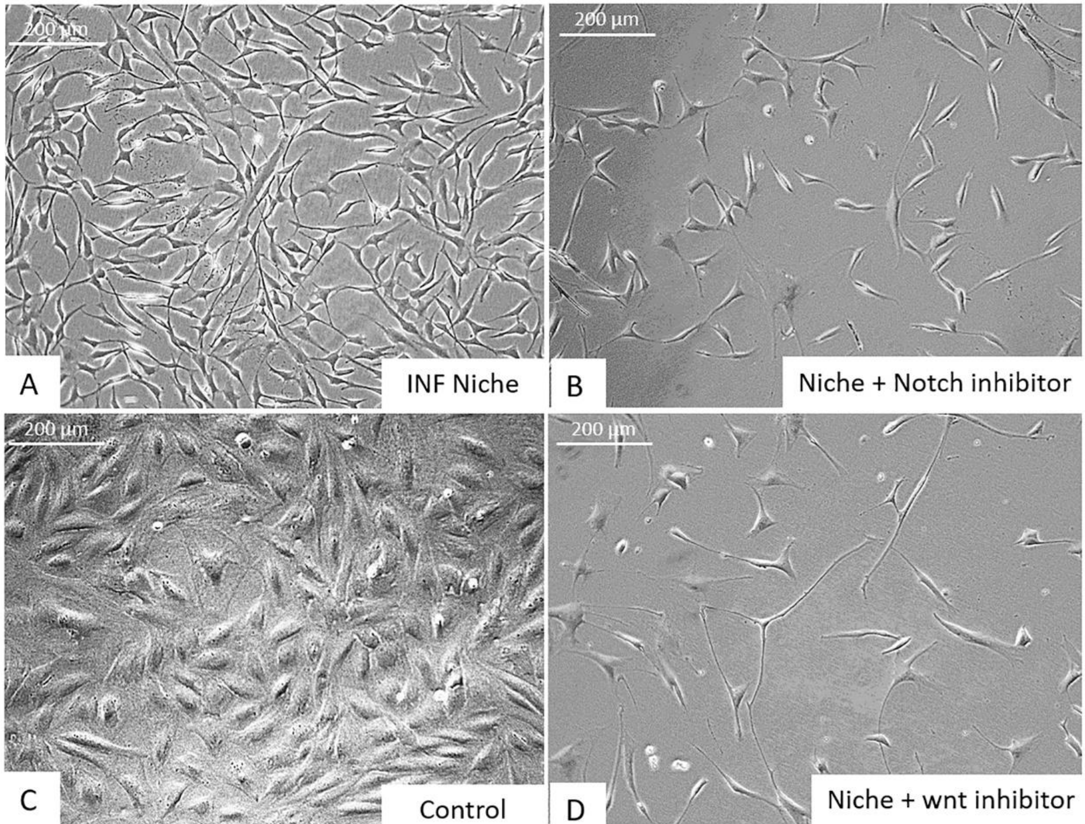


Fig.28 Phase-contrast micrograph showing the morphological effect of signal inhibitors in OPC induction: (A) INF, NPCs induced to OPCs in fibrin niche showing OPC like morphology by day 4 (B) Morphology of notch inhibitor (20 µM/ml) added cultures by day 4 (C) Control hADMSCs grown on bare TCPS on day 4 (D) Thin elongated neuron-like morphology of cells induced on fibrin niche added with wnt inhibitor (40 µM/ml).

4.2.7 Action of inhibitors on Proliferation and OPC gene expression

qRT-PCR analysis indicated a variation in gene expression of OPC markers, OLIG 2 and PDGFRα, and proliferation marker PCNA in different culture conditions (Fig.29). The addition of specific inhibitors to the niche significantly reduced OLIG 2 expression in signal inhibited cultures. Significant upregulation of OLIG 2 mRNA levels was observed in INF culture, PDGFRα expression level was comparable in induced INF culture and specific inhibitor added cultures. However, wnt signal inhibited culture showed downregulation in PDGFRα expression with respect to INF gene expression. PCNA expression was also stable in the upregulated conditions in all three groups.

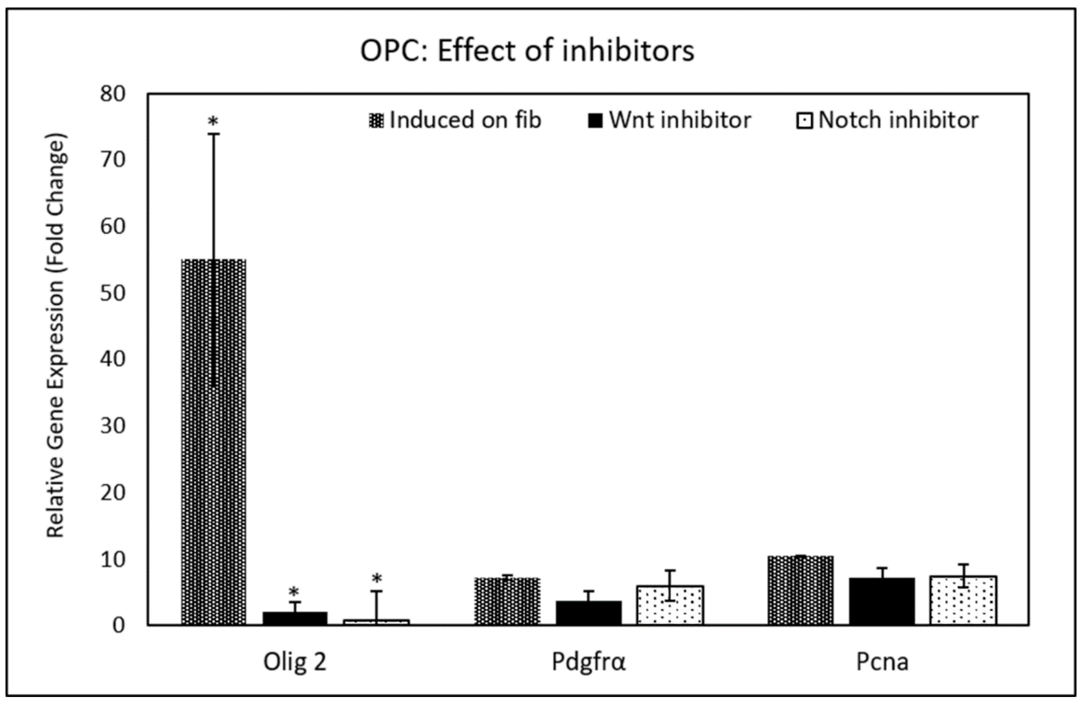


Fig. 29 Graphical representation of qRT-PCR analysis of OPC markers: Analysis of OLIG 2, PDGFRα and PCNA genes after 4 days of OPC induction; ADMSC grown on bare TCPS used as experimental control and GAPDH used as Housekeeping gene; ANOVA: Control, INF, wnt & notch inhibitor; OLIG 2: P=0.02(n=3); '*' (P<0.05) '**' (P<0.01) '***' (P<0.001).

4.2.8 Effect of signaling inhibitors on gene expression of notch downstream molecules.

The qRT-PCR analysis done after 4 days of OPC induction and signal inhibition using 20 µM/ml of notch inhibitor and 40 µM/ml of wnt inhibitor indicated changes in the level of gene expression of various notch downstream markers (Fig 30). NGN 2 gene expression is upregulated in inhibitor added culture compared to that of INF cells indicating active notch signaling in the OPC INF culture. Hes 1 expression is significantly downregulated in inhibitor added culture compared to cells induced on niche confirming active notch signaling elucidated in the niche. The TCF 4 expression was comparatively upregulated in both INF and INF added with inhibitor conditions. Although, a significant effect was not observed in the INF or inhibitor added conditions.

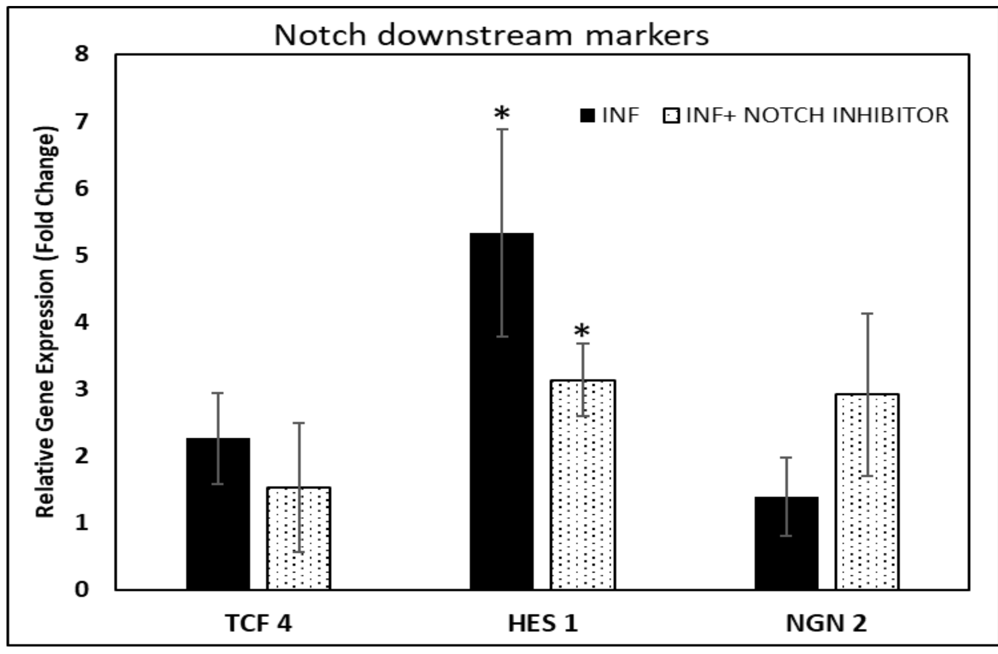


Fig. 30 Graphical representation of qRT-PCR analysis of notch downstream markers: Analysis after 4 days of OPC induction**;** ADMSC grown on bare TCPS used as experimental control and GAPDH used as Housekeeping gene; ANOVA: Control, INF, wnt & notch inhibitor; HES 1: P=0.04(n=3); '*' (P<0.05) '**' (P<0.01) '***' (P<0.001)

4.2.9 Identification of notch and wnt downstream molecules

Active Notch receptor molecule was observed as cyan color in the nucleus by colocalization of notch & DAPI which is indicative of active Notch signaling. The notch is prominent in INF grown cells with a large number of molecules in the nucleus in cyan color (Fig. 31 A). Control ADMSC grown in bare TCPS did not show much active notch molecules in the nucleus (Fig. 31 B). The presence of active notch molecules in the notch is reducing in a concertation dependent manner in the presence of notch inhibitor (10 µM/ml; LC and 20 µM/ml; HC) (Fig 31 C & D).

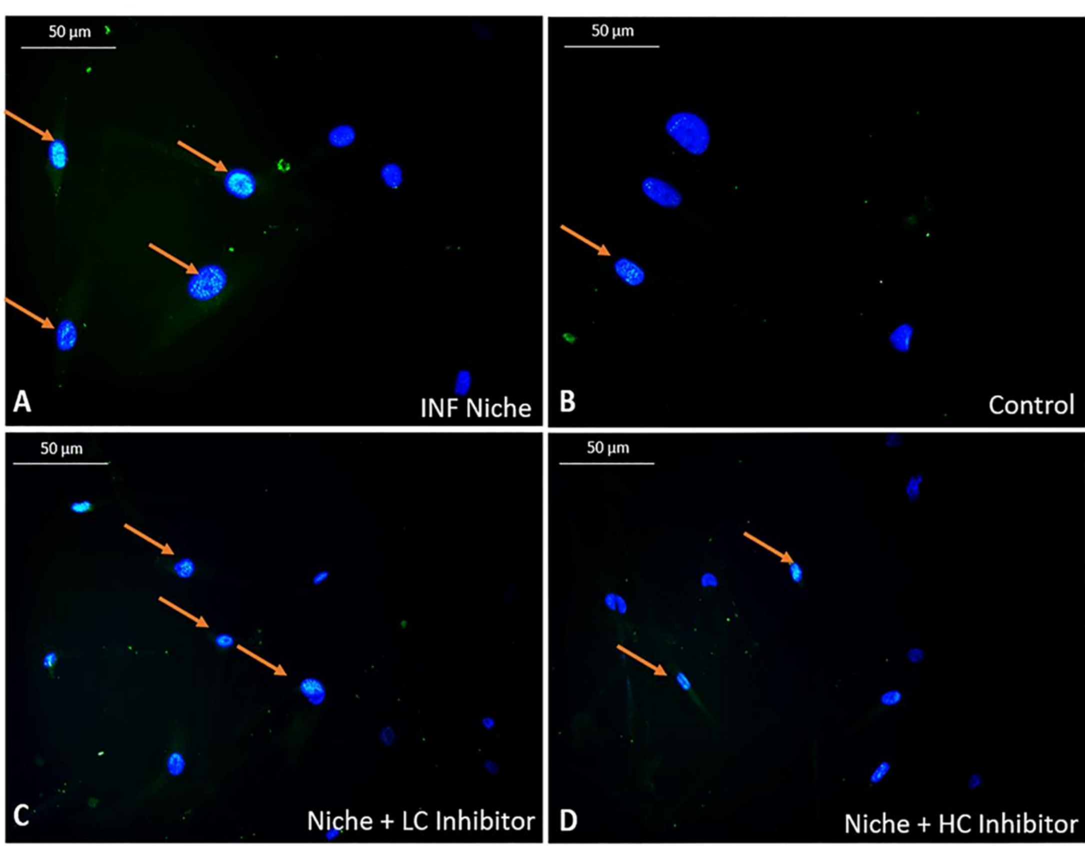


Fig.31 Fluorescence micrograph of OPC immunostained with Notch antibody: (A) Cells induced to OPC in fibrin niche, INF (B) hADMSC control on bare TCPS (C) Cells in niche added with LC (10 µM/ml) inhibitor (D) Cells in niche added with HC (20 µM/ml) inhibitor. DAPI used as nuclear stain. Orange arrows indicate notch molecules in the nucleus colocalized with DAPI.

HES 1 protein was prominently expressed in the nucleus of cells immunostained with HES 1 antibody in all culture conditions. The observable difference was not seen in induced INF cells (Fig. 32 A) and control ADMSCs (Fig.32 B). The induced cells added with notch inhibitor showed a lesser number of DAPI stained nucleus in a concentration-dependent manner indicative of a reduction in cell number (Fig.32 C, D).

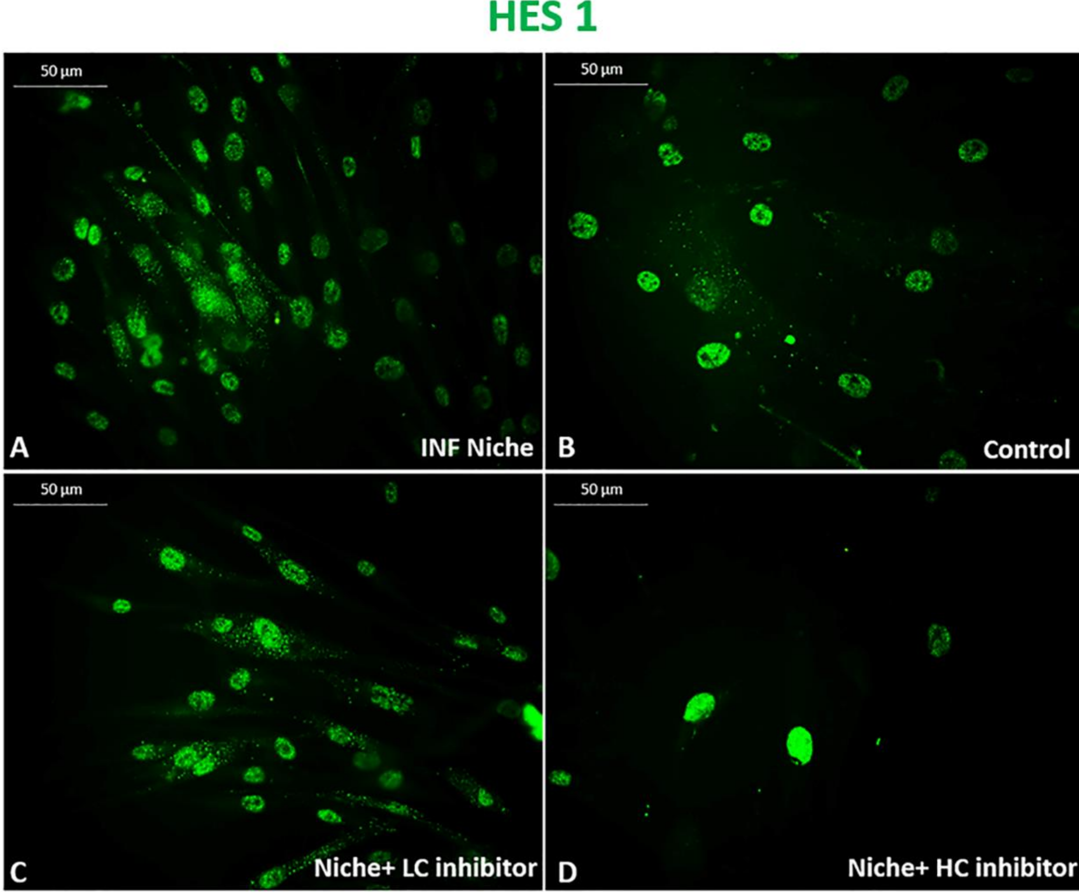


Fig.32 Fluorescence micrograph of induced OPCs immunostained with HES 1 antibody: Cells induced to OPCs showing HES 1transcription factor in the nucleus (A) cells induced in INF (B) hADMSC control on bare TCPS (C)Cells in niche added with a low concentration of inhibitor (LC; 10 µM/ml) (D) Cells in niche added with a higher concentration of inhibitor (HC; 20 µM/ml).

Immunostaining of the induced cells in fibrin and wnt inhibitor added cells showed expression of wnt 3a molecules in ECM and cytoplasm. Wnt 3a is prominently expressed in OPC induced in fibrin niche or INF cells (Fig.33 A) and it was not evident in ADMSCs grown on bare TCPS (Fig.33 B). Inhibitor added cultures showed the disappearance of wnt molecule from the extracellular matrix of the cells (Fig.33 C, D).

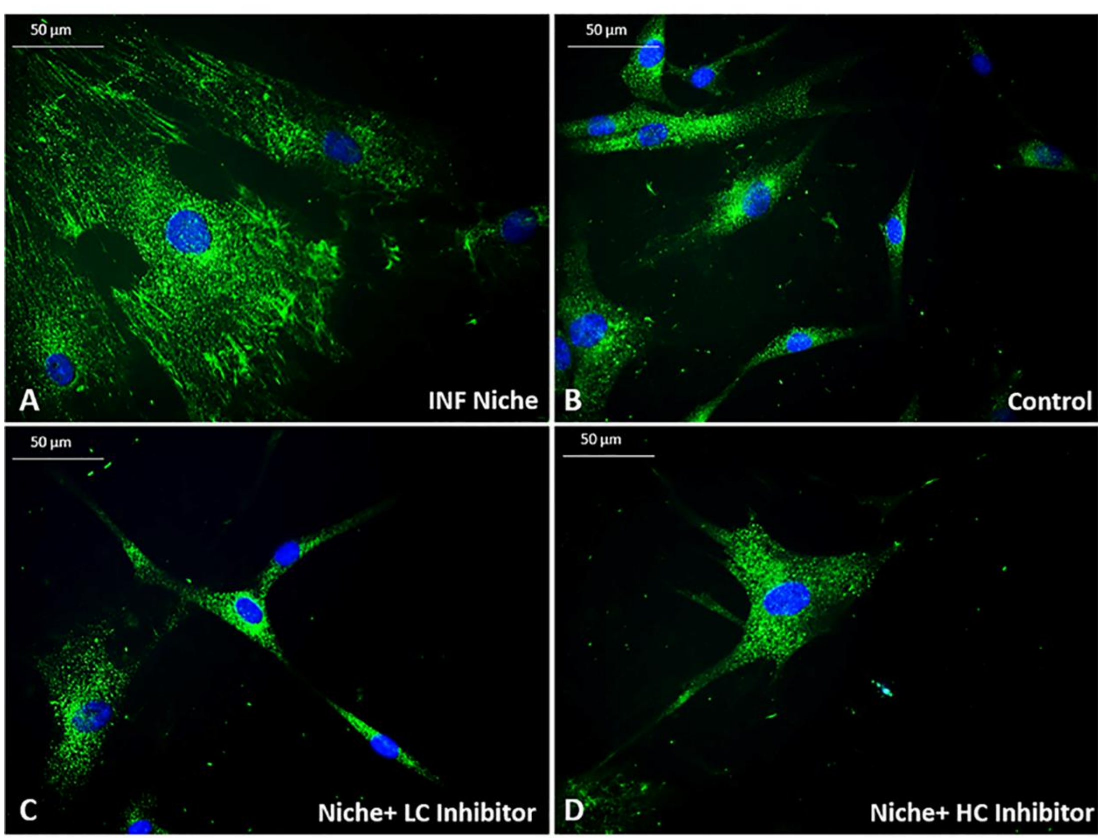


Fig.33 Fluorescence micrograph of cells immunostained with wnt 3a antibody: Cells induced to OPC expressing wnt 3a molecules in (A) cells induced in the niche, INF (B) hADMSC control on bare TCPS (C) Niche added with a low concentration of inhibitor (LC; 20 µM/ml) (D) Niche added with a higher concentration of inhibitor (HC; 40 µM/ml). DAPI used as the nuclear stain.

Immunostaining of induced OPC cells using the β-catenin antibody showed prominent expression of β-catenin in the nucleus of INF cells. Active β-catenin colocalized with DAPI stain in the nucleus and observed in cyan color (Fig.34 A). Active beta-catenin

molecules were observed in a few of the immunostained ADMSC controls. (Fig. 34 B). Inhibitor treated niche lacks prominently active β-catenin in the nucleus (Fig. 34 C, D). Red arrows indicate cyan-colored β-catenin molecules in the nucleus.

β-Catenin/DAPI

Fig.34 Fluorescence micrograph of cells immunostained with Beta-catenin antibody: (A) Cells induced to NS, INF (B) hADMSC control on bare TCPS (C)Niche added with a low concentration of inhibitor (LC; 20 µM/ml) (D) Niche added with a higher concentration of inhibitor (HC; 40 µM/ml). DAPI used as the nuclear stain. Active β-catenin in nucleus colocalized with DAPI indicated in orange arrows

4.3 Transplantation of rADMSC derived NPCs and OPCs in rat SCI models.

This section includes isolation of rADMSC (SCT/IAEC-220/MARCH/2017/9), its differentiation to NPCs and OPCs using protocol standardized using human ADMSCs in niche and transplantation to the rat SCI contusion site

4.3.1 rADMSC isolation and induction to NS

Rat ADMSCs (rADMSC) were isolated using protocol standardized for human ADMSC isolation (#4.1.1).

Cells were plastic adherent with spindle-shaped ADMSC morphology. The rADMSCs induced to form NS in fibrin niche started grouping by day 3 (Fig 35 A). These groups became denser and formed spheres by day 5 (Fig.35 B). A large number of spheres were formed in the culture. DAPI staining of the NS showed the presence of a large number of the nucleus in the sphere (Fig. 35 C).

Quantitative analysis of NES gene expression in induced NS using qRT-PCR showed upregulation in the NES expression at mRNA level compared to ADMSC grown in bare TCPS (Fig.36).

This was confirmed at the protein level using immunocytochemistry of the NS derived from rADMSC. The Nestin protein was prominently expressed in the NS cells (Fig.35 D). Lower magnification of NS (10x) stained with the NES antibody is shown as an inset.

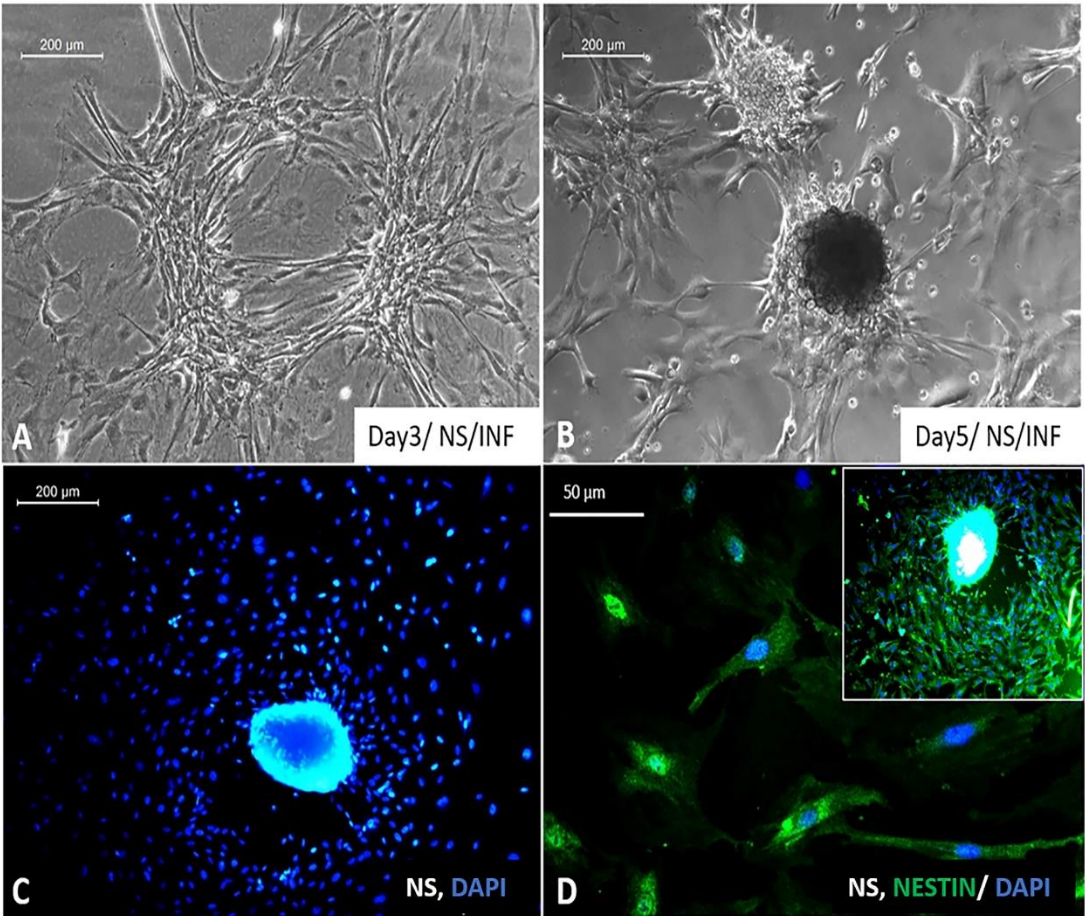


Fig. 35 Analysis of rat NS derived from rADMSC: (A) Phase-contrast micrograph showing the grouping of induced rADMSCs by day 3 in fibrin niche (B) NS derived from rADMSC by day 5 (C) Fluorescent micrograph showing NS stained with DAPI, nuclear stain (D) Nestin immunostained NS (40x magnification of cells in the periphery of NS in the inset) Smaller magnification (10x) in the inset.

Quantitative estimation of Nestin immunostained cells in rat NS induced in fibrin niche showed ~20-40% positivity. Flow cytometric analysis data from replicate experiments (n=3) has been compiled and presented as individual histograms and bar diagram (Fig.36 B).

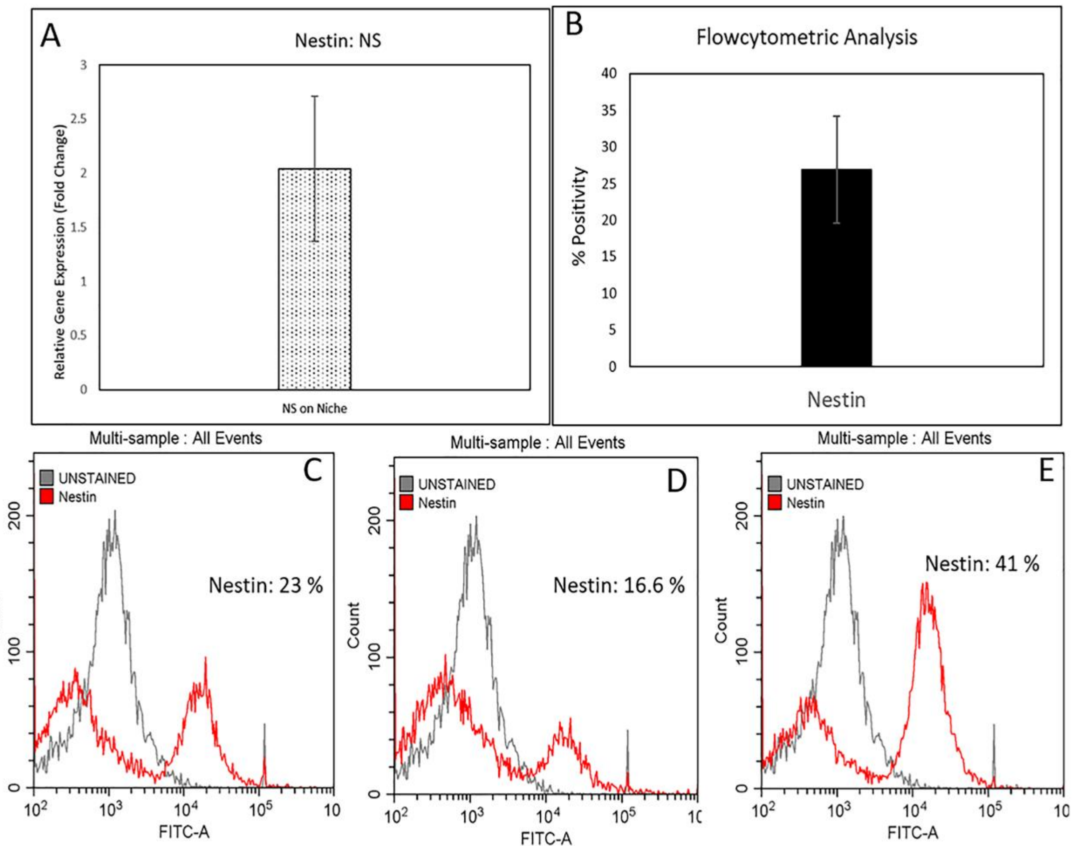


Fig.36 Expression of Nestin in rat NS: (A) Graphical representation showing qRT-PCR analysis of NES gene expression in rat NS. rADMSC in bare TCPS used as experimental control and GAPDH used as the housekeeping gene, n=3. **Flow cytometric analysis of induced NS:** (B)Graphical representation of the flow cytometry data(n=3). The error bar represents SEM. The histograms (CytExpert software) representing Nestin positivity in induced NS in fibrin (C) Donor 1 (D) Donor 2 (E) Donor 3. Unstained ADMSC used as a control as the primary antibody is conjugated.

4.3.2 Reseeding of rADMSC derived NS

Rat ADMSCs cultured in NS induction medium formed 20-30 spheres per 1.75 cm^2 by day 7 (Fig. 37 A). Manually picked and reseeded NS attached and started proliferating after 24 h (Fig.37 B). By day 4 the NS derived cells became confluent in 1.75 cm^2 dishes (Fig. 37 C)

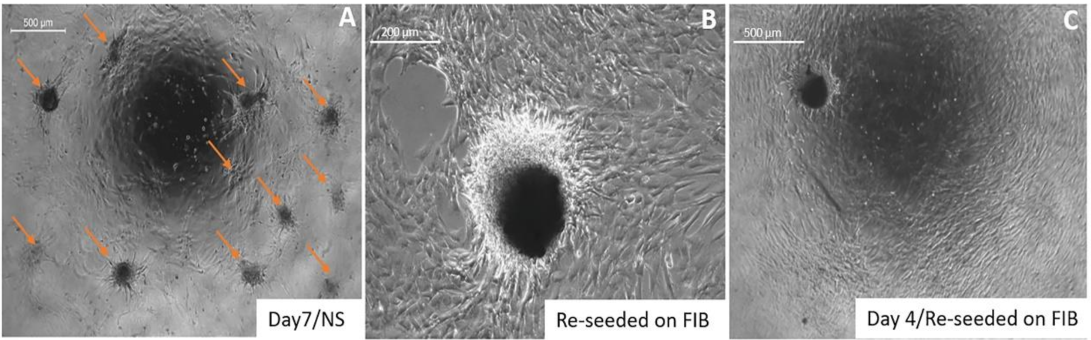


Fig. 37 Phase-contrast micrograph of NS formation and reseeding: (A) NS formation upon induction in the niche (B) Reseeded niche in fibrin niche (C) Reseeded NS confluent in 4 days (4x magnification).

4.3.3 Induction of rat NS to NPCs

rADMSC derived NS induced to NPCs in fibrin niche in NPC induction medium showed NPC like thin elongated morphology in 4 days (Fig.38 A). The induced cells were immunopositive for Nestin antibody and immunostaining implied that a major population of the induced cells were nestin-positive (Fig. 38 B).

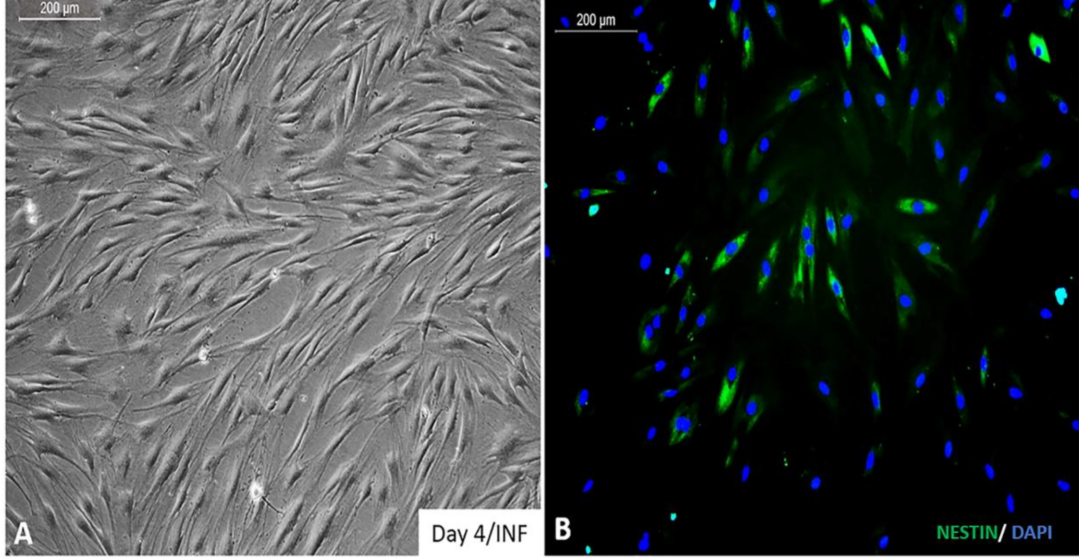


Fig.38 Induction of rat NS to NPCs in fibrin niche:(A) Phase-contrast micrograph of NPCs derived from rat NS (B) Fluorescence micrograph of NPCs stained with nestin. DAPI used as the nuclear stain.

The results of immunocytochemistry were further established with flow cytometry using TUJ 1 antibody staining. ~50% of the population were TUJ 1 positive (n=3) confirming findings obtained from ICC (Fig. 39)

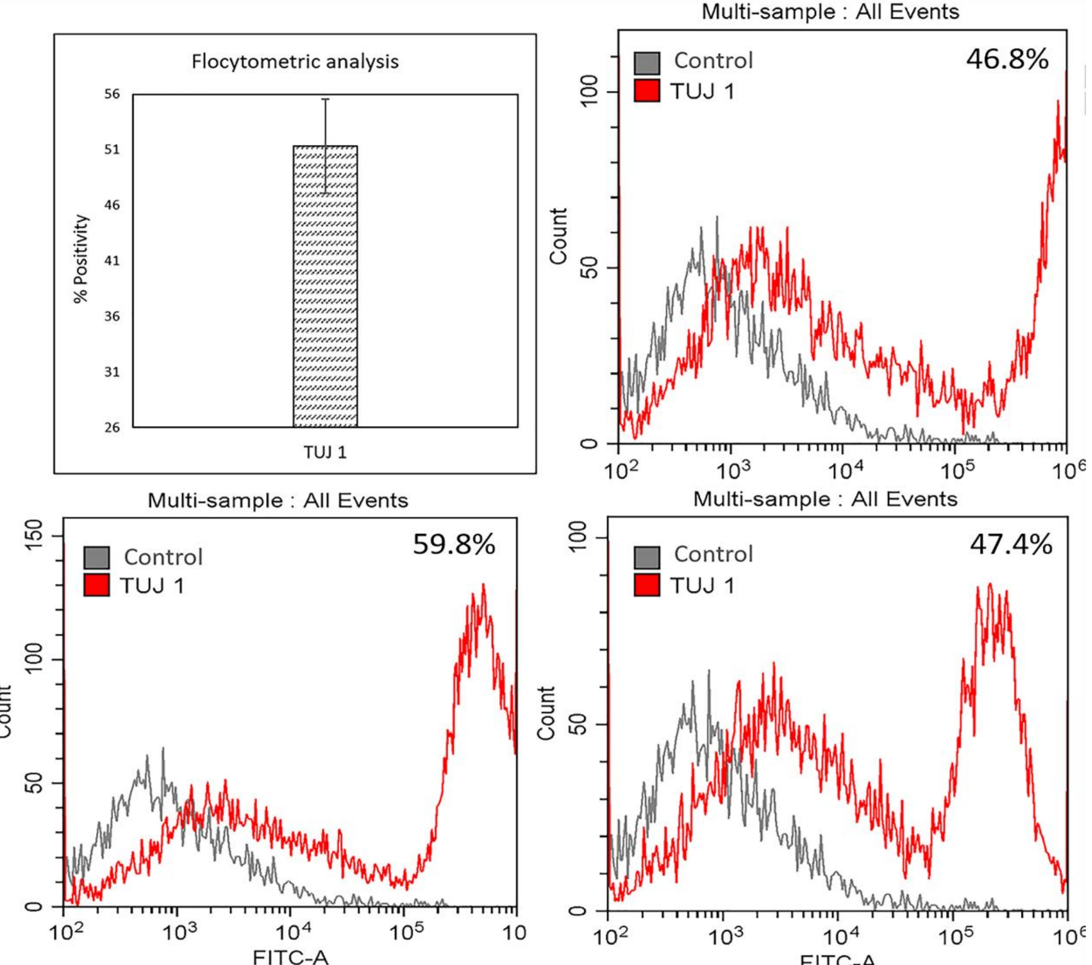


Fig. 39 Histograms showing TUJ1 positive population in NPCs induced from rat NPCs: (A)Graphical representation of the flow cytometry data(n=3). The error bar represents SEM. The histograms (CytExpert software) representing TUJ 1 positivity in induced NPCs in fibrin (B) Donor 1 (C) Donor 2 (D) Donor 3. Secondary antibody alone stained induced cells used as control.

4.3.4 Induction of rat NPCs to OPCs

A change in morphology was observed in the induced OPC culture and typical OPC like cells started appearing in culture after the induction period of 4 days (Fig. 40 A).

qRT-PCR analysis of OPC markers, OLIG 2, and PDGFRα showed an upregulation of these specific markers at mRNA level compared to normal ADMSCs grown on bare TCPS (Fig 40 B). Also, the immuno-staining of specific OPC markers with OLIG 2 and PDGFRα antibody confirmed the expression of these markers at the protein level. Prominent expression of OLIG 2 in the nucleus (Fig. 40 C) and PDGFRα in the cell surface membrane (Fig. 40 D) was observed in the induced cell culture.

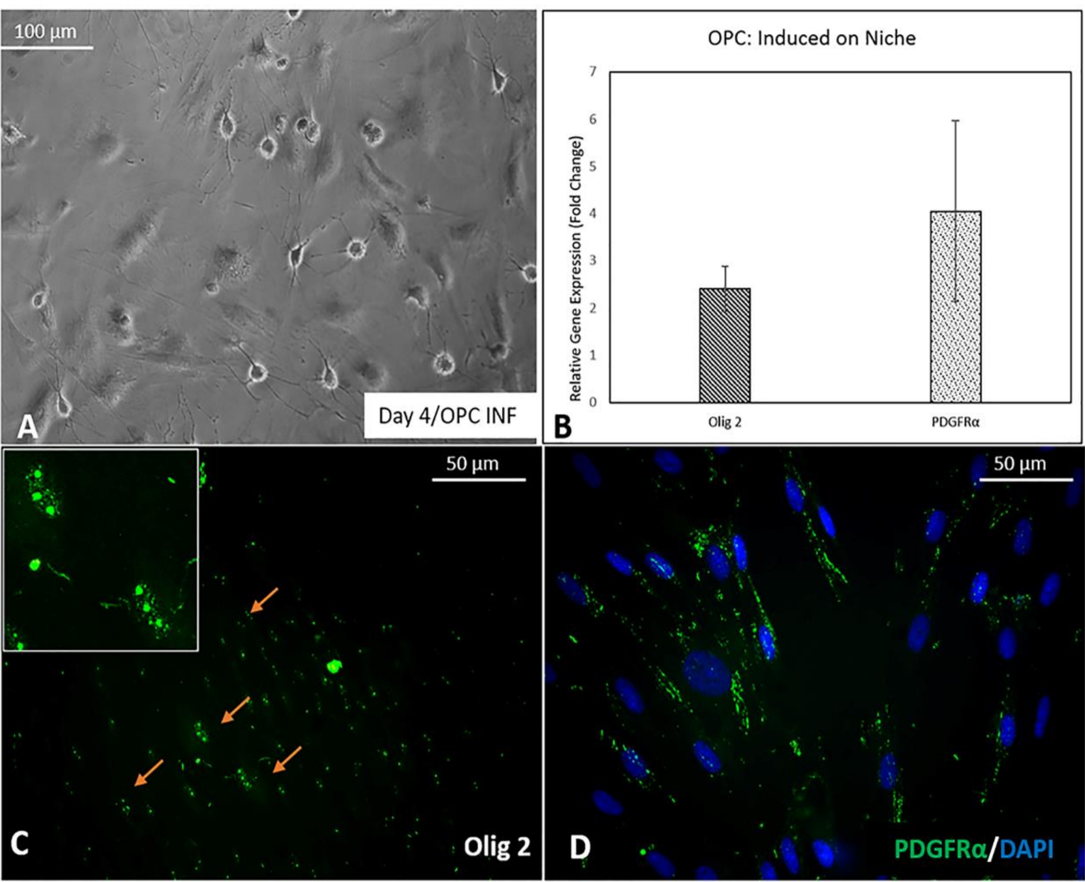


Fig.40 Induction of rat NPC to OPCs in fibrin niche:(A) Phase-contrast micrograph of induced OPC (B) Graphical representation of qRT-PCR analysis of the OPC markers (n=3). rADMSC cultured in bare TCPS used as experimental control and GAPDH used as Housekeeping gene. (C) Fluorescence micrograph of OPCs stained with OLIG 2 antibody. (D) Fluorescence micrograph of OPCs stained with PDGFRα antibody. Nucleus stained with DAPI.

4.3.5 Terminal differentiation of OPCs to OLGs in the niche.

Upon induction of terminal differentiation, the OPCs showed a change in morphology towards OLGs. Typical OLG like morphology with elongated processes started appearing in the induced cells in 48 h (Fig.41). After 3-4 days the cells started detaching from the culture plates and the cell numbers started reducing.

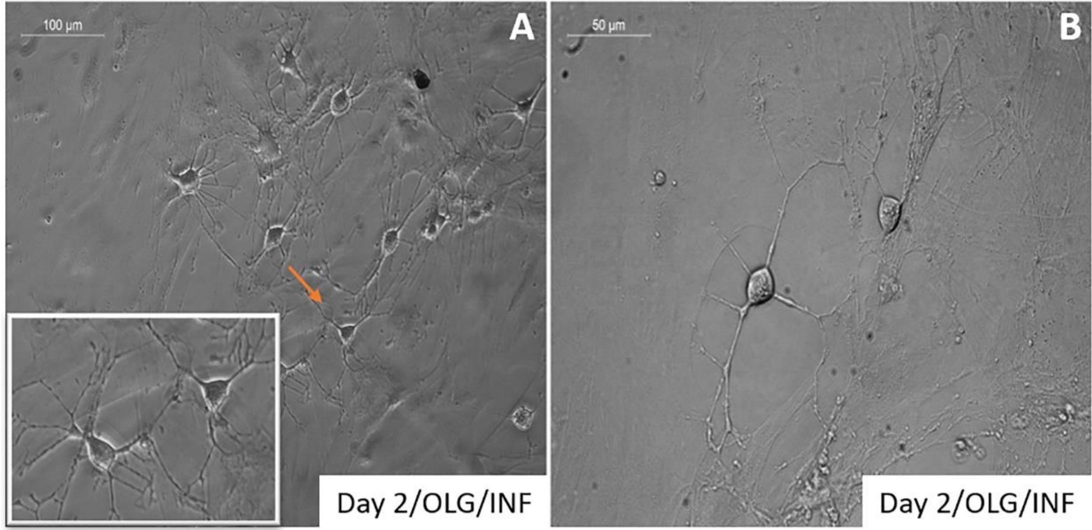


Fig. 41 Phase-contrast micrograph of OLGs derived from induced OPCs: (A)Grayscale phase-contrast Micrograph, 20X magnification of induced OLGs. Cells showing typical OLG morphology indicated in yellow arrow (ROI) and ROI is shown as inset (B) Higher magnification of induced cell showing typical OLG morphology (40 x magnification).

The quantitative analysis of OLG mRNA levels using RT-PCR showed significant upregulation in gene expression of OLG markers, MBP, and OSP. Both MBP and OSP markers, associated with functional OLGs were showing an over 5-fold increase in mRNA levels (Fig. 42 A). The gene expression data of MBP was comparable to that of the qualitative protein expression data. The cells stained with MBP antibody showed prominent expression of MBP protein (Fig. 42 B).

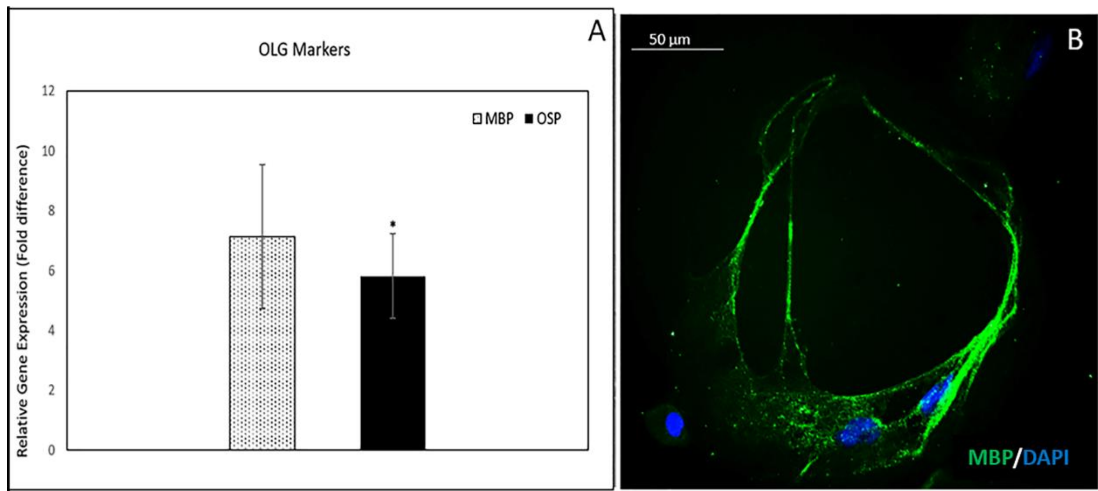


Fig. 42 Analysis of OLGs derived from induced OPCs in fibrin niche: (A) Graphical representation of qRT-PCR data upregulation of gene expression of MBP and OSP; student's t-test: Control, INF, OSP; P=0.04(n=3); rADMSC grown in bare TCPS used as experimental control and GAPDH used as Housekeeping gene. ('*' (P<0.05)) ('*' (P<0.05) '**' (P<0.01) '***' (P<0.001)) (B) Fluorescence micrograph of OLGs stained with MBP antibody. Nucleus stained with DAPI.

4.3.6 Creation of SCI model and *In vivo* Cell Transplantation

Laminectomy was done to expose the rat spinal cord at the Thoracic 10 (T10) position (Fig. 43 D). Then a custom-made impactor was used to provide contusion injury (150 kilodyne) (Fig. 43 E). Response to the contusion injury or the impact was observed as tail twitching of the anesthetized animal. The hematoma was observed at the site of injury (Fig. 43 F; Yellow arrow). The cells/media was given as an injection (15 µL) to the site of injury.

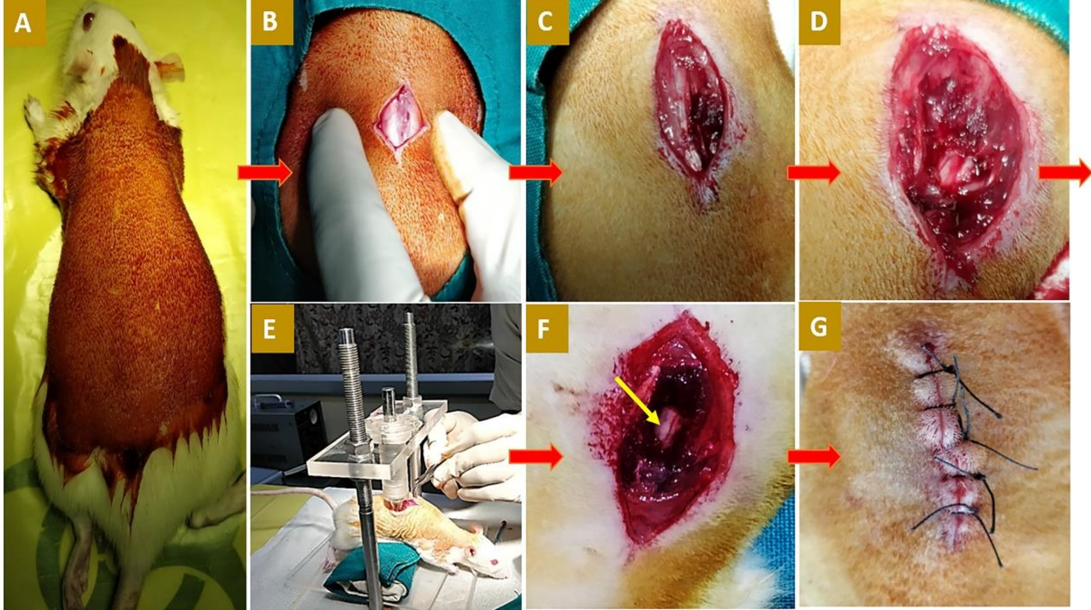


Fig. 43 SCI contusion model development: (A-C) Incision was made and the paravertebral muscles were removed at T10 position of the vertebrae (D) Laminectomy was done to expose spinal cord (E) Contusion injury was made using 150 kilodyne impact using impactor (F) Hematoma development was observed at the injury site (G) After cell/ media transplantation by 15 µl injection, incisions were sutured with non-absorbable silk thread.

4.3.7 Clinical symptom development in SCI animals

All the 28 animals developed paraplegia after the injury. Hind limb movement was restricted in all animals (Fig 44 A). The animals with SCI showed urine retention in the bladder (Fig. 44 C). Digital evacuation of urine was done twice a day. The period of observation was 28 days. All animals survived till 28 days and the explant was collected after the period of observation (Fig 44 B).

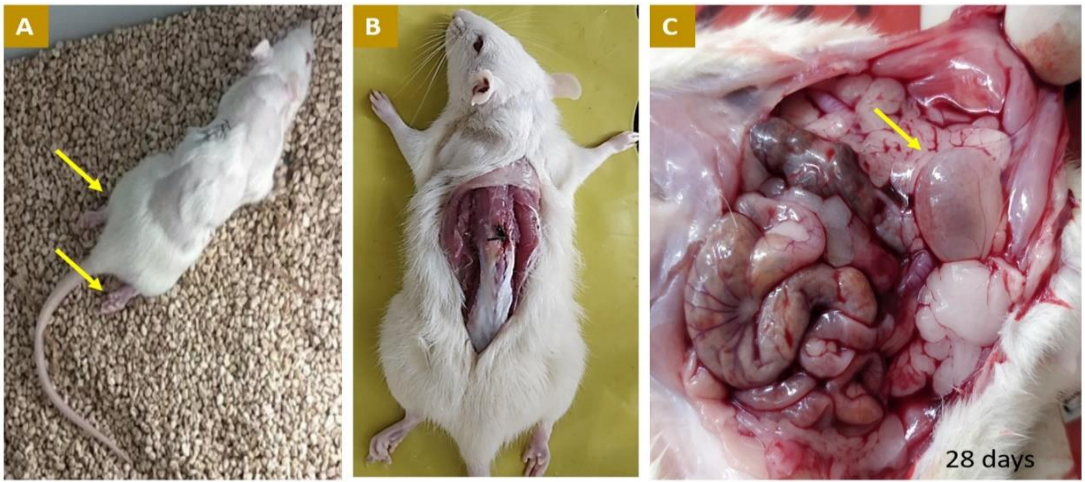
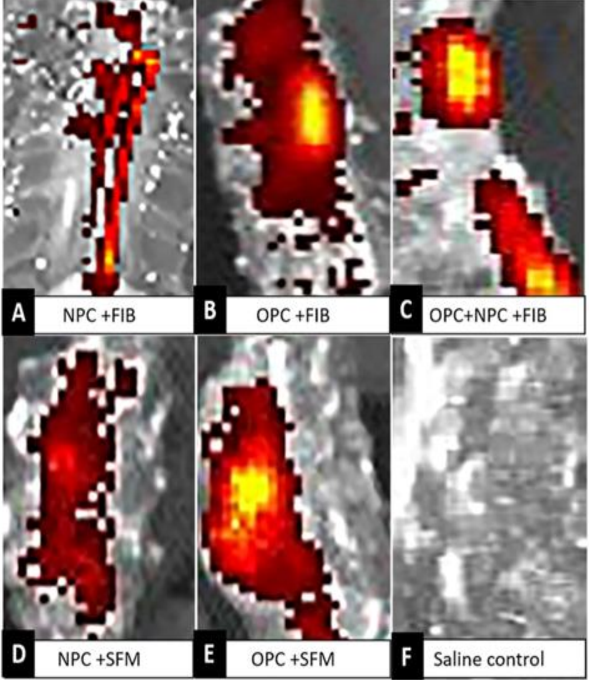


Fig. 44 Clinical symptoms developed in SCI animals: (A) SCI animal with paraplegia (B) After 28 days explant was collected for further analysis (C) Urine retention in the bladder indicating paraplegia.

4.3.8 Survival and differentiation of transplanted cells

Cells survival was assessed by IVIS imaging of the explant collected after 28 days and it indicated that the transplanted cells were retained until 28 days in all groups at the site of injury. However, the transplanted OPCs in SFM (Fig 45 E) /Fibrin (Fig. 45 B) showed high-intensity signals compared to transplanted NPCs in fibrin (Fig 38 A) and SFM (Fig 45 D). OPCs and NPCs transplanted together in the Fibrin matrix also showed high-intensity signals (Fig. 45 C). Negative control used in the imaging was the saline-injected group with no tracking dye or fluorescence (Fig. 45 F)



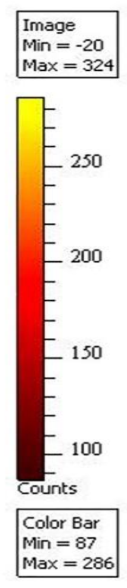


Fig.45 IVIS imaging of the transplanted cells: Yellow color indicates high-intensity fluorescence from the cells retained at the site of injury after 28 days in (A) NPC in fibrin matrix (B) OPC in fibrin matrix (C) OPC and NPC together in fibrin matrix (D) NPC in serum-free media (E) OPC in serum-free media. (F) The negative control, Saline alone injected group

Fluorescence microscopy of the stained sections indicated the co-localization of nuclear stain DAPI and PKH26, confirming the survival of transplanted cells (Fig. 46). The results observed were consistent with that of the IVIS imaging results. The negative control group did not show any fluorescence assuring the specificity of the tracking dye used. In tissue sections of animals received OPC and NPC together in fibrin appearance of PKH 26 labeled cells colocalized with DAPI was more prominent compared to other groups.

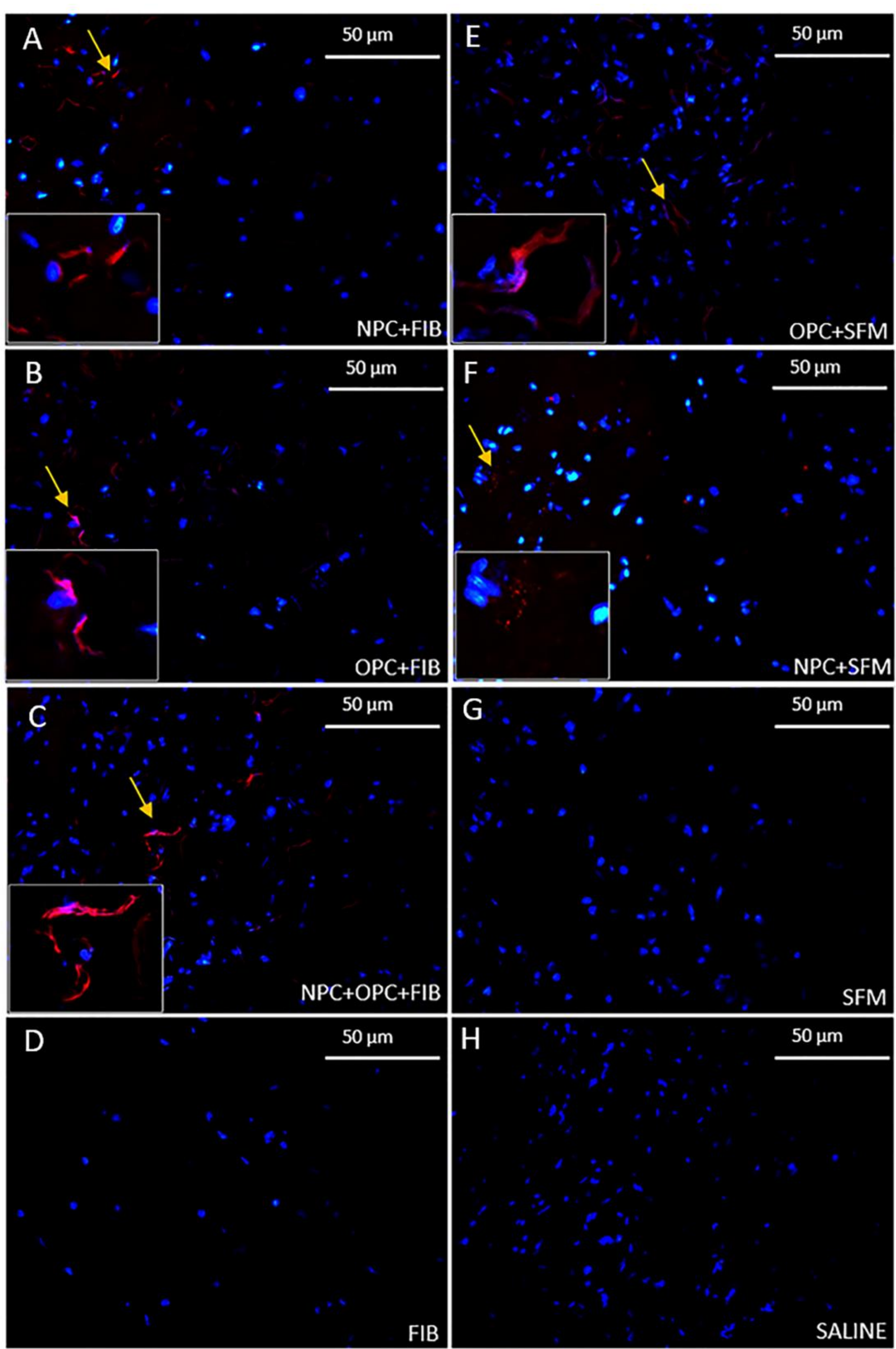


Fig. 46 Fluorescence micrographs tissue showing cells transplanted at SCI: DAPI stained nucleus in blue color and PKH26 labeled cells in red color (A) NPC transplanted in fibrin (B)OPC in fibrin (C)NPC and OPC together in fibrin (D) Fibrin alone injected, Negative control (E) OPC transplanted in SFM (F) NPC in SFM (G) Saline & (H) SFM alone injected, Negative controls.

The MBP immunostaining of the cryosection of the injury site of OPC+ Fibrin treated animals showed the colocalization of PKH26 with MBP in orange color which is indicative of the differentiation of the transplanted cells (Fig 47 D).

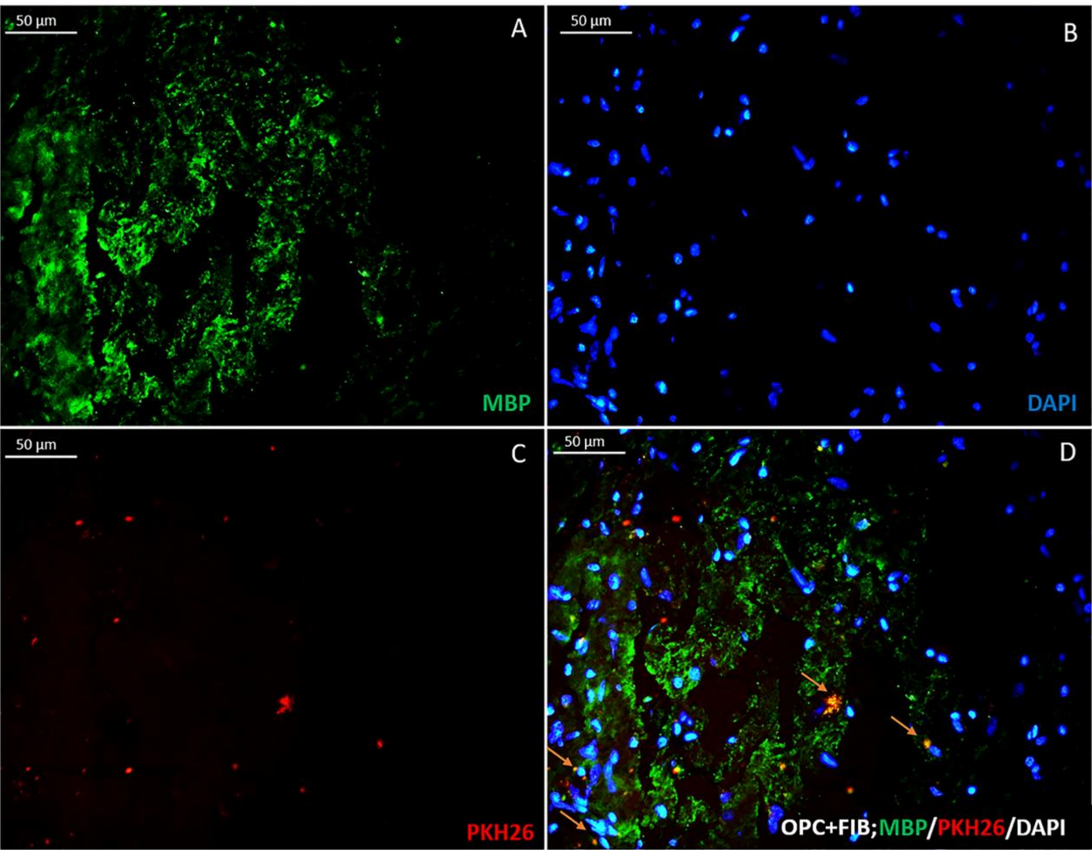


Fig. 47 Immunohistochemistry of the SCI contusion site: Fluorescent micrograph showing injury site stained with MBP antibody in OPC+ Fibrin treated group (A) MBP immunostained tissue (B) DAPI used as nuclear stain (C) PKH26 tracker dye stained cells (D) The merger image showing PKH26-MBP co-staining in orange color (Indicated by orange arrows).

4.3.9 Clinical outcome of cell transplantation in SCI

In all the animals, weight loss was observed post-injury and after the 2nd or 3rd week, the body weight was stable in all groups (Fig. 48). Some of the animals showed a gain in body weight after 2nd week. None of the animals regained their initial body weight after the injury.

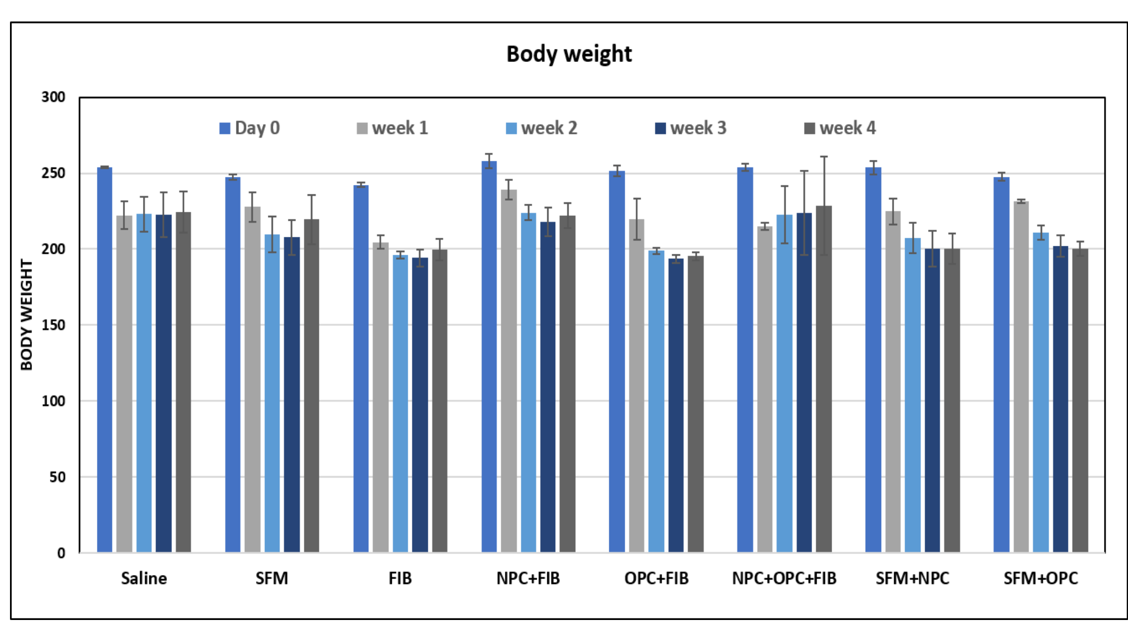


Fig.48 Change in body weight over 4 weeks after SCI induction in rats

The post-injury locomotory function was assessed using the Basso, Beattie, and Bresnahan (BBB) scoring (Fig 49). Compared to the first week all the animals improved their BBB score by 4th week. Significant improvement was observed in the fibrin alone treated group. By the fourth week, the BBB scoring of the Fibrin alone treated group was significantly higher than that of the untreated saline-injected group. However, based on the average BBB score, none of the animals regained the locomotory functions. Animal to animal variation was very high in each group.

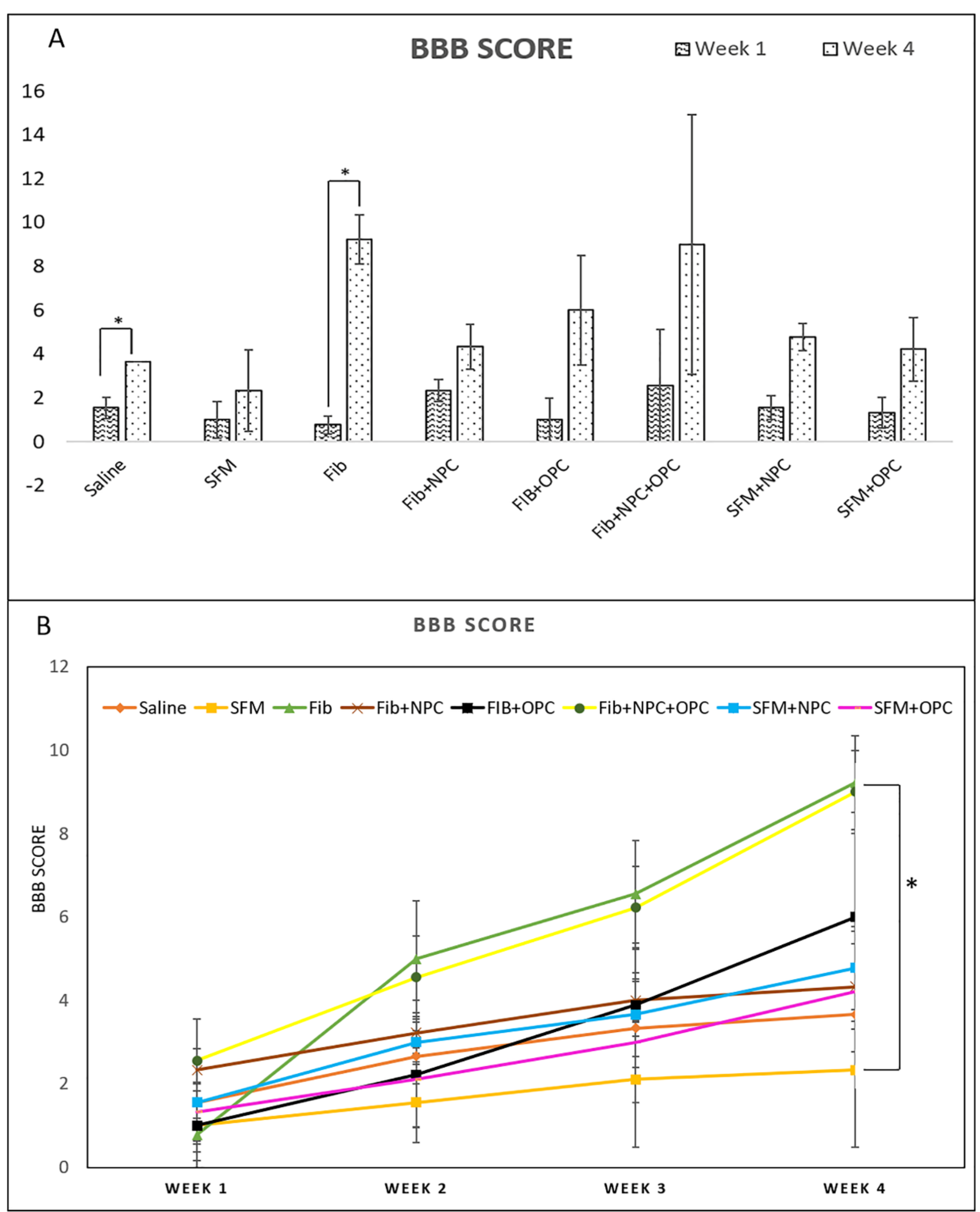


Fig 49: BBB scoring of the SCI animals (A) BBB score 1st and 4th week: BBB scoring of animals calculated after every 7 days. T-test was done to calculate the statistical significance between first- and fourth-week BBB scores of each group (n=3). Error bar represents SEM (B) BBB score 1st to 4th week. T-test was done to calculate the statistical significance between the saline group and other groups at 4th week. (n=3). Error bar represents SEM. ('*' (P<0.05)) ('*' (P<0.05) '**' (P<0.01) '***' (P<0.001)).

4.3.10 Histopathological Evaluation of the Tissue

The grossing of the formaldehyde-fixed injured spinal cord revealed the extent of contusion injury and tissue loss in each sample (Fig.50). Contusion injury was developed in all animals. The animals received OPC and NPC in the fibrin matrix (Fig 50 C) and NPC in SFM (Fig. 50 G) showed comparatively lower tissue loss than other groups. The control group, a saline injection given animal shows complete contusion injury (Fig. 50 A).

Fig. 50 Gross image of rat spinal cord after 28 days of observation: (A) saline control (B) SFM control (C) OPC and NPC transplanted together in fibrin matrix (D) Fibrin alone (E) OPC injected in fibrin matrix (F) NPC transplanted in Fibrin matrix (G) NPC transplanted in SFM (H) OPC transplanted in SFM.

The H and E staining of the injured tissue from different groups indicated tissue loss and degeneration of cells followed by injury (Fig.51). Injury section from the control groups, Saline control, SFM control, and Fibrin control showed larger cell loss and degeneration compared to cell treated one (Fig. 51 A, B, E). Moderate cavitation was observed in the Saline and Fibrin control. The SFM control, OPC+ SFM and NPC +SFM treated groups (Fig.51 B-D) showed more pronounced cavitation which was

absent in the animals that used fibrin as the cell delivery matrix (Fig. 51 F-H). The tissue loss was also moderate in the groups that used cells and fibrin delivery matrix together.

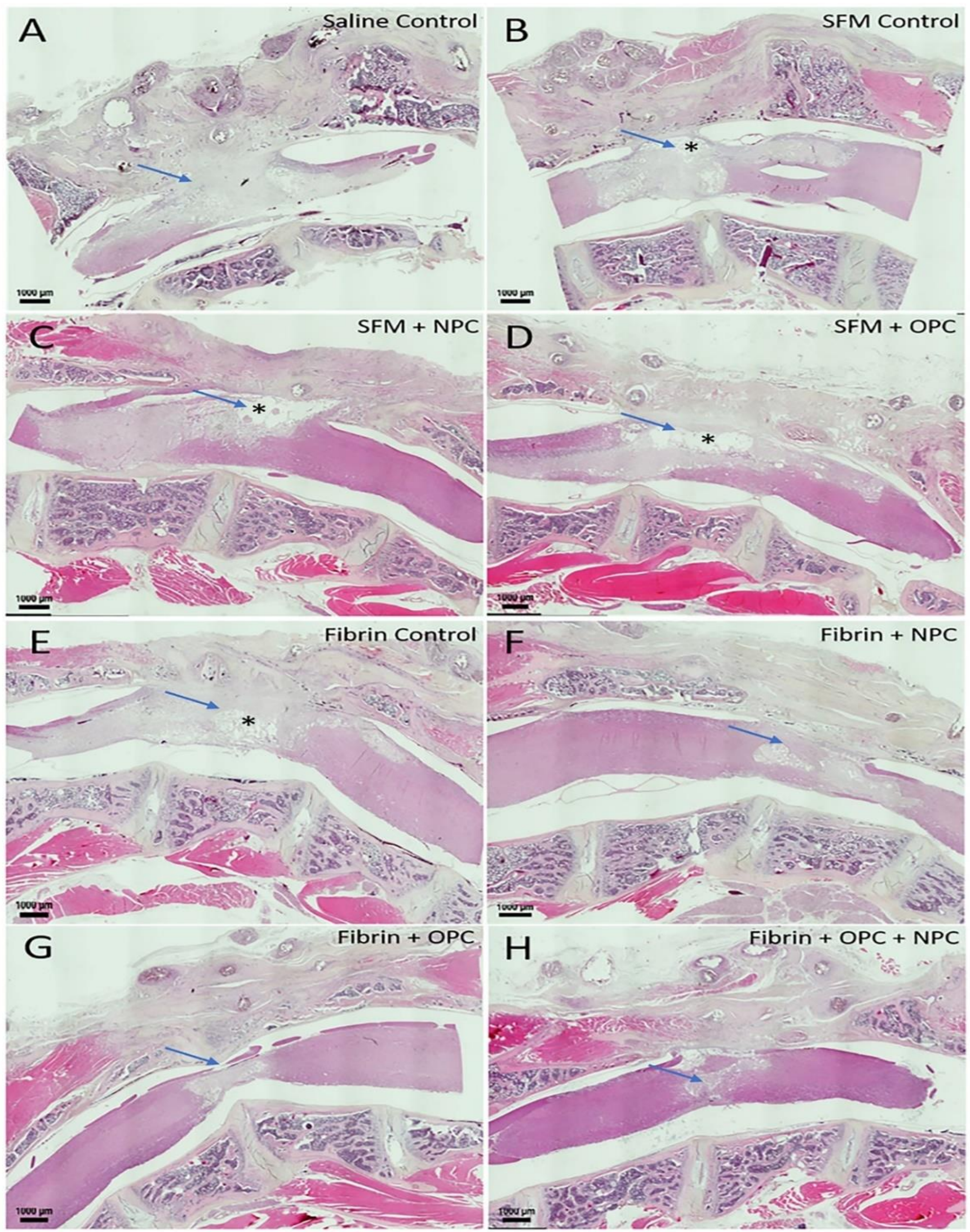


Fig. 51 Micrographs showing H & E staining of the injured tissue: (A)Saline control (B) SFM (C) SFM+NPC (D) SFM+OPC (E) Fibrin control (F) Fibrin+NPC (G) Fibrin +OPC (H) Fibrin+NPC+OPC; Injury region with tissue loss indicated in blue arrows. '*' indicates Cavitation.

The ED1 macrophages, Kupffer cells, monocytes, giant cells, or activated microglia in the injury sections were stained positive for CD 68 immunostaining (Fig.52). In the treatment groups, saline control, SFM control, SFM + NPC, and SFM + OPC the number of ED1 positive cell numbers was high (Fig.52 A-D). The groups which received fibrin as the delivery matrix showed a lesser number of CD 68 positive cells. The immunomodulatory effect of fibrin was evident in the groups- Fibrin alone, Fibrin + OPC, Fibrin +NPC, and Fibrin +OPC+ NPC (Fig.52 E-H).

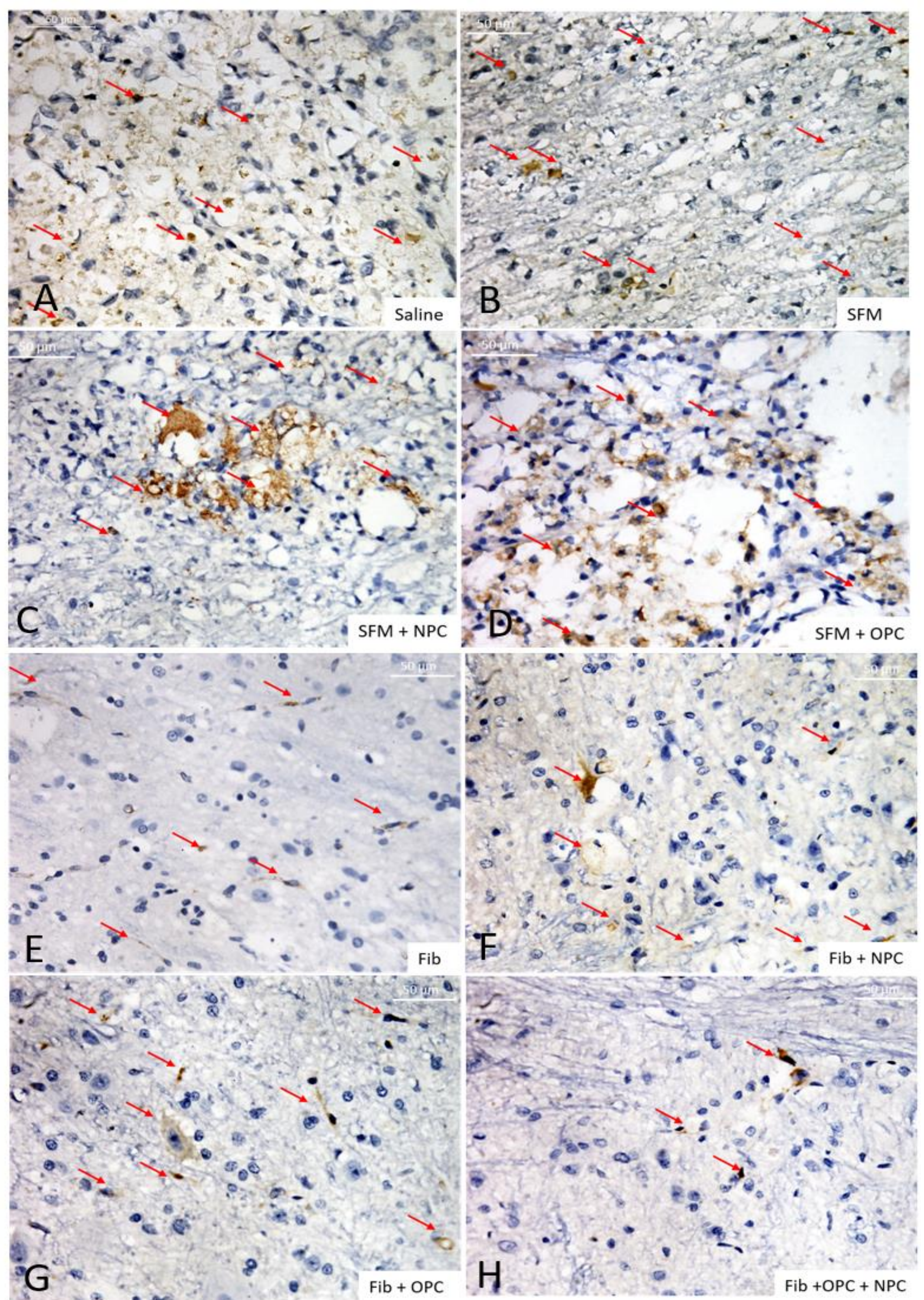


Fig. 52 Micrographs showing CD 68 staining of the injured tissue: (A)Saline control (B) SFM (C) SFM+NPC (D) SFM+OPC (E) Fibrin control (F) Fibrin + NPC (G) Fibrin +OPC (H) Fibrin + NPC + OPC; CD 68 positive cells (ED1 macrophages, Kupffer cells, monocytes, and activated microglia) indicated in red arrows.

Immunohistochemical analysis indicated GFAP hypertrophy in almost all injured tissues (Fig.53). Severe Astrogliosis was observed in the saline control, SFM control and SFM+ NPC treated groups (Fig.53 A-C). Comparatively lesser numbers of reactive astrocytes were present in SFM+ OPC, Fibrin control, NPC+ Fibrin and OPC+ Fibrin treated groups (Fig.53 D-F). Moderate astrogliosis was seen in these animals. In the OPC+NPC+ Fibrin treated group minimal numbers of reactive astrocytes were observed.

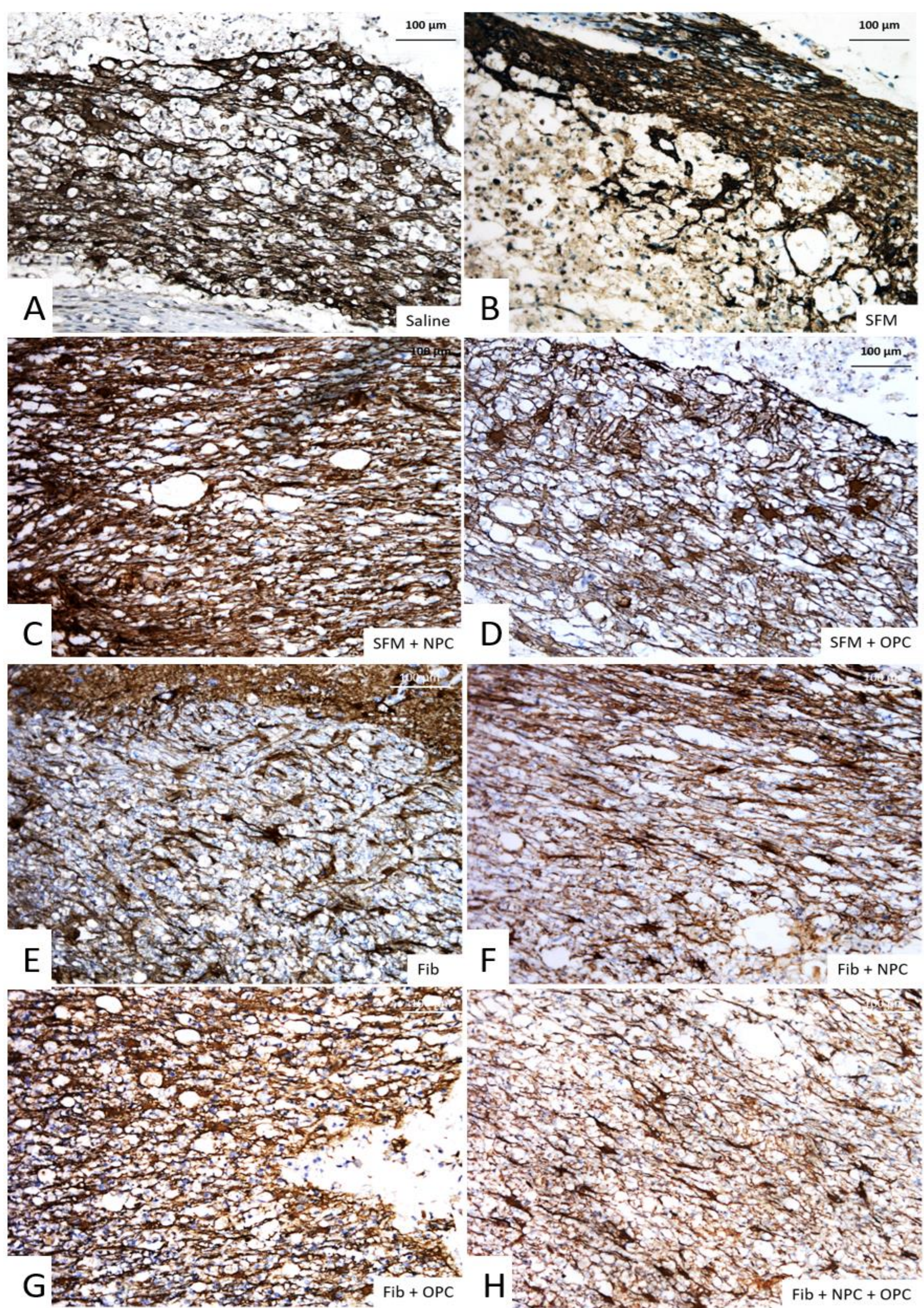


Fig. 53. Micrographs showing GFAP staining of the injured tissue: (A)Saline control (B) SFM (C) SFM+NPC (D) SFM+OPC (E) Fibrin control (F) Fibrin + NPC (G) Fibrin +OPC (H) Fibrin + NPC + OPC; GFAP positive cells in dark brown color.

CHAPTER 5

5. DISCUSSION

The results illustrated in chapter 4 are discussed in this section. Outcomes and findings from this study are highlighted in the light of published literature. Based on recent studies in this field, the current status is compared, and the significance of the results is assessed and discussed in this section. Interpretations are made on major findings enlightening the scope and limitations of the study.

5.1 Influence of niche on hADMSC differentiation to neural cells

Stem cells have been considered for cell-based regenerative medicine in recent years. The multipotency of hADMSC is an important motivation for attempting to derive different kinds of functional cells. Since adipose tissue has heterogeneous cell types, characterization using surface markers is the primary step to ensure that the MSCs isolated from adipose tissue is devoid of other contaminant cells. Even after confirming surface markers, multipotency needs to be confirmed by deriving mesodermal cells such as osteogenic, adipogenic, and chondrogenic lineages, thus meeting the international guidelines suggested by ISCT (Dominici et al. 2006). Since the study is focused on deriving neural lineage cells including oligodendrocytes and neurons, it is important to confirm that the MSCs possess multipotency. In this study, both primary characterizations using surface markers and trilineage differentiation was confirmed. Many recent studies have reported the potential of MSCs of various origins like bone marrow-derived MSCs (BM-MSC) to differentiate to endodermal and ectodermal lineage (Ullah, Subbarao, and Rho 2015). It has been reported that hADMSCs have higher neuronal or glial differentiation potential when compared to BM-MSC (Krampera et al. 2007). Almost all neural differentiation protocols involve the induction using chemical agents, growth factors, or coculture with neuronal cells. Chemical agent-induced differentiation undergoes only a transient morphological change in induced cells with up-regulation of some neural markers whereas, growth factors induce more specific, prolonged stable neural differentiation (Anghileri et al. 2008). Therefore, this study aimed to achieve the sustainable and reproducible

conversion of hADMSC to obtain subtypes of CNS cells such as oligodendrocytes and neurons, for translational purposes.

Autologous stem cells have a high potential for cell-based therapy in the context of reducing allogenic cell rejection and ethical issues. Patient-specific MSCs could be isolated for autologous transplantation because sourcing adipose tissue is relatively easier in most of the disease conditions irrespective of the age. Healthy human adipose tissue provides a large number of stem cells residing in the stromal vascular fraction. However, in degenerative disease and those in higher age groups are known to have relatively fewer numbers of hADMSC in the SVF (M. Liu et al. 2017). Therefore, there is a need to expand the isolated hADMSCs to make sufficient cell numbers to perform effective cell transplantation for tissue regeneration. The proliferation of hADMSC is dependent on the signaling from the milieu (Kusuma et al. 2017). Hence the maintenance of the proliferation rate of hADMSC could be beneficial for cell-based therapy.

In this study, healthy hADMSC was used to standardize conditions of cell proliferation and SCI was taken as an example to demonstrate stem cell potential in CNS functional recovery. In the context of neural regeneration, an important concern is that the hADMSCs are mesodermal origin and lineage conversion to ectodermal lineage is essential. Therefore, direct transplantation of hADMSCs to a niche that has limited ability to support cell differentiation may not be successful in obtaining the desired cell type for CNS regeneration. Therefore, special attention is required to create a suitable niche for sustainable & stable lineage conversion of hADMSC to neural progenitor cells (NPC). Embryonic stem cell research has demonstrated that the NPCs can be further differentiated to specialized cells such as oligodendrocytes and neurons by modifying the GF composition/concentration. Therefore, once the hADMSC derived stable NPCs are obtained, these cells may be utilized for *in vivo* transplantation as these progenitors may possess both proliferation and differentiation potential.

Considering the importance of niche for achieving hADMSC proliferation and differentiation to the desired lineage such as NPCs, fibrin was considered as a preferred matrix for inducing cell proliferation and differentiation. In this study, the fibrin niche provided the better attachment of NS and up-regulated gene expression of the Nestin

marker compared to bare TCPS. The presence of fibronectin and transglutaminase (factor XIII) in the cryoprecipitate used for fibrin preparation has been reported to benefit primary endothelial cell proliferation and maintenance of cell phenotype upon repeated passaging (Prasad Chennazhy and Krishnan 2005). The factor XIII enables the crosslinking of fibronectin to fibrin in the niche. It is well known that the fibronectin plays a major role in cell adhesion, migration, and proliferation (Kolachala et al. 2007) and retention of exogenously added GF within the matrix (Lin et al. 2011). Fibrin niche comprising fibronectin seems to provide better NS adhesion. The laminin is known to be an ideal adhesion matrix for neural cell growth and differentiation. Fibrin plays the role of cross-linker for the retention of other adhesive proteins and GFs, which are the major components of the niche. Both laminin and fibronectin present in the fibrin niche have been shown to support cell adhesion, proliferation and neural differentiation elicited by various signaling molecules (Tara & Krishnan 2015; Tara and Krishnan 2019). However, in the previous study, circulating peripheral blood mononuclear cells (PBMNCs) were grown on a fibrin niche to promote selective adhesion of NPCs which is already ectodermal, and their further differentiation to neurons. In this study, modification of niche was considered as a major requirement because, in the case of ADMSC, mesodermal cells need to be committed to ectodermal cells before they are further differentiated to NPCs and turn into glial cells and neurons. Therefore, specific GFs were added into the niche for inducing mesodermal to ectodermal conversion.

In the current study, EGF and bFGF used are associated with the trans-induction of ADMSCs into the ectodermal lineage. These GFs have been also reported to promote neural differentiation in higher doses (Hu et al. 2013). When hADMSCs were seeded on fibrin and grown in the induction medium, the cell growth was remarkably high as compared to the cells cultured on bare TCPS using normal DMEM/F12 medium. The cells grouped and lost the contact inhibition property of MSCs when the induction was initiated using GFs. The faster proliferation and formation NS were evident in the fibrin-based niche with GF. When hADMSCs were grown in TCPS and induced with GF, effective NS formation was evident. However, the latter showed poor attachment to the culture dish and NS detached by 10 days. Though morphologically the NS

appeared similar, the detachment with time indicates that proper signals for cell survival are absent. Attempt to reseed such detached NS was not quite successful perhaps the cells may have lost focal contact and were unrecoverable. This preliminary observation indicated that fibrin could play a major role in maintaining the NS viability and promote growth in culture.

It has been reported that the NS comprising NSCs have self-renewal capacity and can be sub-cultured by mild trypsinization (Casarosa, Zasso, and Conti 2013). The identification of nestin-positive cells in the hADMSC derived NS suggests the presence of the NSC population. Upon enzymatic digestion and reseeding, the cells multiplied with more nestin-positive cells; therefore, the differentiation is stable. Nearly, 50% in the induced NS culture were nestin-positive. But the percentage of cells that are nestin-positive in NS alone is not clear because, upon trypsinization, all cells in the culture well, including undifferentiated ADMSCs are also harvested. The NS cells picked and seeded in bare TCPS formed secondary NS with EGF and bFGF more readily as compared to those grown on TCPS without GFs. The secondary NS formation is also influenced by the GF. It is consistent with the finding that the extrinsic factors- EGF along with bFGF- even in the absence of fibrin could sustain the symmetrical self-renewing divisions of NS cells (Conti et al. 2005).

The Nestin^{+ve} NS were readily transformed to NPC lineage using induction media which contains a lower concentration of bFGF and EGF, as these growth factors at lower concentrations can significantly increase stem cell proliferation *in vitro* (Hu et al. 2013). The NPCs derived from NS in fibrin niche were proliferating progenitor cells with up-regulated PCNA, TUJ 1, and NES expression. The neurons induced from NPCs showed up-regulated mature neuronal marker MAP2 and NSE which agrees with the previous finding that the fibronectin cross-linked with fibrin plays a role in promoting the differentiation of Nestin positive neural progenitors to neurons *in vitro* (Jose and Krishnan 2010). The addition of KCl in the medium helped in improving the neurite outgrowth and thereby achieving typical neuronal morphology (Tara & Krishnan 2015). The synaptophysin expression at the protein level was relatively low, as compared to other markers, in the neuronal culture which may be due to less density or reduced cell to cell contact. The expression and appearance of synaptophysin are

dependent on the cell to cell contact (Fletcher et. al, 1991). Some of the induced cells were positive for dopaminergic neuron marker, TH. Wnt / Catenin signaling and FGF signaling pathways are key players in dopaminergic neurogenesis (F. Yang et al. 2014). The soluble bFGF added to the niche may have elicited the FGF signaling in the niche which enables the differentiation of NPCs to dopaminergic neurons. In addition to that, a previous study using PBMNC-derived NPCs demonstrated the role of the fibrin-based niche in neuronal differentiation by eliciting Wnt like neural inducing signals (Tara and Krishnan 2019). However, electrophysiological studies were not conducted to confirm the functionality of the induced neuron-like cells.

The same fibrin-based niche with additional growth factors was used to induce ADMSC-derived NPCs to proliferate OPCs with upregulated expression of PCNA, OLIG 2, and PDGFRα. bFGF and PDGF AA used for induction have a role in OPC proliferation and inhibition of terminal differentiation to OLGs (Bögler et al. 1990). The induction to OPC stage was better on fibrin niche in terms of OPC marker gene expression and cell proliferation compared to cells induced on bare TCPS. The induced cells in fibrin niche also showed O4 antigen, Olig 2, and PDGFRα protein expression on immunostaining. ~30% NPCs were differentiated to Olig 2 $^{+ve}$ OPCs in the niche. Induced OPCs were terminally differentiated to OLG like cells with sprouting out processes. The T3 hormone used as an inducer of terminal differentiation blocks the proliferation of OPCs and causes the morphological differentiation of oligodendrocytes as indicated by the elongation of oligodendrocyte processes (Baas et al. 1997). The differentiated cells showed upregulated OLG marker expression at the genetic and protein level. The OPCs were able to differentiate to OLGs devoid of the presence of fibrin. Based on this result it is obvious that the OPCs once injected to the injury site can differentiate to OLGs with or without the influence of fibrin, while induction to progenitor is found to be enhanced by fibrin.

In this study, a stage-wise slow differentiation is achieved using GF induction in the fibrin niche. During developmental neurogenesis, neurons and glial cells are derived from the same neural progenitors. Similarly, in this study, had MSc derived NS which further transformed to NPCs gave rise to both neural and glial cells. The major focus of this approach is to demonstrate stable differentiation of the same stem cells into

different neuronal populations in a controlled biomimetic environment for translational purposes. The stage-wise differentiation protocol used for the induction of hADMSCs to OPCs and NPCs took only 15-16 days which is much less when compared to previously published 5 weeks protocol for hADMSC trans-differentiation to OPCs (Ghasemi 2018). The number of GFs used is also relatively lesser than other protocols.

Neuronal signaling plays an inevitable role in regulating the proliferation, differentiation, and survival of oligodendrocytes *in vivo* (Simons and Trajkovic 2006). The biomimetic niche based on fibrin is able to carry out the same interaction between neurons and OPCs derived from hADMSCs. The co-culture of neurons and OPCs promoted differentiation of OPCs to OLGs without any additional inducer on fibrin niche as confirmed by qRT-PCR analysis and immunocytochemistry of the co-cultured cells. The immature and mature neuron markers as well as OLG markers were up-regulated in the co-culture with no expression of GFAP marker. The observed results were consistent with the previous results that the dopaminergic neuronal expression is higher in fibrin gel-based culture system and the softer fibrin gels with a lower concentration of fibrin (~5 mg/ml) reduce the GFAP positive cells in the culture (Mooney, Tawil, and Mahoney 2009). Here the terminal differentiation of OPCs was under the sole control of neuronal signals in the niche as seen in the *in vivo* conditions.

The differentiation studies indicate that the fibrin-based matrix is a potent biomimetic niche for exploiting the multipotency of hADMSC for neural tissue engineering. However, variability in marker expression was observed in different donors. It has been reported that the proliferation and differentiation capacity of hADMSCs significantly varies among different donors (Mohamed-Ahmed et al. 2018). A fibrin-based biomimetic niche to promote the differentiation of hADMSC to neural cells has immense translational potential in regenerating conditions like SCI. The biomimetic niche incorporated with GFs enables a stable differentiation of the stem cells, unlike the transient chemical induction. Moreover, it is also a potent natural scaffold molecule which could be used as an injectable degradable cell delivery matrix for the translational purpose (Y. Li et al. 2015).

5.2 Biomimetic signaling by fibrin-based niche

The fibrin niche seemed to elicit signals for both proliferation and differentiation of hADMSC to NS and on TCPS the process did not result in survival of formed NS. Also, the NS to NPC and OPC stage was much benefited by the presence of fibrin matrix. The biomimetic signaling from fibrin was established using *in vitro* cell signaling studies. The platelet-rich fibrin is a reservoir of growth factors and cytokines; those playing a vital role in cell signaling (Y.-H. Kang et al. 2011). The in-house prepared fibrin may not contain a significant quantity of platelet factors; however, wnt like signaling from the niche was observed during the neural differentiation of PBMNC-derived NPC to neurons (Tara and Krishnan 2019). In physiology, both wnt and notch signaling molecules have an important role in glial differentiation. Canonical Wnt pathway or Wnt/b-catenin pathway is a key regulator of oligodendrocyte development (Tawk et al. 2011) and notch signaling inhibits the neurogenin expression which promotes the up-regulation of Olig 2 transcription factor (Tsigelny et al. 2016). Therefore, in the derivation of NS and OPCs from hADMSC in the presence of fibrin-based niche, the involvement of both Wnt and Notch signaling was explored.

The role of specific signaling pathways in NS formation and OPC induction was studied by adding known inhibitors of the notch and wnt signaling to the niche. The Wnt inhibitor (PNU 74654) used is known to be specific to the canonical wnt pathway and in this study also it inhibited the β catenin component of the pathway. During active wnt signaling, the cytosolic β-catenin is transported to the nucleus and by binding to the T-cell factor/lymphoid enhancer-binding factor, transcription of the Wnt target genes is initiated. The PNU 74654 binds beta-catenin and hinders its interaction with TCF/LEF and thereby inhibits the wnt signaling (Lyou et al. 2017).

The Notch receptors 1 to 4 are associated with mammalian signaling system where the ligand binding to the receptors initiates the cleavage of the intracellular domain of receptor by γ-secretase. This Notch intracellular domain (NICD) translocates to the nucleus and activates the transcriptional Notch target genes. The Notch inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)- L-alanyl]-S-phenylglycine t-butyl ester) used in this study specifically inhibits the gamma-secretase enzyme component of the

classical notch signaling pathway which results in continuing the inactivated state of the cells (L. Jiang et al. 2011).

The hADMSCs may show toxic responses to chemical inhibitors. The standard MTT assay indicated minimal cytotoxicity of the notch and wnt chemical inhibitors added to the hADMSC induction niche; therefore, poor proliferation or differentiation is not related to the toxicity of the chemical. The cells induced in the niche without inhibitor showed high cell to cell contact and cell density upon induction. During NS induction, cells in niche added with the notch or wnt inhibitor did not show any sphere formation or grouping and associated minimal cell to cell contact as compared to NS induced in the normal niche. A similar effect of DAPT or notch signal inhibitor was seen in a human embryonic stem cell-based study, where neural rosette formation was negatively affected and premature neural differentiation was observed (Elkabetz et al. 2008). These results indicate an association of active notch signals with higher levels of symmetric divisions and reduced cell death of neural stem cells, which results in rosette or sphere formation (Elkabetz et al. 2008). Wnt signaling also has a substantial role in regulating the symmetrical division of NSCs in the brain and it has been shown to promote NS formation by NSCs *in vitro* and *in vivo* (Piccin and Morshead 2011).

The morphological analysis of the hADMSCs induced in the niche and those induced on inhibited niche clearly shows the vital role of the active notch and wnt signaling for sphere formation. The lineage conversion of hADMSCs and NS formation involves biomimetic niche elicited signals. This is similar to those involved in NS formation in ESCs or ectodermal NSCs as confirmed by significant downregulation of neural stem cell marker, NES in both wnt and notch signal inhibited cultures. More prominent effect or down-regulation was seen in wnt inhibited cultures suggesting a greater role for wnt signals in NS formation. The analysis of notch downstream molecules using qRT-PCR revealed a reduced level TCF 4 expression together with HES 5 up-regulation which indicates an active notch signaling elicited in the fibrin niche. Ngn is a pro-neuronal molecule and its expression is inversely proportional to the up-regulation of notch signaling (Z.-D. Zhou et al. 2010). Here the significant upregulation of ngn signaling in the inhibited culture indicated a lack of active signals. The dose-response of both chemical inhibitors was not systematically studied;

therefore, if anyone of the inhibitors can cause complete inhibition is not clear in this study.

The protein level expression of Wnt and beta-catenin molecules involved in the canonical wnt signaling pathway and the notch 1 molecules in the classical notch pathway supported the role of these mechanisms in NS formation upon hADMSC induction by the niche. The wnt 3a molecules in the ECM of induced cells indicated an active paracrine wnt signaling to occur in the fibrin matrix which was sparingly observed in inhibited cultures.

The Hes 1 gene expression remained unchanged in the induced and inhibited culture. Similarly, the expression of HES 1 protein was equally present in the nucleus of hADMSCs, inhibitor added cells, and induced cells. This is suggestive of the presence of an active non-classical notch signaling pathway which has a key role *in vitro* differentiation of hADMSCs (Sanalkumar, Dhanesh, and James 2010). Moreover, HES 1 plays an important role in the interplay between the β-catenin signaling and Notch classical signaling pathway (Peignon et al. 2011).

NPC to OPC transformation was also reduced by the addition of both Wnt and Notch inhibitors to the niche. Reduced cell number with minimal cell to cell contact was observed in inhibited cells upon induction; whereas, OPC like morphology with high cell numbers was seen in non-inhibited control. The Olig2 gene expression is inhibited significantly upon the addition of inhibitors to the niche without affecting PDGFRα or PCNA, indicating the specificity of inhibitor action. The expression of downstream molecules also indicated the presence of the active notch and wnt signaling in the niche. Although Hes 1 gene expression was found to be up-regulated in the fibrin niche induced OPCs, at protein level much difference wasn't observed.

5.3 Survival of transplanted progenitors in rat SCI

It has been well-established that terminally differentiated cells of CNS do not withstand trypsin-digestion, the harvested cells losing the ability to attach and grow. Therefore, transplantation of progenitors is preferable for regenerative therapy as they possess both proliferation and terminal differentiation potential. This study focused on obtaining neural progenitors exploiting 2 advantages: (i) they may proliferate even after transplantation and (ii) their multipotency is reduced by *in vitro* lineage

commitment and could differentiate preferentially to one of the CNS cells upon transplantation *in vivo*. Rat SCI was selected as a model to establish the survival of progenitors at the transplanted site. To reduce problems related to xenogenic cells, adipose tissue from the inbred rat colony was used to obtain ADMSC-derived progenitors for transplantation. Rat ADMSCs induced to OPCs and NPCs in the fibrin matrix showed a quicker response as compared to the hADMSC upon induction in the niche. A large number of nestin-positive NS were formed upon induction of rADMSCs in fibrin niche and were used for the transplantation in SCI contusion rat models. Accordingly, the consistency of the induction protocol using a fibrin-based niche was confirmed to achieve both human and rat ADMSC lineage commitment to NPCs. The study establishes the advantage of this protocol for effective use in more preclinical and clinical studies since both rat and human progenitors showing similar properties are achieved using fibrin as the induction matrix. Rat SCI model is well accepted globally for studying regeneration. Therefore, achieving rat ADMSC derived NPCs for cell-based therapy is a significant outcome of this study. If required autologous rADMSC can also be collected; but in this study cells from an inbred rat, the colony was used.

Homing, survival, and proliferation of transplanted cells have been a concern in the field of regenerative medicine. This is mainly because the tissues needing cell transplantation is always under degenerative changes. Therefore, introducing cells along with a fresh biomimetic matrix that has cell attachment and signaling properties could be an appropriate solution for improving the survival of the transplanted cells. Fibrin could play a dual role in stem cell-based therapy, as an extracellular matrix-based niche for *in vitro* differentiation (Tara & Krishnan 2015) and as a potential cell delivery matrix improving the survival and migration of the bone marrow stromal cells *in vivo* in SCI models (Itosaka et al. 2009). The advantage of fibrin is that cells can be mixed with fibrinogen and upon injection along with a low concentration of thrombin, cells could be encapsulated in the solidified fibrin network. This study exploited the dual role of fibrin; like an *in vitro* differentiation niche and as an injectable cell delivery vehicle for creating an *in vivo* niche. Both roles of a fibrin-based niche for ADMSC derived NPC derivation and transplantation has been already known. However, a

specific composition of fibrin had to be standardized for cell delivery (unpublished; patented by Krishnan and Sudhadevi, 2016) The composition of thrombin and fibrinogen was adjusted in such a way to increase the porosity of the *in situ* solidified gel for cells to survive and get sufficient nutrients, the fibrin strands are broad for cells to attach & spread. The enzyme concentration is low to prevent thrombin-inducible apoptosis of NPCs.

In the current study, all the animals recovered after T10 contusion injury, but symptoms of SCI persisted and the experiment was terminated on the 28th day. Based on the fluorescence signal from the injury site, the cell survival was found to be superior in OPC transplanted groups with or without the NPCs as compared to those with NPCs alone. The cell survival by co-localization of DAPI with the tracking dye confirmed PKH26 was specifically seen in spinal cord sections transplanted with cells in medium or fibrin matrix. The negative controls did not show auto-fluorescence from the tissue indicating that the fluorescence seen in cell transplanted tissues are specifically due to the stably bound PKH26. Both OPC in fibrin and OPC+NPC in fibrin transplanted groups showed a good number of PKH26-DAPI co-labeled cells suggesting a significant role of fibrin matrix in cell survival. One of the major challenges in using MSCs for *in vivo* application in SCI has been reported to be a low survival rate of grafted cells in the range of 5–10% of the transplanted cell (J. Li and Lepski 2013). Quantification of the survived cell was not attempted in this study; however, the advantage of fibrin as a cell delivery vehicle improving the frequency of transplanted cells is clearly demonstrated using fluorochrome tagged cells. An additional observation of the study is OPCs showed improved survival because when equal numbers of NPCs alone were transplanted, the frequency of PKH26 cells in the tissue sections was not comparable with that in OPC transplanted site. The PKH26 labeled cells usually exhibit good retention of the fluorescent signals with less toxicity when compared to widely used CFDA dye. Whereas, the CFDA has lower retention in cell and it easily exists the cell. However, CFDA-SE is a good alternative to PKH26 in terms of its retention in the cells. The CFDA-SE labeled lymphocytes can be tracked in vivo for more than 8 weeks/ up to 8 cell divisions while PKH26 can be tracked for 100 days (AU - Agrawal et al. 2017).

Single-dose administration of cells to the injury site was done which limited the number of cells transplanted to $1x10^6$; however, this was enough to assess the cell survival in the inhibitory environment of nervous tissue injury associated degeneration. Few transplanted cells were differentiated to OLGs as indicated by immunostaining. However, the locomotory functions assessed by BBB scoring indicated that none of the animals recovered completely in 28 days. It may be because the transplanted cell number was not enough, or the time period was not enough to achieve the complete regeneration of injured tissue.

The cavitation associated with SCI was minimal in the fibrin received animals as the cell delivery matrix. A similar reduction in the number of ED1 inflammatory cells was observed in the region of injury in which fibrin + cells were administered. The ED1 macrophages and cavitation are directly connected as a drastic reduction in cavitation are observed upon reduced inflammation (Gorio et al. 2002). The astrogliosis was moderate in OPC treated groups regardless of the delivery vehicle. Similarly, reduced levels of reactive astrocytes were observed in SCI that received fibrin as a cell delivery matrix which implies an immunomodulatory function of fibrin when given in conjunction with neural or glial progenitors. Minimal astrogliosis, reduced ED1 cells, and absence of cavitation were observed in OPC+NPC+ Fibrin treated SCI, which suggests that this strategy is better than using OPC or fibrin or NPC alone. It is highly debated whether the outcome of neuroinflammation is neurotoxic or neuroprotective. But a balanced infiltration of inflammatory cells is necessary for removal of tissue debris and for the attenuation of the inflammatory responses (Okada 2016), which is observed when fibrin is used as a delivery matrix.

In summary, this proof of concept study establishes the advantage of using the fibrin-based biomimetic niche in providing better support for differentiation and proliferation of both neural and glial progenitors. Improved survival of transplanted OPCs signifies the role of OPC or OPC derived factors in cell survival in the SCI *milieu*. The reduced inflammatory responses and cavitation provided by the fibrin used as a cell delivery matrix are also promising. Therefore, the study directs extension to further evaluations using ADMSC-derived OPCs and NPCs using fibrin as a delivery matrix. Exploring the dose-response in terms of cell numbers for longer periods to establish the cell

numbers and time required for complete regeneration and functional recovery would be of importance for translational purposes.

5.4 Limitations of the study

The major lacuna of the study is that the functional status of neurons derived from hADMSCs was not evaluated. The electrical excitability is the key feature of neurons and patch-clamp technique needs to be used to establish terminal differentiation to neurons. The differentiation was established based on the presence of certain stage-specific molecular markers at predefined time periods. However, the best time period is not known for this study. If the test frequency is closer, the marker expression and lineage commitment path would be understood more systematically. The *in vitro* signaling studies were established using chemical inhibitors which provided only partial inhibition to notch or wnt signaling pathways. The use of siRNA based signaling studies could be conducted to identify complete signal inhibition. The animal studies conducted show promising observations regarding cell survival, but it was not assessed quantitatively. The effect of dose-response (varying cell number) on transplantation was also not evaluated. The preliminary studies conducted were mainly focused on cell survival rather than tissue regeneration. However, the effect of varying doses of increased cell numbers and for a long period of time on the regeneration of the injured nervous tissue was not evaluated.

CHAPTER 6

6. SUMMARY AND CONCLUSION

Degenerative diseases of the central nervous system (CNS) is a major health problem. Many such conditions have no pharmacologic remedy currently. Stem cell-based regenerative medicine provides a ray of hope to treat some of these diseases using autologous MSCs. However, stem cells are unlikely to survive in the degenerating *milieu* because of inflammation and matrix degradation causing a lack of cell survival and proliferation. Even when stem cells survive post-transplantation they may not undergo desired differentiation in the *milieu* as required for tissue regeneration due to two reasons (i) MSCs are multipotent and hence may be directed to any lineage depending on signals in the *milieu*; (ii) the *milieu* may not support any differentiation at all due to lack of adhesive proteins and growth factors. This study proposed a potential strategy for use of stem cells effectively to promote neuro-regeneration by *in vitro* controlled derivation of neural progenitors that can differentiate into both glial cells and neurons upon transplantation. The *in vitro* differentiation is relatively easier as the conditions in the culture niche may be regulated using known factors required for specific lineage commitment. This study aimed differentiation of multipotent hADMSC to bi-potent neural progenitor cells (NPC) which can differentiate into oligodendrocyte progenitor cells (OPC) or neural precursor cells (NPCs) expressing neural markers. Once the cells are lineage-committed, chances of their differentiation to undesirable lineage reduces automatically. However, the progenitors should be stable with proliferation potential so that even after transplantation they can multiply to populate the tissue and regenerate appropriately. Stable differentiation requires physiological-like stimuli so that the differentiation process undergo required biochemical pathways through the stimulus-response coupling. Spontaneous differentiation using chemical inducers can only produce unstable progenitors or mature cells. To obtain such balanced and stable NPC for transplantation the primary requirement is to use biomimetic pathways of differentiation so that cells can be harvested and transplanted without affecting the phenotype or proliferation potential.

This study proposed fibrin-based matrix coated tissue culture polystyrene could promote cell proliferation and the GFs may be supplemented in the medium to create a biomimetic niche to develop NPCs & OPCs. Even when most appropriate cell type is obtained for tissue regeneration, the transplantation often fails because the damaged tissue presents a degenerating milieu with poor conditions for cell survival, proliferation, and differentiation. In most of the cases, the ECM is biochemically different with no proper cell adhesion molecule for permitting cell survival and growth. This study proposed to use an injectable biomimetic matrix into which cells can be encapsulated before transplantation. This study proposed that NPCs/OPCs may be mixed with liquid components of the modified composition of fibrin sealant. When cell-encapsulated fibrinogen is mixed with a small quantity of thrombin and injected to the treatment site, liquid to the solid transition of fibrin components *in situ* can enable retention of the cells within the matrix. The fibrin matrix may also protect the degenerative molecules in the *milieu* from attacking the transplanted cells and thus preventing immune response. In summary two major aspects of the study were: (i) use of fibrin as *in vitro* niche for producing NPCs from ADMSCs; and (ii) to demonstrate that fibrin-based cell delivery may improve post-transplantation cell survival.

6.1. Summary of Results:

The hADMSCs isolated from lipoaspirates were expanded into >90% homogeneous population of stem cells which underwent tri-lineage differentiation proving multipotency. A biomimetic fibrin-based neuron-specific niche was created in the tissue culture polystyrene surface (TCPS). Upon the culture of hADMSCs, both on TCPS and fibrin coated TCPS, the GFs induced NS formation; however, the former did not support long term survival of NS. The NS reseeded on fibrin coated surface resulted in faster cell growth, secondary NS formation, and upon reducing GF concentration the cells differentiated into NPCs. The NPCs were further differentiated into OPCs, OLGs, and neurons by differently modifying the GF composition. It is evident from the results that the fibrin-based niche is able to support the differentiation of hADMSCs to ectodermal lineages and also supports the proliferation of the progenitor population. The OPCs derived from NS were further differentiated to OLGs on bare TCPS or fibrin niche, similarly, the NPCs were differentiated to neurons using

a previously standardized KCL based protocol. At all steps, transcriptional and translational level neural/glial marker expressions were achieved consistently. Moreover, the fibrin matrix has the potential to maintain various neuronal and glial cells in co-culture. The time frame required for the expansion of ADMSCs to neural progenitors was relatively short as compared to other neural induction protocols described in the literature. The transplantable OPCs and NPCs were obtained in the culture within 14 days of induction. The stage-wise and stable induction of cells demonstrated using adhesive fibrin matrix and GFs in this study protocol scores over the widely used chemical inducer-based differentiation. Upon arresting 2 different physiological signaling pathways using chemical inhibitors, NS formation, and differentiation to NPCs/OPCs were almost completely lacking. Therefore, both Wnt and Notch signaling elicited by fibrin-based niche are responsible for well regulated, steady differentiation of hADMSCs to NS/NPCs/OPCs. Thus, it is proven that the signaling pathways elicited by the niche are similar to that of *in vivo* neuro-gliogenesis giving stable progenitors suitable for transplantation.

The consistency and reliability of the induction protocol are confirmed by obtaining NPCs from rat ADMSCs as well, using fibrin niche-based induction protocol. The NPCs and OPCs obtained from rat also expressed stage-specific markers and were harvested easily for transplantation. The time required for rat ADMSC differentiation to NPC was shorter than the time required for hADMSCs. By using fluorescent tagging and imaging technology, the survival of transplanted cells until the 28th day of the experiment was proven. Furthermore, few of the transplanted cells also differentiated into OLGs at the site of injury. The inflammation was minimal in those injury sites which received fibrin with or without cells indicated by reduced cavitation, astrogliosis, and ED1 inflammatory cells.

Survival of OPCs transplanted with or without NPCs was found to be better upon delivering cells using fibrin as compared with that transferred in SFM. This study indicates that the fibrin promotes cell retention, homing, and differentiation at the site of injury. A significant difference in motor functions as compared to sham/control was evidenced only in the fibrin administered group; but not in FIB+ Cell injected SCI rats.

A more detailed study is required to understand why fibrin alone is showing a better score but not fibrin + NPCs/OPCs.

To summarize, stem cells were differentiated *in vitro* using a protocol involving fibrin-based biomimetic niche, proving the feasibility of deriving stable neural progenitor cells from human and rat ADMSCs. The process is less time consuming, produces progenitors that pass through different stages, resulting in mature neurons and oligodendrocytes. Each stage of transition is distinguishable based on respective markers. The physiological-like Wnt and Notch signaling could be arrested using chemical inhibitors to stop the processes of differentiation, proving that signals originating from fibrin play a role in differentiation. Fibrin is also found to be a suitable delivery matrix which protects site of injury from immune response and inflammation when injected with or without NPCs/OPCs.

6.2 Conclusion

1. *In vitro* niche was designed by depositing insoluble fibrin on TCPS providing adhesive proteins and by supplementing with GFs in the medium, to induce ADMSC to neural lineage.
2. Fibrin based niche is essential for the long-term survival of NS and for further differentiation into NPCs/OPCs.
3. On fibrin niche, NPCs and OPCs differentiated into neurons and oligodendrocytes respectively, suggesting uni-potent differentiation potential of both progenitors in the niche.
4. Both ADMSC derived neurons and OPCs co-existed in the fibrin niche.
5. Physiological-like Wnt and Notch signaling pathways are elicited by fibrin niche to produce stable neural progenitors.
6. The validity and stability of fibrin niche-based differentiation are reproducible and applicable for other species cells such as rat ADMSC differentiation.
7. Demonstrated better survival of OPCs and NPCs when transplanted to rat SCI using fibrin as a delivery vehicle, as compared to SFM.
8. Few transplanted cells differentiated *in vivo* into OLGs.
9. Cavitation was minimum in SCI tissue on the 28th day of the experiment in the cases of cells delivered in fibrin.
10. The remarkable immune-modulatory effect is seen upon using fibrin as a cell delivery vehicle.

6.3 Future perspective

1. Conduct patch-clamp experiments to demonstrate the functionality of the neurons derived from hADMSCs.
2. Evaluate cell-cell interaction using Neurons-OPCs co-culture on fibrin niche to understand mutual support for cell growth and myelination
3. Investigate the effect of dose-response of varying cell numbers on transplantation outcomes in SCI rat models.
4. Establish cell survival in fibrin matrix post-transplantation, quantitatively.
5. Study the effect of NPC & OPC transplantation on the regeneration of SCI and functional outcome after long term such as 3/6 months after the experiment
6. Explore translational potential by initiating human limited clinical trial by transplantation of autologous NPCs & OPCs using fibrin as a delivery matrix.

BIBLIOGRAPHY

Abbaszadeh, Hojjat-Allah, Taki Tiraihi, AliReza Delshad, Majid Saghedizadeh, Taher Taheri, Hadi Kazemi, and Hayder K. Hassoun. 2014. "Differentiation of Neurosphere-Derived Rat Neural Stem Cells into Oligodendrocyte-like Cells by Repressing PDGF-α and Olig2 with Triiodothyronine." *Tissue & Cell* 46 (6): 462–69. https://doi.org/10.1016/j.tice.2014.08.003.

Ahuja, Christopher S., Allan R. Martin, and Michael Fehlings. 2016. "Recent Advances in Managing a Spinal Cord Injury Secondary to Trauma." *F1000Research* 5 (May). https://doi.org/10.12688/f1000research.7586.1.

Alizadeh, Arsalan, Scott M. Dyck, and Soheila Karimi-Abdolrezaee. 2015. "Myelin Damage and Repair in Pathologic CNS: Challenges and Prospects." *Frontiers in Molecular Neuroscience* 8 (July). https://doi.org/10.3389/fnmol.2015.00035.

Alizadeh, Arsalan, and Soheila Karimi-Abdolrezaee. 2016. "Microenvironmental Regulation of Oligodendrocyte Replacement and Remyelination in Spinal Cord Injury." *The Journal of Physiology* 594 (13): 3539–52. https://doi.org/10.1113/JP270895.

Almad, Akshata, F. Rezan Sahinkaya, and Dana M. McTigue. 2011. "Oligodendrocyte Fate after Spinal Cord Injury." *Neurotherapeutics* 8 (2): 262–73. https://doi.org/10.1007/s13311-011-0033-5.

Altschuler, Richard A., Yehoash Raphael, Su-Hua Sha, Jochen Schacht, David C. Martin, and Josef M. Miller. 2014. "Chapter 65 - Protection and Repair of Hearing." In *Principles of Tissue Engineering (Fourth Edition)*, edited by Robert Lanza, Robert Langer, and Joseph Vacanti, 1375–95. Boston: Academic Press. https://doi.org/10.1016/B978-0-12-398358-9.00065-3.

Anghileri, Elena, Silvia Marconi, Angela Pignatelli, Pierangelo Cifelli, Mirco Galié, Andrea Sbarbati, Mauro Krampera, Ottorino Belluzzi, and Bruno Bonetti. 2008. "Neuronal Differentiation Potential of Human Adipose-Derived Mesenchymal Stem Cells." *Stem Cells and Development* 17 (5): 909–16. https://doi.org/10.1089/scd.2007.0197.

Anwar, M. Akhtar, Tuqa S. Al Shehabi, and Ali H. Eid. 2016. "Inflammogenesis of Secondary Spinal Cord Injury." *Frontiers in Cellular Neuroscience* 10 (April). https://doi.org/10.3389/fncel.2016.00098.

Aranda-Anzaldo, Armando. 2012. "The Post-Mitotic State in Neurons Correlates with a Stable Nuclear Higher-Order Structure." *Communicative & Integrative Biology* 5 (2): 134–39. https://doi.org/10.4161/cib.18761.

Aravind, S. R., and Lissy K. Krishnan. 2016. "Curcumin-Albumin Conjugates as an Effective Anti-Cancer Agent with Immunomodulatory Properties." *International Immunopharmacology* 34 (May): 78–85. https://doi.org/10.1016/j.intimp.2016.02.010.

Arezoumand, Khatereh Saei, Effat Alizadeh, Younes Pilehvar-Soltanahmadi, Mohammad Esmaeillou, and Nosratollah Zarghami. 2017. "An Overview on Different Strategies for the Stemness Maintenance of MSCs." *Artificial Cells, Nanomedicine, and Biotechnology* 45 (7): 1255–71. https://doi.org/10.1080/21691401.2016.1246452.

Arkudas, Andreas, Jimmy Tjiawi, Oliver Bleiziffer, Lucia Grabinger, Elias Polykandriotis, Justus P Beier, Michael Stürzl, Raymund E Horch, and Ulrich Kneser. 2007. "Fibrin Gel-Immobilized VEGF and BFGF Efficiently Stimulate Angiogenesis in the AV Loop Model." *Molecular Medicine* 13 (9–10): 480–87. https://doi.org/10.2119/2007-00057.Arkudas.

AU - Agrawal, Rupesh, Praveen Kumar AU - Balne, Sai Bo Bo AU - Tun, Yeo AU - Sia Wey, Neha AU - Khandelwal, and Veluchamy A. AU - Barathi. 2017. "Fluorescent Dye Labeling of Erythrocytes and Leukocytes for Studying the Flow Dynamics in Mouse Retinal Circulation." *JoVE*, no. 125 (July): e55495. https://doi.org/10.3791/55495.

Baas, D., D. Bourbeau, L. L. Sarliève, M. E. Ittel, J. H. Dussault, and J. Puymirat. 1997. "Oligodendrocyte Maturation and Progenitor Cell Proliferation Are Independently Regulated by Thyroid Hormone." *Glia* 19 (4): 324–32.

Baumann, Nicole, and Danielle Pham-Dinh. 2001. "Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System." *Physiological Reviews* 81 (2): 871–927. https://doi.org/10.1152/physrev.2001.81.2.871.

Becerra-Calixto, Andrea, and Gloria P. Cardona-Gómez. 2017. "The Role of Astrocytes in Neuroprotection after Brain Stroke: Potential in Cell Therapy." *Frontiers in Molecular Neuroscience* 10 (April). https://doi.org/10.3389/fnmol.2017.00088.

Bergles, Dwight E., and William D. Richardson. 2016. “Oligodendrocyte Development and Plasticity.” *Cold Spring Harbor Perspectives in Biology* 8 (2). https://doi.org/10.1101/cshperspect.a020453.

Bez, Alessandra, Elena Corsini, Daniela Curti, Marco Biggiogera, Augusto Colombo, Roberto Francesco Nicosia, Stefano Filippo Pagano, and Eugenio Agostino Parati. 2003. “Neurosphere and Neurosphere-Forming Cells: Morphological and Ultrastructural Characterization.” *Brain Research* 993 (1): 18–29. https://doi.org/10.1016/j.brainres.2003.08.061.

Bögler, O., D. Wren, S. C. Barnett, H. Land, and M. Noble. 1990. “Cooperation between Two Growth Factors Promotes Extended Self-Renewal and Inhibits Differentiation of Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitor Cells.” *Proceedings of the National Academy of Sciences* 87 (16): 6368–72. https://doi.org/10.1073/pnas.87.16.6368.

Bradl, Monika, and Hans Lassmann. 2010. “Oligodendrocytes: Biology and Pathology.” *Acta Neuropathologica* 119 (1): 37–53. https://doi.org/10.1007/s00401-009-0601-5.

Bunnell, Bruce A., Mette Flaat, Christine Gagliardi, Bindiya Patel, and Cynthia Ripoll. 2008. “Adipose-Derived Stem Cells: Isolation, Expansion and Differentiation.” *Methods (San Diego, Calif.)* 45 (2): 115–20. https://doi.org/10.1016/j.ymeth.2008.03.006.

Busch, Sarah A., and Jerry Silver. 2007. “The Role of Extracellular Matrix in CNS Regeneration.” *Current Opinion in Neurobiology* 17 (1): 120–27. https://doi.org/10.1016/j.conb.2006.09.004.

Cao, Q. L., Y. P. Zhang, R. M. Howard, W. M. Walters, P. Tsoulfas, and S. R. Whittemore. 2001. “Pluripotent Stem Cells Engrafted into the Normal or Lesioned Adult Rat Spinal Cord Are Restricted to a Glial Lineage.” *Experimental Neurology* 167 (1): 48–58. https://doi.org/10.1006/exnr.2000.7536.

Cardozo, Marcos Julian, Karolina S. Mysiak, Thomas Becker, and Catherina G. Becker. 2017. “Reduce, Reuse, Recycle – Developmental Signals in Spinal Cord Regeneration.” *Developmental Biology* 432 (1): 53–62. https://doi.org/10.1016/j.ydbio.2017.05.011.

Casarosa, Simona, Jacopo Zasso, and Luciano Conti. 2013. "Systems for Ex-Vivo Isolation and Culturing of Neural Stem Cells." *Neural Stem Cells - New Perspectives*, April. https://doi.org/10.5772/55137.

Conti, Luciano, Steven M. Pollard, Thorsten Gorba, Erika Reitano, Mauro Toselli, Gerardo Biella, Yirui Sun, et al. 2005. "Niche-Independent Symmetrical Self-Renewal of a Mammalian Tissue Stem Cell." *PLoS Biology* 3 (9): e283. https://doi.org/10.1371/journal.pbio.0030283.

Deng, Junhao, Yiling Zhang, Yong Xie, Licheng Zhang, and Peifu Tang. 2018. "Cell Transplantation for Spinal Cord Injury: Tumorigenicity of Induced Pluripotent Stem Cell-Derived Neural Stem/Progenitor Cells." Research article. Stem Cells International. 2018. https://doi.org/10.1155/2018/5653787.

Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, Fc Marini, Ds Krause, Rj Deans, A. Keating, Dj Prockop, and Em Horwitz. 2006. "Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement." *Cytotherapy* 8 (4): 315–17. https://doi.org/10.1080/14653240600855905.

Doucette, R. 2002. *Olfactory Ensheathing Cells: Past, Present and Future*. Vol. 16.

Dumont, R. J., D. O. Okonkwo, S. Verma, R. J. Hurlbert, P. T. Boulos, D. B. Ellegala, and A. S. Dumont. 2001. "Acute Spinal Cord Injury, Part I: Pathophysiologic Mechanisms." *Clinical Neuropharmacology* 24 (5): 254–64.

Egawa, Naohiro, Josephine Lok, Kazuo Washida, and Ken Arai. 2017. "Mechanisms of Axonal Damage and Repair after Central Nervous System Injury." *Translational Stroke Research* 8 (1): 14–21. https://doi.org/10.1007/s12975-016-0495-1.

Elkabetz, Yechiel, Georgia Panagiotakos, George Al Shamy, Nicholas D. Socci, Viviane Tabar, and Lorenz Studer. 2008. "Human ES Cell-Derived Neural Rosettes Reveal a Functionally Distinct Early Neural Stem Cell Stage." *Genes & Development* 22 (2): 152–65. https://doi.org/10.1101/gad.1616208.

Emery, Ben. 2010. "Regulation of Oligodendrocyte Differentiation and Myelination." *Science (New York, N.Y.)* 330 (6005): 779–82. https://doi.org/10.1126/science.1190927.

Erlichman, J. S., and J. C. Leiter. 2010. "Glia Modulation of the Extracellular Milieu as a Factor in Central CO2 Chemosensitivity and Respiratory Control."

Journal of Applied Physiology (Bethesda, Md.: 1985) 108 (6): 1803–11. https://doi.org/10.1152/japplphysiol.01321.2009.

Faigle, Roland, and Hongjun Song. 2013. “Signaling Mechanisms Regulating Adult Neural Stem Cells and Neurogenesis.” *Biochimica et Biophysica Acta* 1830 (2): 2435–48. https://doi.org/10.1016/j.bbagen.2012.09.002.

Fatemeh Atashi, Veronique Serre. 2015. “Platelet Rich Plasma Promotes Proliferation of Adipose Derived Mesenchymal Stem Cells via Activation of AKT and Smad2 Signaling Pathways.” *Journal of Stem Cell Research & Therapy* 05 (08). https://doi.org/10.4172/2157-7633.1000301.

Frei, Eveline, Isabel Klusman, Lisa Schnell, and Martin E. Schwab. 2000. “Reactions of Oligodendrocytes to Spinal Cord Injury: Cell Survival and Myelin Repair.” *Experimental Neurology* 163 (2): 373–80. https://doi.org/10.1006/exnr.2000.7379.

Fuchs, Elaine, Tudorita Tumbar, and Geraldine Guasch. 2004. “Socializing with the Neighbors: Stem Cells and Their Niche.” *Cell* 116 (6): 769–78. https://doi.org/10.1016/S0092-8674(04)00255-7.

Gazdic, Marina, Vladislav Volarevic, C. Randall Harrell, Crissy Fellabaum, Nemanja Jovicic, Nebojsa Arsenijevic, and Miodrag Stojkovic. 2018. “Stem Cells Therapy for Spinal Cord Injury.” *International Journal of Molecular Sciences* 19 (4). https://doi.org/10.3390/ijms19041039

.

Ghasemi, Nazem. 2018. “Transdifferentiation of Human Adipose-Derived Mesenchymal Stem Cells into Oligodendrocyte Progenitor Cells.” *Iranian Journal of Neurology* 17 (1): 24–30.

Ghiasi, Mahdieh, Reza Tabatabaei Qomi, Naser Kalhor, and Mohsen Sheykhhasan. 2016. “Adipose-Derived Stem Cells: An Optimized Protocol for Isolation and Proliferation.” *Acta Medica International* 3 (1): 116. https://doi.org/10.5530/ami.2016.1.25.

Ghosh, Hiyaa S. 2019. “Adult Neurogenesis and the Promise of Adult Neural Stem Cells.” *Journal of Experimental Neuroscience* 13 (June). https://doi.org/10.1177/1179069519856876.

Gimble Jeffrey M., Katz Adam J., and Bunnell Bruce A. 2007. “Adipose-Derived Stem Cells for Regenerative Medicine.” *Circulation Research* 100 (9): 1249–60. https://doi.org/10.1161/01.RES.0000265074.83288.09.

Gorio, A., N. Gokmen, S. Erbayraktar, O. Yilmaz, L. Madaschi, C. Cichetti, A. M. Di Giulio, E. Vardar, A. Cerami, and M. Brines. 2002. "Recombinant Human Erythropoietin Counteracts Secondary Injury and Markedly Enhances Neurological Recovery from Experimental Spinal Cord Trauma." *Proceedings of the National Academy of Sciences* 99 (14): 9450–55. https://doi.org/10.1073/pnas.142287899.

Hamill, Cecily E., Anna Goldshmidt, Olivier Nicole, Robert J. McKeon, Daniel J. Brat, and Stephen F. Traynelis. 2005. "Special Lecture: Glial Reactivity after Damage: Implications for Scar Formation and Neuronal Recovery." *Clinical Neurosurgery* 52: 29–44.

Haylock-Jacobs, Sarah, Michael B. Keough, Lorraine Lau, and V. Wee Yong. 2011. "Chondroitin Sulphate Proteoglycans: Extracellular Matrix Proteins That Regulate Immunity of the Central Nervous System." *Autoimmunity Reviews* 10 (12): 766–72. https://doi.org/10.1016/j.autrev.2011.05.019.

Hu, Feihu, Xiu Wang, Gaofeng Liang, Lanxin Lv, Yanliang Zhu, Bo Sun, and Zhongdang Xiao. 2013. "Effects of Epidermal Growth Factor and Basic Fibroblast Growth Factor on the Proliferation and Osteogenic and Neural Differentiation of Adipose-Derived Stem Cells." *Cellular Reprogramming* 15 (3): 224–32. https://doi.org/10.1089/cell.2012.0077.

Huang, Hao, Xiao-Feng Zhao, Kang Zheng, and Mengsheng Qiu. 2013. "Regulation of the Timing of Oligodendrocyte Differentiation: Mechanisms and Perspectives." *Neuroscience Bulletin* 29 (2): 155–64. https://doi.org/10.1007/s12264-013-1314-2.

Huebner, Eric A., and Stephen M. Strittmatter. 2009. "Axon Regeneration in the Peripheral and Central Nervous Systems." *Results and Problems in Cell Differentiation* 48: 339–51. https://doi.org/10.1007/400_2009_19.

Irioda, Ana Carolina, Rafael Cassilha, Larissa Zocche, Julio Cesar Francisco, Ricardo Correa Cunha, Priscila Elias Ferreira, Luiz Cesar Guarita-Souza, et al. 2016. "Human Adipose-Derived Mesenchymal Stem Cells Cryopreservation and Thawing Decrease A4-Integrin Expression." Research article. Stem Cells International. 2016. https://doi.org/10.1155/2016/2562718.

Ishibashi, Tomoko, Kelly A. Dakin, Beth Stevens, Philip R. Lee, R. Douglas Fields, Serguei V. Kozlov, and Colin L. Stewart. 2006. "Astrocytes Promote Myelination in Response to Electrical Impulses." *Neuron* 49 (6): 823–32. https://doi.org/10.1016/j.neuron.2006.02.006.

Itosaka, Hiroyuki, Satoshi Kuroda, Hideo Shichinohe, Hiroshi Yasuda, Shunsuke Yano, Shintaro Kamei, Ryoichi Kawamura, Kazutoshi Hida, and Yoshinobu Iwasaki. 2009. "Fibrin Matrix Provides a Suitable Scaffold for Bone Marrow Stromal Cells Transplanted into Injured Spinal Cord: A Novel Material for CNS Tissue Engineering." *Neuropathology* 29 (3): 248–57. https://doi.org/10.1111/j.1440-1789.2008.00971.x.

Jessen, Kristján R., Rhona Mirsky, and Alison C. Lloyd. 2015. "Schwann Cells: Development and Role in Nerve Repair." *Cold Spring Harbor Perspectives in Biology* 7 (7). https://doi.org/10.1101/cshperspect.a020487.

Jessica C. F. Kwok, Elena Vecino. 2016. *The Extracellular Matrix in the Nervous System: The Good and the Bad Aspects*. Intech.

Jiang, Li-yu, Xiao-lei Zhang, Ping Du, and Jian-hua Zheng. 2011. "γ-Secretase Inhibitor, DAPT Inhibits Self-Renewal and Stemness Maintenance of Ovarian Cancer Stem-like Cells In Vitro." *Chinese Journal of Cancer Research* 23 (2): 140–46. https://doi.org/10.1007/s11670-011-0140-1.

Jiang, Peng, Vimal Selvaraj, and Wenbin Deng. 2010. "Differentiation of Embryonic Stem Cells into Oligodendrocyte Precursors." *Journal of Visualized Experiments : JoVE*, no. 39 (May). https://doi.org/10.3791/1960.

Jose, Anumol, and Lissy K. Krishnan. 2010. "Effect of Matrix Composition on Differentiation of Nestin-Positive Neural Progenitors from Circulation into Neurons." *Journal of Neural Engineering* 7 (3): 036009. https://doi.org/10.1088/1741-2560/7/3/036009.

Kang, Soo-Kyung, Myung-Joo Shin, Jin Sup Jung, Yong Geun Kim, and Cheul-Hong Kim. 2006. "Autologous Adipose Tissue-Derived Stromal Cells for Treatment of Spinal Cord Injury." *Stem Cells and Development* 15 (4): 583–94. https://doi.org/10.1089/scd.2006.15.583.

Kang, Young-Ho, Soung Hoo Jeon, Joo-Young Park, Jong-Hoon Chung, Yun-Hoon Choung, Han-Wool Choung, Eun-Suk Kim, and Pill-Hoon Choung. 2011. "Platelet-Rich Fibrin Is a Bioscaffold and Reservoir of Growth Factors for Tissue Regeneration." *Tissue Engineering. Part A* 17 (3–4): 349–59. https://doi.org/10.1089/ten.TEA.2010.0327.

Kim, Yoon-Chung, Young-Hoon Kim, Jang-Woon Kim, and Kee-Yong Ha. 2016. "Transplantation of Mesenchymal Stem Cells for Acute Spinal Cord Injury in Rats: Comparative Study between Intralesional Injection and Scaffold Based Transplantation." *Journal of Korean Medical Science* 31 (9): 1373–82. https://doi.org/10.3346/jkms.2016.31.9.1373.

Kim, Young-Hoon, Kee-Yong Ha, and Sang-Il Kim. 2017. "Spinal Cord Injury and Related Clinical Trials." *Clinics in Orthopedic Surgery* 9 (1): 1–9. https://doi.org/10.4055/cios.2017.9.1.1.

Kjell, Jacob, and Lars Olson. 2016. "Rat Models of Spinal Cord Injury: From Pathology to Potential Therapies." *Disease Models & Mechanisms* 9 (10): 1125–37. https://doi.org/10.1242/dmm.025833.

Kolachala, Vasantha L., Rahul Bajaj, Lixin Wang, Yutao Yan, Jeff D. Ritzenthaler, Andrew T. Gewirtz, Jesse Roman, Didier Merlin, and Shanthi V. Sitaraman. 2007. "Epithelial-Derived Fibronectin Expression, Signaling, and Function in Intestinal Inflammation." *Journal of Biological Chemistry* 282 (45): 32965–73. https://doi.org/10.1074/jbc.M704388200.

Kolar, Mallappa K., Paul J. Kingham, Liudmila N. Novikova, Mikael Wiberg, and Lev N. Novikov. 2014. "The Therapeutic Effects of Human Adipose-Derived Stem Cells in a Rat Cervical Spinal Cord Injury Model." *Stem Cells and Development* 23 (14): 1659–74. https://doi.org/10.1089/scd.2013.0416.

Kolf, Catherine M., Elizabeth Cho, and Rocky S. Tuan. 2007. "Mesenchymal Stromal Cells: Biology of Adult Mesenchymal Stem Cells: Regulation of Niche, Self-Renewal and Differentiation." *Arthritis Research & Therapy* 9 (1): 204. https://doi.org/10.1186/ar2116.

Krampera, Mauro, Silvia Marconi, Annalisa Pasini, Mirco Galiè, Gino Rigotti, Federico Mosna, Martina Tinelli, et al. 2007. "Induction of Neural-like Differentiation in Human Mesenchymal Stem Cells Derived from Bone Marrow, Fat, Spleen and Thymus." *Bone* 40 (2): 382–90. https://doi.org/10.1016/j.bone.2006.09.006.

Kriegstein, Arnold, and Arturo Alvarez-Buylla. 2009. "The Glial Nature of Embryonic and Adult Neural Stem Cells." *Annual Review of Neuroscience* 32: 149–84. https://doi.org/10.1146/annurev.neuro.051508.135600.

Krishnan, Lissy, and Tara Sudhadevi. n.d. Biomimetic fibrin gel composite injectable to spinal cord injury for prevention of immune reaction and delivery of neural progenitors. 201641015636, filed May 5, 2016.

Kusuma, Gina D., James Carthew, Rebecca Lim, and Jessica E. Frith. 2017. "Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine Signaling: Opportunities to Engineer the Therapeutic Effect." *Stem Cells and Development* 26 (9): 617–31. https://doi.org/10.1089/scd.2016.0349.

Kwok, Jessica C. F., Simona Foscarin, and James W. Fawcett. 2015. “Perineuronal Nets: A Special Structure in the Central Nervous System Extracellular Matrix.” In *Extracellular Matrix*, 23–32. Neuromethods. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-2083-9_3.

Lee, Jae K., and Binhai Zheng. 2012. “Role of Myelin-Associated Inhibitors in Axonal Repair after Spinal Cord Injury.” *Experimental Neurology* 235 (1): 33–42. https://doi.org/10.1016/j.expneurol.2011.05.001.

Lei, Lei, WeiMing Liao, PuYi Sheng, Ming Fu, AiShan He, and Gang Huang. 2007. “Biological Character of Human Adipose-Derived Adult Stem Cells and Influence of Donor Age on Cell Replication in Culture.” *Science in China. Series C, Life Sciences* 50 (3): 320–28. https://doi.org/10.1007/s11427-007-0019-z.

Leite, Cristiana, N. Tatiana Silva, Sandrine Mendes, Andreia Ribeiro, Joana Paes de Faria, Tânia Lourenço, Francisco dos Santos, et al. 2014. “Differentiation of Human Umbilical Cord Matrix Mesenchymal Stem Cells into Neural-Like Progenitor Cells and Maturation into an Oligodendroglial-Like Lineage.” *PLOS ONE* 9 (10): e111059. https://doi.org/10.1371/journal.pone.0111059.

Li, Jun, and Guilherme Lepski. 2013. “Cell Transplantation for Spinal Cord Injury: A Systematic Review.” *BioMed Research International* 2013. https://doi.org/10.1155/2013/786475.

Li, Ning, and Gilberto K. K. Leung. 2015. “Oligodendrocyte Precursor Cells in Spinal Cord Injury: A Review and Update.” *BioMed Research International* 2015. https://doi.org/10.1155/2015/235195.

Li, Yuting, Hao Meng, Yuan Liu, and Bruce P. Lee. 2015. “Fibrin Gel as an Injectable Biodegradable Scaffold and Cell Carrier for Tissue Engineering.” *The Scientific World Journal* 2015. https://doi.org/10.1155/2015/685690.

Lin, Fubao, Xiang-Dong Ren, Zhi Pan, Lauren Macri, Wei-Xing Zong, Marcia G. Tonnesen, Miriam Rafailovich, Dafna Bar-Sagi, and Richard A.F. Clark. 2011. “Fibronectin Growth Factor-Binding Domains Are Required for Fibroblast Survival.” *The Journal of Investigative Dermatology* 131 (1): 84–98. https://doi.org/10.1038/jid.2010.253.

Liu, Meichen, Hua Lei, Ping Dong, Xin Fu, Zhigang Yang, Ying Yang, Jiguang Ma, Xia Liu, Yilin Cao, and Ran Xiao. 2017. “Adipose-Derived Mesenchymal Stem Cells from the Elderly Exhibit Decreased Migration and Differentiation

Abilities with Senescent Properties." *Cell Transplantation* 26 (9): 1505–19. https://doi.org/10.1177/0963689717721221.

Liu, Tong Ming. 2017. "Stemness of Mesenchymal Stem Cells." *Journal of Stem Cell Therapy and Transplantation* 1 (1): 071–073. https://doi.org/10.29328/journal.jsctt.1001008.

Loane, David J., and Kimberly R. Byrnes. 2010. "Role of Microglia in Neurotrauma." *Neurotherapeutics* 7 (4): 366–77. https://doi.org/10.1016/j.nurt.2010.07.002.

Lourenço, Tânia, Joana Paes de Faria, Christian A. Bippes, João Maia, José A. Lopes-da-Silva, João B. Relvas, and Mário Grãos. 2016. "Modulation of Oligodendrocyte Differentiation and Maturation by Combined Biochemical and Mechanical Cues." *Scientific Reports* 6 (February): 21563. https://doi.org/10.1038/srep21563.

Louvi, Angeliki, and Spyros Artavanis-Tsakonas. 2006. "Notch Signalling in Vertebrate Neural Development." *Nature Reviews Neuroscience* 7 (2): 93–102. https://doi.org/10.1038/nrn1847.

Lowry, Natalia Abramova, and Sally Temple. 2007. "Making Human Neurons from Stem Cells after Spinal Cord Injury." *PLoS Medicine* 4 (2). https://doi.org/10.1371/journal.pmed.0040048.

Lukovic, Dunja, Miodrag Stojkovic, Victoria Moreno-Manzano, Pavla Jendelova, Eva Sykova, Shomi S. Bhattacharya, and Slaven Erceg. 2015. "Concise Review: Reactive Astrocytes and Stem Cells in Spinal Cord Injury: Good Guys or Bad Guys?: Stem Cell and Astrogliosis." *STEM CELLS* 33 (4): 1036–41. https://doi.org/10.1002/stem.1959.

Lyou, Yung, Amber N Habowski, George T Chen, and Marian L Waterman. 2017. "Inhibition of Nuclear Wnt Signalling: Challenges of an Elusive Target for Cancer Therapy." *British Journal of Pharmacology* 174 (24): 4589–99. https://doi.org/10.1111/bph.13963.

Maio, Roberto Di, Kommu Naga Mohan, Brianna Heath, and Leonardo D'Aiuto. 2011. "Spontaneous Generation of Neurospheres from Mouse Embryonic Stem Cells." *Stem Cells in Clinic and Research*, August. https://doi.org/10.5772/24454.

Maynard, Frederick M, B Ducker, Susan L Garber, Ralph J Marino, Samuel L Stover, Charles H Tator, Robert L Waters, Jack E Wilberger, and Wise Young. n.d. "International Standards for Neurological and Functional Classi®cation of Spinal Cord Injury," 10.

Meirelles, Lindolfo da Silva, Arnold I. Caplan, and Nance Beyer Nardi. 2008. "In Search of the In Vivo Identity of Mesenchymal Stem Cells." *STEM CELLS* 26 (9): 2287–99. https://doi.org/10.1634/stemcells.2007-1122.

Menorca, Ron M. G., Theron S. Fussell, and John C. Elfar. 2013. "Peripheral Nerve Trauma: Mechanisms of Injury and Recovery." *Hand Clinics* 29 (3): 317–30. https://doi.org/10.1016/j.hcl.2013.04.002.

Michalski, John-Paul, and Rashmi Kothary. 2015. "Oligodendrocytes in a Nutshell." *Frontiers in Cellular Neuroscience* 9 (September). https://doi.org/10.3389/fncel.2015.00340.

Moeendarbary, Emad, Isabell P. Weber, Graham K. Sheridan, David E. Koser, Sara Soleman, Barbara Haenzi, Elizabeth J. Bradbury, James Fawcett, and Kristian Franze. 2017. "The Soft Mechanical Signature of Glial Scars in the Central Nervous System." *Nature Communications* 8 (March): 14787. https://doi.org/10.1038/ncomms14787.

Mohamed-Ahmed, Samih, Inge Fristad, Stein Atle Lie, Salwa Suliman, Kamal Mustafa, Hallvard Vindenes, and Shaza B. Idris. 2018. "Adipose-Derived and Bone Marrow Mesenchymal Stem Cells: A Donor-Matched Comparison." *Stem Cell Research & Therapy* 9 (1): 168. https://doi.org/10.1186/s13287-018-0914-1.

Mooney, Rachael, Bill Tawil, and Melissa Mahoney. 2009. "Specific Fibrinogen and Thrombin Concentrations Promote Neuronal Rather Than Glial Growth When Primary Neural Cells Are Seeded Within Plasma-Derived Fibrin Gels." *Tissue Engineering Part A* 16 (5): 1607–19. https://doi.org/10.1089/ten.tea.2009.0372.

Mothe, Andrea J., and Charles H. Tator. 2012. "Advances in Stem Cell Therapy for Spinal Cord Injury." *The Journal of Clinical Investigation* 122 (11): 3824–34. https://doi.org/10.1172/JCI64124.

Ng, Felicia, Shayne Boucher, Susie Koh, Konduru S. R. Sastry, Lucas Chase, Uma Lakshmipathy, Cleo Choong, et al. 2008. "PDGF, TGF-β, and FGF Signaling Is Important for Differentiation and Growth of Mesenchymal Stem Cells

(MSCs): Transcriptional Profiling Can Identify Markers and Signaling Pathways Important in Differentiation of MSCs into Adipogenic, Chondrogenic, and Osteogenic Lineages." *Blood* 112 (2): 295–307. https://doi.org/10.1182/blood-2007-07-103697.

Noori, Alireza, Seyed Jamal Ashrafi, Roza Vaez-Ghaemi, Ashraf Hatamian-Zaremi, and Thomas J. Webster. 2017. "A Review of Fibrin and Fibrin Composites for Bone Tissue Engineering." International Journal of Nanomedicine. July 12, 2017. https://doi.org/10.2147/IJN.S124671.

Norris, Erin H., and Sidney Strickland. 2017. "Fibrinogen in the Nervous System: Glia Beware." *Neuron* 96 (5): 951–53. https://doi.org/10.1016/j.neuron.2017.11.021.

Oh, Jin Soo, Keung Nyun Kim, Sung Su An, William A. Pennant, Hyo Jin Kim, So-Jung Gwak, Do Heum Yoon, Mi Hyun Lim, Byung Hyune Choi, and Yoon Ha. 2011. "Cotransplantation of Mouse Neural Stem Cells (MNSCs) with Adipose Tissue-Derived Mesenchymal Stem Cells Improves MNSC Survival in a Rat Spinal Cord Injury Model." *Cell Transplantation* 20 (6): 837–49. https://doi.org/10.3727/096368910X539083.

Okada, Seiji. 2016. "The Pathophysiological Role of Acute Inflammation after Spinal Cord Injury." *Inflammation and Regeneration* 36 (October). https://doi.org/10.1186/s41232-016-0026-1.

Oliveira, Sophia L. B., Micheli M. Pillat, Arquimedes Cheffer, Claudiana Lameu, Telma T. Schwindt, and Henning Ulrich. 2013. "Functions of Neurotrophins and Growth Factors in Neurogenesis and Brain Repair." *Cytometry Part A* 83A (1): 76–89. https://doi.org/10.1002/cyto.a.22161.

Oyinbo, Charles Aidemise. n.d. "Secondary Injury Mechanisms in Traumatic Spinal Cord Injury: A Nugget of This Multiply Cascade," 19.

Park, H.-C. 2003. "Delta-Notch Signaling Regulates Oligodendrocyte Specification." *Development* 130 (16): 3747–55. https://doi.org/10.1242/dev.00576.

Patrikoski, Mimmi, Bettina Mannerström, and Susanna Miettinen. 2019. "Perspectives for Clinical Translation of Adipose Stromal/Stem Cells." Research article. Stem Cells International. 2019. https://doi.org/10.1155/2019/5858247.

Peignon, Grégory, Aurélie Durand, Wulfran Cacheux, Olivier Ayrault, Benoît Terris, Pierre Laurent-Puig, Noah F. Shroyer, et al. 2011. "Complex Interplay between β-Catenin Signalling and Notch Effectors in Intestinal Tumorigenesis." *Gut* 60 (2): 166–76. https://doi.org/10.1136/gut.2009.204719.

Phinney, Donald G., and Darwin J. Prockop. 2009. "Concise Review: Mesenchymal Stem/Multipotent Stromal Cells: The State of Transdifferentiation and Modes of Tissue Repair—Current Views." *STEM CELLS* 25 (11): 2896–2902. https://doi.org/10.1634/stemcells.2007-0637.

Piccin, David, and Cindi M. Morshead. 2011. "Wnt Signaling Regulates Symmetry of Division of Neural Stem Cells in the Adult Brain and in Response to Injury." *STEM CELLS* 29 (3): 528–38. https://doi.org/10.1002/stem.589.

Pires-Neto, M. A., S. Braga-de-Souza, and R. Lent. 1999. "Extracellular Matrix Molecules Play Diverse Roles in the Growth and Guidance of Central Nervous System Axons." *Brazilian Journal of Medical and Biological Research* 32 (5): 633–38. https://doi.org/10.1590/S0100-879X1999000500017.

Popko, Brian. 2003. "Notch Signaling: A Rheostat Regulating Oligodendrocyte Differentiation?" *Developmental Cell* 5 (5): 668–69. https://doi.org/10.1016/S1534-5807(03)00331-9.

Prasad Chennazhy, Krishna, and Lissy K. Krishnan. 2005. "Effect of Passage Number and Matrix Characteristics on Differentiation of Endothelial Cells Cultured for Tissue Engineering." *Biomaterials* 26 (28): 5658–67. https://doi.org/10.1016/j.biomaterials.2005.02.024.

prokop, Reichardt. 2011. "Introduction: The Role of Extracellular Matrix in Nervous System Development and Maintenance."https://onlinelibrary.wiley.com/doi/pdf/10.1002/dneu.20975.

P dev, and Fletcher TL al et. n.d. "The Distribution of Synapsin I and Synaptophysin in Hippocampal Neurons Developing in Culture. - PubMed - NCBI." Accessed January 10, 2020. https://www.ncbi.nlm.nih.gov/pubmed/1904480.

Purves, Dale, George J. Augustine, David Fitzpatrick, Lawrence C. Katz, Anthony-Samuel LaMantia, James O. McNamara, and S. Mark Williams. 2001. "The Initial Formation of the Nervous System: Gastrulation and Neurulation." *Neuroscience. 2nd Edition*. https://www.ncbi.nlm.nih.gov/books/NBK10993/.

Qu, Jing, and Huanxiang Zhang. 2017. “Roles of Mesenchymal Stem Cells in Spinal Cord Injury.” Research article. Stem Cells International. 2017. https://doi.org/10.1155/2017/5251313.

“Review: Manipulating the Extracellular Matrix and Its Role in Brain and Spinal Cord Plasticity and Repair - Burnside - 2014 - Neuropathology and Applied Neurobiology - Wiley Online Library.” n.d. Accessed October 15, 2018. https://onlinelibrary.wiley.com/doi/abs/10.1111/nan.12114.

Rust, Ruslan, and Julia Kaiser. 2017. “Insights into the Dual Role of Inflammation after Spinal Cord Injury.” *The Journal of Neuroscience* 37 (18): 4658–60. https://doi.org/10.1523/JNEUROSCI.0498-17.2017.

Sanalkumar, Rajendran, Sivadasan Bindu Dhanesh, and Jackson James. 2010. “Non-Canonical Activation of Notch Signaling/Target Genes in Vertebrates.” *Cellular and Molecular Life Sciences* 67 (17): 2957–68. https://doi.org/10.1007/s00018-010-0391-x.

Schweigreiter, Rüdiger, and Christine E. Bandtlow. 2006. “Nogo in the Injured Spinal Cord.” *Journal of Neurotrauma* 23 (3–4): 384–96. https://doi.org/10.1089/neu.2006.23.384.

Sedý, Jirí, Lucia Urdzíková, Pavla Jendelová, and Eva Syková. 2008. “Methods for Behavioral Testing of Spinal Cord Injured Rats.” *Neuroscience and Biobehavioral Reviews* 32 (3): 550–80. https://doi.org/10.1016/j.neubiorev.2007.10.001.

Sekhon, Lali H.S., and Michael G. Fehlings. 2001. “Epidemiology, Demographics, and Pathophysiology of Acute Spinal Cord Injury:” *Spine* 26 (Supplement): S2–12. https://doi.org/10.1097/00007632-200112151-00002.

Sembulingam, K., and Prema Sembulingam. 2012. *Essentials of Medical Physiology*. JP Medical Ltd. 6th edition; pg: 659-774

Shen, Yingjie, Alan P. Tenney, Sarah A. Busch, Kevin P. Horn, Fernando X. Cuascut, Kai Liu, Zhigang He, Jerry Silver, and John G. Flanagan. 2009. “PTPσ Is a Receptor for Chondroitin Sulfate Proteoglycan, an Inhibitor of Neural Regeneration.” *Science (New York, N.Y.)* 326 (5952): 592–96. https://doi.org/10.1126/science.1178310.

Shi, Zhongju, Hongyun Huang, Hongyun Huang, and Shiqing Feng. 2017. "Stem Cell-Based Therapies to Treat Spinal Cord Injury: A Review." Journal of Neurorestoratology. July 3, 2017. https://doi.org/10.2147/JN.S139677.

Silva, Nuno A., Nuno Sousa, Rui L. Reis, and António J. Salgado. 2014. "From Basics to Clinical: A Comprehensive Review on Spinal Cord Injury." *Progress in Neurobiology* 114 (March): 25–57. https://doi.org/10.1016/j.pneurobio.2013.11.002.

Silver, Jerry, and Jared H. Miller. 2004. "Regeneration beyond the Glial Scar." *Nature Reviews Neuroscience* 5 (2): 146–56. https://doi.org/10.1038/nrn1326.

Simons, Mikael, and Klaus-Armin Nave. 2016. "Oligodendrocytes: Myelination and Axonal Support." *Cold Spring Harbor Perspectives in Biology* 8 (1). https://doi.org/10.1101/cshperspect.a020479.

Simons, Mikael, and Katarina Trajkovic. 2006. "Neuron-Glia Communication in the Control of Oligodendrocyte Function and Myelin Biogenesis." *Journal of Cell Science* 119 (21): 4381–89. https://doi.org/10.1242/jcs.03242.

Singh, Roop, Sansar Chand Sharma, Rajeev Mittal, and Ashwini Sharma. 2003. "TRAUMATIC SPINAL CORD INJURIES IN HARYANA : AN EPIDEMIOLOGICAL STUDY," Indian J Commun Med 28:184–186

Sofroniew, Michael V. 2009. "Molecular Dissection of Reactive Astrogliosis and Glial Scar Formation." *Trends in Neurosciences* 32 (12): 638–47. https://doi.org/10.1016/j.tins.2009.08.002.

Sofroniew Michael V. 2015. "Astrocyte Barriers to Neurotoxic Inflammation." *Nature Reviews Neuroscience* 16 (5): 249–63. https://doi.org/10.1038/nrn3898.

"Spinal Cord Injury: Hope Through Research | National Institute of Neurological Disorders and Stroke." n.d. Accessed March 25, 2018. https://www.ninds.nih.gov/Disorders/Patient-Caregiver-Education/Hope-Through-Research/Spinal-Cord-Injury-Hope-Through-Research.

Sreerekha, P. R., P. Divya, and L. K. Krishnan. 2006. "Adult Stem Cell Homing and Differentiation in Vitro on Composite Fibrin Matrix." *Cell Proliferation* 39 (4): 301–12. https://doi.org/10.1111/j.1365-2184.2006.00389.x.

Sun, Yan, Chong-Chong Xu, Jin Li, Xi-Yin Guan, Lu Gao, Li-Xiang Ma, Rui-Xi Li, Yu-Wen Peng, and Guo-Pei Zhu. 2013. "Transplantation of Oligodendrocyte Precursor Cells Improves Locomotion Deficits in Rats with Spinal Cord Irradiation Injury." Edited by Brahim Nait-Oumesmar. *PLoS ONE* 8 (2): e57534. https://doi.org/10.1371/journal.pone.0057534.

Tao, Helen, Renuka Rao, and David D. F. Ma. 2005. "Cytokine-Induced Stable Neuronal Differentiation of Human Bone Marrow Mesenchymal Stem Cells in a Serum/Feeder Cell-Free Condition." *Development, Growth & Differentiation* 47 (6): 423–33. https://doi.org/10.1111/j.1440-169X.2005.00810.x.

Tara, S, and Lissy Krishnan. 2015. "Bioengineered Fibrin-Based Niche to Direct Outgrowth of Circulating Progenitors into Neuron-like Cells for Potential Use in Cellular Therapy." *Journal of Neural Engineering* 12 (May): 036011. https://doi.org/10.1088/1741-2560/12/3/036011.

Tara, S., and Lissy K. Krishnan. 2019. "Differentiation of Circulating Neural Progenitor Cells in Vitro on Fibrin-Based Composite -Matrix Involves Wnt- β-Catenin-like Signaling." *Journal of Cell Communication and Signaling* 13 (1): 27–38. https://doi.org/10.1007/s12079-018-0467-1.

Tawk, Marcel, Joelle Makoukji, Martin Belle, Cosima Fonte, Amalia Trousson, Thomas Hawkins, Huiliang Li, Said Ghandour, Michael Schumacher, and Charbel Massaad. 2011. "Wnt/β-Catenin Signaling Is an Essential and Direct Driver of Myelin Gene Expression and Myelinogenesis." *Journal of Neuroscience* 31 (10): 3729–42. https://doi.org/10.1523/JNEUROSCI.4270-10.2011.

Terzic, Dino, Jacob R. Maxon, Leah Krevitt, Christina DiBartolomeo, Tarini Goyal, Walter C. Low, James R. Dutton, and Ann M. Parr. 2016. "Directed Differentiation of Oligodendrocyte Progenitor Cells From Mouse Induced Pluripotent Stem Cells." *Cell Transplantation* 25 (2): 411–24. https://doi.org/10.3727/096368915X688137.

Tsigelny, Igor F., Valentina L. Kouznetsova, Nathan Lian, and Santosh Kesari. 2016. "Molecular Mechanisms of OLIG2 Transcription Factor in Brain Cancer." *Oncotarget* 7 (33): 53074–101. https://doi.org/10.18632/oncotarget.10628.

Ullah, Imran, Raghavendra Baregundi Subbarao, and Gyu Jin Rho. 2015. "Human Mesenchymal Stem Cells - Current Trends and Future Prospective." *Bioscience Reports* 35 (2). https://doi.org/10.1042/BSR20150025.

Urbán, Noelia, and François Guillemot. 2014. "Neurogenesis in the Embryonic and Adult Brain: Same Regulators, Different Roles." *Frontiers in Cellular Neuroscience* 8. https://doi.org/10.3389/fncel.2014.00396.

Varma, Abhay K., Arabinda Das, Gerald Wallace, John Barry, Alexey A. Vertegel, Swapan K. Ray, and Naren L. Banik. 2013. "Spinal Cord Injury: A Review of Current Therapy, Future Treatments, and Basic Science Frontiers." *Neurochemical Research* 38 (5): 895–905. https://doi.org/10.1007/s11064-013-0991-6.

Velasco, Manuel, Joselyn Rojas-Quintero, Mervin Chávez-Castillo, Milagros Rojas, Jordan Bautista, María Sofía Martínez, Juan Salazar, Rafael Mendoza, and Valmore Bermúdez. 2017. "Excitotoxicity: An Organized Crime at The Cellular Level." *Journal of Neurology and Neuroscience* 8 (3). https://doi.org/10.21767/2171-6625.1000193.

Villageois, Phi, Brigitte Wdziekonski, Laure-Emmanuelle Zaragosi, Magali Plaisant, Tala Mohsen-Kanson, Nadège Lay, Annie Ladoux, Pascal Peraldi, and Christian Dani. 2011. "Regulators of Human Adipose-Derived Stem Cell Self-Renewal." *American Journal of Stem Cells* 1 (1): 42–47.

Wang, Hai-feng, Xing-kai Liu, Rui Li, Ping Zhang, Ze Chu, Chun-li Wang, Hua-rui Liu, et al. 2017. "Effect of Glial Cells on Remyelination after Spinal Cord Injury." *Neural Regeneration Research* 12 (10): 1724–32. https://doi.org/10.4103/1673-5374.217354.

Watson, Robert A, and Trevor M Yeung. 2011a. "What Is the Potential of Oligodendrocyte Progenitor Cells to Successfully Treat Human Spinal Cord Injury?" *BMC Neurology* 11 (September): 113. https://doi.org/10.1186/1471-2377-11-113.

Wei, Xin, Xue Yang, Zhi-peng Han, Fang-fang Qu, Li Shao, and Yu-fang Shi. 2013. "Mesenchymal Stem Cells: A New Trend for Cell Therapy." *Acta Pharmacologica Sinica* 34 (6): 747–54. https://doi.org/10.1038/aps.2013.50.

Wheeler, Natalie A., and Babette Fuss. 2016. "Extracellular Cues Influencing Oligodendrocyte Differentiation and (Re)Myelination." *Experimental Neurology* 283 (September): 512–30. https://doi.org/10.1016/j.expneurol.2016.03.019.

"WHO | Spinal Cord Injury." n.d. WHO. Accessed March 12, 2018. http://www.who.int/mediacentre/factsheets/fs384/en/.

Xiao, Mei-Juan, Zhao Han, Bei Shao, and Kunlin Jin. 2009. "Notch Signaling and Neurogenesis in Normal and Stroke Brain." *International Journal of Physiology, Pathophysiology and Pharmacology* 1 (2): 192–202.

Yang, Fan, Yunhui Liu, Jie Tu, Jun Wan, Jie Zhang, Bifeng Wu, Shanping Chen, Jiawei Zhou, Yangling Mu, and Liping Wang. 2014. "Activated Astrocytes Enhance the Dopaminergic Differentiation of Stem Cells and Promote Brain Repair through BFGF." *Nature Communications* 5 (December): 5627. https://doi.org/10.1038/ncomms6627.

Yang, Jin, Liu-Lin Xiong, You-Cui Wang, Xiang He, Ling Jiang, Song-Jun Fu, Xue-Fei Han, Jia Liu, and Ting-Hua Wang. 2018. "Oligodendrocyte Precursor Cell Transplantation Promotes Functional Recovery Following Contusive Spinal Cord Injury in Rats and Is Associated with Altered MicroRNA Expression." *Molecular Medicine Reports* 17 (1): 771–82. https://doi.org/10.3892/mmr.2017.7957.

Yiu, Glenn, and Zhigang He. 2006. "Glial Inhibition of CNS Axon Regeneration." *Nature Reviews. Neuroscience* 7 (8): 617–27. https://doi.org/10.1038/nrn1956.

Zannettino, A. C. W., S. Paton, A. Arthur, F. Khor, S. Itescu, J. M. Gimble, and S. Gronthos. 2008. "Multipotential Human Adipose-Derived Stromal Stem Cells Exhibit a Perivascular Phenotype in Vitro and in Vivo." *Journal of Cellular Physiology* 214 (2): 413–21. https://doi.org/10.1002/jcp.21210.

Zaragosi, Laure-Emmanuelle, Gérard Ailhaud, and Christian Dani. 2006. "Autocrine Fibroblast Growth Factor 2 Signaling Is Critical for Self-Renewal of Human Multipotent Adipose-Derived Stem Cells." *Stem Cells (Dayton, Ohio)* 24 (11): 2412–19. https://doi.org/10.1634/stemcells.2006-0006.

Zhang, Jiawei. 2019. "Secrets of the Brain: An Introduction to the Brain Anatomical Structure and Biological Function." *ArXiv:1906.03314 [Cs, q-Bio]*, May. http://arxiv.org/abs/1906.03314.

Zhou, Xiang, Xijing He, and Yi Ren. 2014. "Function of Microglia and Macrophages in Secondary Damage after Spinal Cord Injury." *Neural Regeneration Research* 9 (20): 1787–95. https://doi.org/10.4103/1673-5374.143423.

Zhou, Zhi-Dong, Udhaya Kumari, Zhi-Cheng Xiao, and Eng-King Tan. 2010. "Notch as a Molecular Switch in Neural Stem Cells." *IUBMB Life* 62 (8): 618–23. https://doi.org/10.1002/iub.362.

Zhu, Yanxia, Tianqing Liu, Kedong Song, Xiubo Fan, Xuehu Ma, and Zhanfeng Cui. 2008a. "Adipose-Derived Stem Cell: A Better Stem Cell than BMSC." *Cell Biochemistry and Function* 26 (6): 664–75. https://doi.org/10.1002/cbf.1488.

LIST OF PUBLICATIONS

1. Chandrababu, K Senan M Krishnan, L K Exploitation of fibrin based signaling niche for deriving progenitors from human adipose derived mesenchymal stem cells towards potential neural engineering applications Sci Rep 10 7116 2020

   https :://doi org/ 10 1038 /s 41598 020 63445 2

2. Krishnapriya Chandrababu Harikrishnan VS, Tara S, Arya Anil, Sabareeswaran A Lissy K.K, In vivo engineering of neural tissue with adipose derived mesenchymal stem cells using fibrin based niche for pre differentiation and transplantation (2020)(under review)

CURRICULUM VITAE

Personal details

Name : Krishnapriya Chandrababu

Email : bio.krishnapriya@gmail.com

Educational qualification

Master of Science in Biotechnology:
St. Berchman's College (2012-2014)
Changanacherry (affiliated to M.G University, Kerala)
Score: 3.56/4 ; A Grade

Bachelor of Science in Biotechnology:
Amrita School of Biotechnology (2009-2012)
Amritapuri, Amrita University
Score: 8.56/10

Board of Higher Secondary Examination of Kerala:
Govt. Girls Higher Secondary School (2008)
Haripad, Alapuzha
Score: 95%

Matriculation Exam (Kerala):
Bethany Balikamadom HSS (2006)
Nangiarkulangara, Alapuzha
Score: 94%

Academic project details

1. Title: "Expression, Purification and Refolding of Recombinant Amidase in *Escherichia coli"*
   Done during final semester of B.Sc.under the guidance of Dr. Nandita Mishra & Dr. Sanjay Pal, at Amrita School of Biotechnology, Amritapuri, Kollam.

2. Title : " Gene expression alteration of Alpha synuclein in cardiac tissue during diabetes and Ganoderma extract treatment in rats "
   Done during final semester of M.Sc. under guidance of Dr. S. Balarama Kaimal, Scientist, Neurosurgery and Regenerative Medicine Laboratory, at Pushpagiri Research Centre, Thiruvalla

Awards and achievements:

1. National fellowship: DBT Junior Research Fellowship (2015)
2. 3rd position in Mahatma Gandhi university for M.Sc. Biotechnology (2014, PGCSS scheme)
3. Overseas Student Travel Fellowship, SCSS Symposium 2017
4. IBRO- APRC School 2019 attendee; SCTIMST, Trivandrum, 22ndApril - 6th May

Publications:

1. Chandrababu, K Senan M Krishnan, L K Exploitation of fibrin based signaling niche for deriving progenitors from human adipose derived mesenchymal stem cells towards potential neural engineering applications Sci Rep 10 7116 2020

Conferences:

1. Participated in and organized the seminar - Bioradiance '14 on "Cytogenetics and application" conducted by Pushpagiri research center and Pushpagiri center for virology (3/5/14)
2. Participated in CPCSEA- PIMSRC Workshop on "Use of experimental animal in toxicity studies and research" at Pushpagiri institute of medical science and research center (9/6/2014)
3. Oral presentation, National seminar on Frontiers in Biotechnology, inter-university center for Genomics and Gene Technology, Dept. of Biotechnology, University of Kerala, 1-3 March 2017
4. Poster Presentation, Advances in Cell Therapy, Academia, SGH, Stem Cell Society Singapore Symposium,16-17 November 2017

Areas of interest: Neurobiology, Stem cell biology, Regenerative biology

APPENDIX

1. **PBS** (1000ml) (pH 7.4; filtered and stored at RT).

Reagent	Amount
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g

2. **PBST** was prepared by adding 0.1% Tween-20 to PBS.

Reagent	Amount
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Tween 20	0.1 %

3. **HBSS** (1000ml) (pH 7.4; filtered, autoclaved and stored at 4^0C

Reagent	Amount
KCl	0.4g
KH_2PO_4	0.06g
NaCl	8g
Na_2PO_4	0.0482g

4. **SFM** 50 ml (Filtered and stored at 4^0C)

Reagent	Amount
DMEM: F12	50ml
Antibiotics (10X)	500µl

5. **DMEM F12 (1%)** 50 ml (Filtered and stored at 4^0C)

Reagent	Amount
DMEM: F12	49 ml
FBS	500µl
Antibiotics (10X)	500µl

6. DMEM LG (10%) 50 ml (Filtered and stored at 4^0C)

Reagent	Amount
DMEM: F12	45 ml
FBS	5ml
Antibiotics (10X)	500µl

Document Information

Analyzed document	Krishnapriya- plagiarism.docx (D77738655)
Submitted	8/13/2020 1:18:00 PM
Submitted by	Anugya Bhatt
Submitter email	anugyabhatt@sctimst.ac.in
Similarity	7%
Analysis address	anugyabhatt.sctims@analysis.urkund.com

Sources included in the report

SA	**Sree Chitra Tirunal Institute, Thiruvananthapuram / MANUSCRIPT 17112019.docx** Document MANUSCRIPT 17112019.docx (D59158254) Submitted by: anugyabhatt@sctimst.ac.in Receiver: anugyabhatt.sctims@analysis.urkund.com	65
J	**Oligodendrocyte precursor cells in spinal cord injury: a review and update.(Report)** URL: 95fa41b0-1906-414a-b6aa-0984a904bb6e Fetched: 3/7/2019 3:20:28 PM	1
J	**Differentiation and Purification of Human Pluripotent Stem Cell-derived Neuronal and Glial Cells - graft designing for spinal cord injury repair** URL: 8c1f129d-d672-4163-9cfa-70ac8de0378a Fetched: 2/15/2019 7:14:29 PM	1
SA	**Subathra R.docx** Document Subathra R.docx (D57361543)	1