BIOENGINEERED FIBRIN MATRIX FOR *IN VITRO* DIFFERENTIATION OF CIRCULATING PROGENITOR CELLS TO NEURONS AND FOR THEIR *IN VIVO* SURVIVAL UPON TRANSPLANTATION IN RAT SPINAL CORD INJURY MODEL

A THESIS PRESENTED BY

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CERTIFICATE

I, **Tara S**, hereby certify that I had personally carried out the work depicted in the thesis entitled, ***"Bioengineered fibrin matrix for in vitro differentiation of circulating progenitor cells to neurons and for their in vivo survival upon transplantation in rat spinal cord injury model",*** except where due acknowledgment 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.

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This is to certify that **Ms. Tara S**, in the division of Thrombosis Research Unit 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, **"*Bioengineered fibrin matrix for in vitro differentiation of circulating progenitor cells to neurons and for their in vivo survival upon transplantation in rat spinal cord injury model*"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

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

Thiruvananthapuram
04-01-2017

Dr. Lissy K Krishnan
(Research Supervisor)

DEDICATED TO
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ABBREVIATIONS

2D	: 2- Dimensional
3D	: 3- Dimensional
ACD	: Acid Citrate Dextrose
AGE	: Agarose Gel Electrophoresis
ANOVA	: Analysis Of Variance
ASC	: Adult Stem Cell
ASIA	: American Spinal cord Injury Association
BBB	: Basso Beattie Bresnahan
BDNF	: Brain Derived Neurotrophic Factor
bFGF	: basic Fibroblast Growth Factor
BMDSC	: Bone Marrow Derived Stem Cells
BSA	: Bovine Serum Albumin
C-	: Cervical
C	: Control
L-	: Lumbar
cDNA	: Complimentary Deoxy ribo Nucleic Acid
CF	: Control Fibrin
CM	: Control Medium
CNIM	: Complete Neural Induction Medium
CNS	: Central Nervous System
CPCSEA	: Committee for the Purpose of Control and Supervision of Experiments on Animals
CSPG	: Chondroitin Sulphate Proteoglycan
DAB	: 3,3'-Diaminobenzidine
DAPI	: 4',6-diamidino-2-phenylindole
DCX	: Double Cortin
DEAE	: Diethyl Amino Ethyl
dNTPs	: deoxy Nucleotide Tri Phosphate
ECM	: Extracellular Matrix
EDTA	: Ethylene Diamine Tetra Acetic acid
EGF	: Epidermal Growth Factor
ESC	: Embryonic Stem Cell
E-SEM	: Environmental Scanning Electron Microscopy
FACS	: Fluorescent Activated Cell Sorter
FBS	: Foetal Bovine Serum
FC	: Fibrinogen Composite
FGF	: Fibroblast Growth Factor
Fib	: Fibrinogen
FITC	: Fluorescein isothiocyanate
FN	: Fibronectin
GAG	: Glycosaminoglycan

GAPDH : Glyceraldehyde 3-Phosphate Dehydrogenase
Gel : Gelatin
GF : Growth Factor
GFAP : Glial Fibrillary Acidic Protein
H&E : Hematoxylin and Eosin
HA : Hyaluronic Acid
HBSS : Hank's Balanced Salt Solution
HE : Hypothalamus Extract
HGF : Hepatocyte Growth Factor
HRP : Horseradish Peroxidase
IAEC : Institutional Animal Ethics Committee
IEC : Institutional Ethics Committee
IHC : Immunohistochemistry
Inh : Inhibitor
iPSCs : Induced Pluripotent Stem Cells
KCl : Potassium Chloride
La : Laminin
MAP-2 : Microtubule-Associated Protein 2
MAPC : Multipotent Adult Progenitor Cell
MN : Motor neuron
MSC : Mesenchymal Stem Cells
NaCl : Sodium Chloride
NCAM : Neural Cell Adhesion Molecule
Nes : Nestin
NLC : Neuron-Like Cells
NPC : Neural Progenitor Cell
NSC : Neural Stem Cell
NSE : Neuron Specific Enolase
NT : Neurotrophin
OSP : Oligodendrocyte Specific Protein
PAS-AB : Periodic acid-Schiff-Alcian Blue
PBMNC : Peripheral Blood Mononuclear Cell
PBS : Phosphate Buffered Saline
PCNA : Proliferating Cell Nuclear Antigen
PDL : Poly-D-Lysine
PGF : Platelet Growth Factor
PNS : Peripheral Nervous System
PRP : Platelet Rich Plasma
PTAH : Phospho Tungstic Acid-Hematoxylin
PVDF : Poly Vinylidene Di Fluoride
qRT-PCR : Quantitative Real-time Polymerase Chain Reaction
RA : Retinoic Acid
RBC : Red Blood Cell

RNA	: Ribo Nucleic Acid
ROS	: Reactive Oxygen Species
RT	: Room Temperature
RT-PCR	: Reverse-Transcriptase Polymerase Chain Reaction
S-	: Sacral
SC	: Stem Cells
SCI	: Spinal cord Injury
SD	: Standard Deviation
SDF-1α	: Stromal cell Derived Factor-1alpha
SFM	: Serum Free Medium
SGZ	: Sub Granular Zone
SHH	: Sonic Hedge Hog
Std	: Standard
SVZ	: Sub Ventricular Zone
Syn	: Synaptophysin
T	: Test
T-	: Thoracic
TB	: Toluidine Blue
TBI	: Traumatic Brain Injury
TCPS	: Tissue Culture Poly Styrene
TH	: Tyrosine Hydroxylase
UCB	: Umbilical Cord Blood
UCBSCs	: Umbilical Cord Blood Stem Cells
WHO	: World Health Organization
Wnt	: Wingless
β-III tub	: β-III tubulin

ANNOTATIONS

%	: Percentage
<	: Less than
>	: Greater than
µg	: Microgram
µl	: Microlitre
µm	: Micro molar
d	: Days
h	: Hours
IU	: International Unit
kDa	: Kilo Dalton
mA	: Milli Ampere
min	: Minutes
mM	: Milli Molar
nm	: Nanometers
pmol	: Picomol

SYNOPSIS

The thesis is divided into 5 chapters.

Chapter I describe the research problem based on which the study hypothesis and objectives to be achieved are defined. Spinal cord Injury (SCI) is a universal problem, resulting in either temporary or permanent changes to the normal motor, sensory or autonomic functions of the body. It is estimated that 4.5 million people worldwide suffer from SCI of which ~33% of the population is from India. Current treatment modalities have poor outcome; therefore, various research efforts focus on development of suitable strategy for SCI repair.

Use of stem/progenitor cell for transplantation is considered a promising strategy to promote neuronal regeneration. Out of different stem cell types, autologous ones are considered more appropriate for effective patient-specific therapy because of its genomic stability, low tumorigenicity, absence of immune rejection and adherence to moral and ethical principles. Though several preclinical studies were attempted using bone marrow derived mesenchymal cells for cell-based correction of SCI, cell collection process is painful and cause discomfort. In this context, cells released from bone marrow into circulating blood which is known to include several multipotent progenitor cells (MAPC) could be a potential source of neural progenitor cells (NPC) for regeneration of injured neural tissue.

There are several challenges in using MAPC for regenerative medicine, including its rare occurrence in the peripheral blood mononuclear cell (PBMNC) fraction. Since PBMNC is a heterogenous population with multipotency at various stages of lineage commitment, isolation of patient-specific NPC by *in vitro* processing is crucial for

reducing interference by other cell types to prevent adverse outcomes. Further, isolated NPCs should be committed to neural lineage to prevent transdifferentiation into undesirable cell types after transplantation. The cells must possess proliferation potential at the time of transplantation for effective regeneration because the number of cells required for creating new tissue is not known. Roles of extracellular matrix (ECM) as adhesive matrix and signaling sites are well described in developmental biology. Therefore, employing a composition of different molecules biomimetic to neural tissue ECM may be a suitable approach to culture PBMNC for selection of NPC and their induction into neural lineage. A well-regulated lineage commitment may permit harvesting of NPC with potential to proliferate and differentiate into neuron-like cell (NLC) after transplantation therapy. The efficacy of cell-based therapies may depend on the ability to control proliferation, differentiation and survival.

Irrespective of the source of cell used, the success of cellular therapy depends on the ability of local niche to support homing, proliferation and differentiation. The cells may perish soon after transplantation because of the unfavourable environment at the injury site. Therefore, supplementing a compatible *in vivo* niche at the time of transplantation might facilitate NPC homing and regeneration. Fibrin, which is an injectable hydrogel, is expected to act as a potential cell carrier during transplantation because of its prospective to contain and protect the NPC from the adverse environment at the SCI site, thus promoting survival and effecting regeneration.

With this background, the research hypotheses to test this study has been developed: (i) the composition of a fibrin-based biomimetic niche may be standardized for isolation of NPCs from circulating MAPCs; (ii) the potential of NPCs for

differentiation into immature and mature neurons by the modified culture protocol may be tracked using specific markers; (iii) the involvement of signal transduction mechanism from the constituents of the niche for induction of NPC into NLC using markers of Wnt signaling pathway may be established; and (iv) the fibrin composition may be modified to function as a compatible cell delivery vehicle with better *in vivo* survival of transplanted NPCs in rat SCI model.

To prove the hypothesis, five major objectives have been set which are listed below.

1. To standardise the niche composition for isolation and lineage commitment of NPCs from PBMNCs using a matrix-directed selection strategy.
2. To study the potential of biomimetic niche on programming PBMNC-derived NPCs to different stages of differentiation.
3. To prove that normal signaling in neuronal development is operational in the standardised NPC differentiation program, by selecting Wnt-3a pathway as a model.
4. To identify a compatible composition of injectable 3D fibrin niche for *in vivo* delivery of isolated rat NPC and test transplantation efficiency in rat SCI model.
5. To carry out experiment in rat SCI model and prove better survival of NPCs at the injured site upon cell transplantation using fibrin as the cell carrier.

In chapter 2, literature in the field of research has been reviewed extensively to understand various strategies adopted for *in vitro* differentiation of stem cells in to neurons as well as problems associated with *in vivo* transplantation and its outcome. The topics discussed include an overview of SCI, current strategies to promote spinal

cord regeneration, pre-differentiation of stem cell *in vitro* and signaling pathways involved in neural differentiation. The review also describes development of SCI animal models and method of evaluation of SCI after regeneration.

In chapter 3, the experimental design adopted to achieve the proposed objectives of the study is elaborated. It includes detailed description of materials, experimental protocols and instruments employed for the present study. Western blot and immunodiffusion technique employed for demonstrating important molecules in the composite required for development of neural cells from the progenitors is described. Preparation of PBMNCs from discarded blood bank buffy coats, cell culture protocols, NPC identification and quantification in PBMNC, characterisation of NPC by morphological examination, immunocytochemistry, flow cytometry and Reverse Transcriptase Polymer Chain Reaction (RT-PCR) /qRT-PCR are also elaborated in this chapter. Markers employed at the transcriptional and translational level to demonstrate presence of NPC in circulation, to quantify frequency of cells, to track differentiation into neuron like cells (NLC), to demonstrate *in vitro* proliferation potential and survival after different periods of culture and maturation are explained. Morphological and qRT-PCR analysis with selected markers is described to demonstrate that NPC differentiation is through niche directed signaling with specific emphasis to prove Wnt-3a signaling is operational in mediating this differentiation. Cell labelling with tracking dye to identify survival of transplanted cells, creation of rat SCI model, cell transplantation and evaluation of the injured site using various histochemical staining techniques and functional analysis are also explained in detail.

In chapter 4, the results of the experiments substantiated by figures, tables and graphs are presented. The matrix-directed selection of NPCs, their expansion and differentiation in to NLC was found to be an effective and consistent procedure which is feasible for translation into clinical practice. The progression of NPC differentiation into neurons on optimised niche was found to be a slow and steady process. It resulted in >90% homogenous intermediate neurons which could be harvested without enzyme treatment. As compared to the basic differentiation medium, additional KCl in the niche had profound influence on cell lengthening, cell survival and expression of functional proteins such as synaptophysin and Tyrosine Hydroxylase. Wnt-3a signaling was found to play an important role in the NPC attachment, elongation, survival as well as differentiation. Wnt-3a signals were found to be a collective effect of various niche constituents.

Isolation of NPCs from rat circulating PBMNCs with proliferation and differentiation potential was achieved with the optimised niche. The use of NPC from inbred colony of animals seemed to be a suitable source which behaved similar to autologous cells in terms of immune response upon transplantation into a rat SCI model. The ideal 3D-niche composition that supports optimal neuronal growth for use in *in vivo* cell delivery was standardised. Animal model with clinical signs of contusion SCI was successfully created. The rat NPCs harvested at early period of culture survived for up to 8 days and retained neural markers upon transplantation into rat SCI model as per histology and immunofluorescence staining. The retention and survival of cells was found to be much better when cells were transplanted with fibrin as compared to cells delivered in culture medium as control. The functional

locomotor scoring was also better. Fibrin was also found to exhibit immunomodulatory properties.

In chapter 5, the results obtained during this study are discussed in the light of other similar studies published in the literature. Use of NPCs derived from adult circulating progenitors is found to be a promising strategy. This study appears to be the first one that described the controlled differentiation of circulating NPC that enabled enzyme-free harvesting of cells, suitable for transplantation. Since lineage commitment of NPCs was found to be a programmed process supported by the biomimetic fibrin-based niche, the cells possessing further proliferation and differentiation potential could be obtained at the time of harvest suggesting the prospect for NPC proliferation and differentiation after transplantation. The study demonstrated that the premature NPC harvested from the *in vitro* culture niche could be resuspended in either fibrinogen or in culture medium and transplanted by injection to the sites of SCI. It was proven that fibrinogen delivered with thrombin formed fibrin clot and retained the cells at the injured site. Using *in vitro* experiments, concentrations of fibrinogen and thrombin was optimized for obtaining cytocompatible 3D fibrin niche with suitable fiber density and porosity. The transplanted NPCs were retained more at the injury site even after 7 days when cells were transplanted using fibrin as compared to the delivery using culture medium. The cells thus transplanted were found to maintain neural lineage as demonstrated by the presence of neural markers after seven days of transplantation. The fibrin-based cell delivery strategy standardised in this study is proposed to have unlimited potential in various clinical situations where cell / drug retention at the injured site is a major challenge.

In chapter 6, results of the study are summarised and conclusions are drawn. To summarize, neural progenitors can be obtained from circulating blood for potential use in autologous cell-based therapy for regeneration of neural injuries. The niche directed biological control of differentiation enables collection of progenitors with proliferation and differentiation potential for *in vivo* transplantation. The role of niche-directed Wnt-3a signaling in cell attachment, elongation, proliferation and expression of neural markers was proven. Use of biomimetic niche, composed mainly from constituents of human origin, makes it feasible for translation of this technology into regenerative medicine. Delivery of cells using fibrin as cell carrier is found to be effective for cell survival post transplantation. Use of inbred colony of rats for PBMNC isolation and transplantation is found to be a good model for studying long-term effect of autologous cell-based therapy. The limitations of the study have been identified and future studies are proposed in this chapter to address the gap and make clinical use of the developed technology. The citations are listed in the bibliography section.

Publications

1. Tara S and Krishnan LK. Bioengineered fibrin-based niche to direct outgrowth of circulating progenitors into neuron-like cells for potential use in cellular therapy. J Neural Eng 2015; 12:036011.
2. Tara S, Harikrishnan VS, Easwer HV, Sandhyamani S, Lissy K. Krishnan. Role of fibrin-based niche for effective delivery and survival of autologous neural progenitor cells at spinal cord injury site in a rat model (under communication).
3. Tara S and Lissy K. Krishnan. Wnt signals from fibrin-based niche facilitate β-catenin mediated differentiation of circulating neural progenitor cells to neurons (under communication).
4. Indu S, Tara S and Lissy K.Krishnan. Fibrin-based niche directs bone marrow derived mesenchymal stem cells into neuron like cells (under communication).

CHAPTER 1

1. INTRODUCTION

Spinal cord is the part of the Central Nervous System (CNS) that mediates the transmission of impulses from different parts of the body to brain and vice versa. When Spinal cord injury (SCI) occurs, the associated neuronal loss and degeneration severs this communication between the brain and the body causing a significant impact on the life expectancy and quality of life of the victims (Zhang et al., 2010)(Thuret et al., 2006). The injury/trauma to the spinal cord could be caused due to contusion, compression, hemisection or complete section of the spinal cord resulting in temporary or permanent changes in the normal motor, sensory or autonomic functions of the body. It is estimated that about 4.5 million people worldwide suffer from SCI and about 0.5 million people are added to this list every year (WHO, 2015). In India, 1.5 million people are reported to have SCI and approximately 10,000 people are added to this yearly (data published by Manipal College of Allied Health Sciences, Karnataka/ Dehradun PGI of Biomedical Research).

The current treatment modalities for SCI are aimed to minimise the damage occurred and to restore at least limited abilities to the patient. This is achieved through prevention of further damage by stabilisation of the vertebra, administration of corticosteroids and painkillers, physiotherapy and emotional therapy. However, these approaches lack effectiveness and have drawbacks like side effects and physical-financial burdens to the patient which necessitates requirement for alternative therapies.

Various experimental strategies have been developed to promote axonal regeneration which includes neuroprotection, neurorestoration, neuroregeneration, specific regeneration and neuroconstruction (Schwab et al., 2006). Of these, neuroconstruction which includes cell/tissue replacement is an obvious option for the treatment of spinal cord injury lesions. Use of tissue replacements has been limited these days due to inadequacy, allogenecity and ethical issues and researches are focussed on cell-based therapies. The transplantation of a stem/progenitor cell could be a promising approach to promote neuronal regeneration (Lindvall et al., 2004). Embryonic stem cells (ESCs) and Adult stem cells (ASCs) are the primary focus of stem cell based therapies. The focus of current research is on autologous sourced ASCs. Their genomic stability, low immunogenicity, low tumorigenicity, low chance of immune rejection and adherence to ethical principles make them appropriate for developing effective patient-specific therapy (Ma et al., 2011)(Hsu et al., 2013).

Despite the advantages of ASCs, there are several challenges associated which includes identification of a suitable cell source which is easy to collect without much discomfort or tissue damage; ability to proliferate and differentiate in to specific lineage and the one that it is easily translatable to clinics. Even though stem cells are present in adult brain, its isolation without damaging the nervous system is difficult. Therefore, an alternative ASC source capable of proliferation and differentiation in to specific lineage becomes necessary. Even though, bone marrow remains to be a promising source, its isolation is tedious and painful. Multipotent adult progenitor cells (MAPCs) are reported to be present in peripheral blood that had migrated from bone marrow along with blood cells through the vascular network. It is easily accessible and hence becomes a feasible stem cell source. The number of stem/

progenitor cells in circulation is however very low and heterogeneous. Therefore, *in vitro* expansion and subsequent differentiation in to neuronal lineage becomes important for obtaining sufficient number of cells for transplantation and to prevent undesirable outcomes, which could be achieved using an *in vitro* niche based on natural ECM molecules.

In this study, a biomimetic niche based on fibrin, growth factors and glycosaminoglycans was engineered. It was envisaged that, by adjusting the composition and concentration of specific components of the biomimetic culture matrix and the medium, selection of neural progenitor cells (NPCs) from the peripheral blood mononuclear cell (PBMNC) mixture, their proliferation, survival and differentiation into NLCs with expression of functional proteins might be achieved. It is crucial to use proliferating and immature neurons for transplantation to achieve a successful outcome. Only a gradual differentiation program can enable the collection of immature neural progenitors from culture. Successful *in vitro* differentiation should be a slow and steady process which would enable the harvest of cells at the required level of lineage commitment, which is an area of study to be focussed. Since the differentiation of neural progenitors to neurons involves well-defined signal transduction mechanisms it is important to prove that the pathways are followed while the progenitors differentiate and produce neurons. Since Wnt-3a signaling is one of the known mechanisms of neural development, this pathway may be selected to prove differentiation.

The adult CNS has very limited ability to regenerate. Transplantation of autologous, adult stem/progenitor cell may promote regeneration of injured spinal cord by replacing the damaged cells and regenerating host axons. It was anticipated that rat

NPCs isolated from rat blood using fibrin-based biomimetic niche could be used as an autologous cell source in rat SCI model. Identification of a suitable cell source for the treatment of SCI alone does not solve the problem. There are a number of post-transplantation issues that rises due to the unfavourable environment at the injury site which causes poor cell homing, limited cell survival, deprived cell-cell communication, undesirable differentiation and transplanted cell leakage that impedes the regeneration process. Thus, identification and optimisation of a niche, which can act as a cell carrier during transplantation also becomes necessary. For many cell types fibrin matrix has been found to serve as a promising cell delivery vehicle with the ability to retain and protect the transplanted cells at the site of injury. With this background, it may be expected that peripheral progenitors may be derived from peripheral blood and prospects of their selection as a cell source for regenerating SCI may be explored.

1.1. Peripheral blood as a potential stem/progenitor cell source

The peripheral blood stem cells that originate from bone marrow are multipotent and show biological characteristics similar to bone marrow derived stem cells (BMDSCs) (Zhao et al., 2003). Circulating MAPCs include various lineage-committed progenitors. The capability of the MAPC population to differentiate towards endothelial, myocardial and neural lineages under defined culture conditions has already been demonstrated (Porat et al., 2006). However, the challenge in the use of MAPCs in regenerative medicine is the small numbers of the specific cells present among a heterogenous population in circulation. Mobilization of a larger number of stem/progenitor cells from the bone marrow into circulation may be achieved using FDA approved cytokines such as a colony stimulating factor (Krause et al., 2001).

Neuronal outgrowth cells isolated from PBMNCs of stroke patients were induced into neural lineage which, upon transplantation in ischemic rat brains, survived over six months, suggesting the potential of these cells for neural regeneration (Jung et al., 2008). The homogeneity of the desired cell population achieved in all these cases is low and needs to be improved for a successful cell transplantation therapy.

1.2. Need for a biomimetic niche *in vitro*

The success of cell transplantation depends on the ability of cells to home, proliferate, differentiate and regenerate which is very much dependent on the interaction between the cell and the extra cellular matrix (ECM). The composition of ECM is critical for continuous regulation of stem cell properties during *in vitro* culture in the same way as it is important for transplanted cell behaviour *in vivo* (Zimmermann and Dours-Zimmermann, 2008)(Gevertz and Torquato, 2008). Therefore, constitution of a biomimetic composition of ECM comprising both insoluble and soluble components may be a suitable approach to the culture of PBMNCs and for inducing differentiation of NPCs into the desired phenotype. It has been reported that selective *in vitro* attachment of respective progenitors followed by their expansion to mature endothelial and smooth muscle cell phenotypes is favoured by the cell specific niche (Prasad Chennazhy and Krishnan, 2005)(Sreerekha et al., 2006). The effect of some matrix proteins on circulating NPCs in culture has been reported earlier (Jose and Krishnan, 2010). However, the previous study failed to demonstrate the purity of the resultant neuron-like cells (NLCs), the proliferation potential of NPCs and the expression of specific functional proteins, to confirm differentiation. However, since neural properties were consistently expressed on PBMNC derived NPCs, fine-tuning

of the composed niche may be required to confirm that the progenitors are capable of proliferation and further differentiation to prove their potential for use in regenerative neuro-medicine.

1.3. Bioengineering of improved fibrin based matrix

Components of fibrin, a natural scaffold that promotes wound healing in physiology may be used for constructing an *in vitro* niche. It is a clinically approved surgical sealant which has been used widely in various tissue engineering applications because of its properties to promote cell adhesion, migration and differentiation. It is biocompatible, biodegradable and hemostatic (Sreerekha et al., 2006)(Bensaïd et al., 2003). The use of fibrin has been demonstrated for differentiation of ESCs in to neurons (Willerth et al., 2007). The tumorigenic potential of the ESCs were still a concern. Growth factors play an important role in lineage commitment of stem/progenitor cells in addition to providing a more favourable environment *in vivo* for the cells to sustain at the site of injury. Identification of optimum growth factor effects when hypothalamus extract (HE) and platelet growth factor (PGF) are used for differentiation of peripheral blood progenitor cells to neurons is an important area to be studied.

1.4. Major signaling pathways in neural development

Use of stem/progenitor cells in regenerative medicine is an area of swift development which enabled delineation of the merits and demerits of different types of cells for clinical use. The fate of the cells during *in vitro* lineage commitment and after transplantation may largely depend on the biochemical pathways that direct cell proliferation and differentiation. Only when the developed protocols for

differentiation follows signaling processes similar to the natural signaling mechanisms in neural development and homeostasis, it is likely to be a reproducible, reliable and robust technique for *in vivo* applications. Understanding the regulation of differentiation by signaling is essential for controlling NPC differentiation in stem cell engineering. The efficacy of cell-based therapies may depend on the ability to control the proliferation, differentiation and survival of NPCs.

During nervous system development as well as in the matured CNS, Wnt family of secreted cell signaling proteins play an active role. It has been reported that Wnt-3a signaling promote the acquisition of pluripotency during reprogramming of somatic cells to induced pluripotent stem cells (Marson et al., 2008). Conditioned medium prepared from embryonic day 10-mouse hindbrain/somite/otocyst was found to contain Wnt proteins which were responsible for better sensory neuronal fate specification from mesenchymal stem cells (MSCs) through Tlx3 activation as compared to medium containing neural lineage inducing molecules such as sonic hedgehog (SHH) and retinoic acid (RA) (Kondo et al., 2011). Thus, a major pathway implicated in neuronal differentiation is Wnt signaling. It has been proven that Wnt-3a signaling inhibit the maintenance of neural stem cells, at the same time promoting the differentiation of embryonic neural stem cells into several cell lineages (Muroyama et al., 2004). Activation of Wnt5a/JNK signaling was identified responsible for neural differentiation of human adipose derived tissue and directing Axin/GSK-3ß. Level of Wnt5a was upregulated after neural differentiation whereas Wnt3 was down-regulated (Jang et al., 2015). Thus, whether Wnt signaling maintains stem cells in an undifferentiated and self-renewing state or whether it promotes differentiation remains controversial. A number of external cues as well as intrinsic

cellular programs seemed to be involved in neural development both *in vivo* and *in vitro*. Therefore, a biomimetic approach which elicits Wnt signals may program the cells in a better-controlled manner when stem/progenitor cells are directed to lineage commitment for regenerative purpose. Therefore, any attempt to derive stem/progenitor cells also address the possibility of obtaining bio-similar signals during the *in vitro* culture of the sourced cells for further proliferation, lineage commitment and differentiation which should be continuously available when transplanted as well.

1.5. Challenges due to hostile environment at injured site

To maximize stem cell-based therapeutic efficiency, some critical issues need to be addressed. Exposure to hostile conditions such as oxidative stress can result in considerably low survival rate of transplanted cells thereby reducing the therapeutic efficiency (Bokara et al., 2014). A number of studies have found that the survival of the transplanted NPCs is poor and they undergo undesirable differentiation, which has been attributed to the inflammatory environment of the injured spinal cord, which limits integration with the host tissue at the injury site. The unfavourable niche at the injury site also inhibit NPC differentiation to neurons (Johnson et al., 2010a). Moreover, the lesion site lacks the molecular signals which promote cell survival and differentiation due to the degradation of the ECM. Another consistent problem associated with transplantation is the very low rate of sustained engraftment of transplanted cells due to lack of active adhesion sites.

1.6. Engineering of the injury site for better cell homing

A promising option to solve the injury site associated problems is the use of biomaterials/biodegradable scaffolds in cell transplantation. It has been demonstrated that when embryonic neural progenitors in combination with Gelfoam was transplanted into rat neonatal SCI, cells survived, integrated with host tissue and differentiated into neurons and oligodendrocytes, thus contributing significantly to plasticity and return of lost function (Nakamura et al., 2005). However, being a foreign material, Gelfoam may have limitations for human use. Fibrin is a natural biocompatible biopolymer, suitable for wound healing. It supports population expansion, migration and proliferation of several cell types (Barsotti et al., 2011) including NPCs. The use of fibrin is expected to reduce the overall inhibitory nature of spinal cord lesions and provide a more favorable environment for cell transplantation and nerve regeneration. Researchers have employed fibrin to transplant ESC-derived neurons and found better recovery in rat SCI model (Willerth et al., 2007). Overall, the use of fibrin may be expected to promote survival and differentiation of transplanted PBMNC derived NPC into neurons leading to enhanced regeneration. Central nervous system is a very delicate tissue protected through blood-brain barrier. Therefore, when blood components are applied to the tissue site, care should be taken to ensure that optimal concentrations of the composite may be used to prevent possible adverse effects.

1.7. Optimisation of fibrin gel composition for *in vivo* use

When very high concentrations of fibrinogen is used as soft tissue sealant, fibrinolytic system may not be effective for the matrix degradation to remove the formed clot so that cells can migrate and proceed with fibrosis-free wound healing (LK Krishnan, Biomaterials 2004). Similarly, it has been reported that thrombin causes vascular disruption, inflammatory response, oxidative stress and direct cellular toxicity at higher concentrations. Thrombin is known to be a potent cell activator and excessive concentrations of thrombin may result in neurotoxicity (Donovan et al., 1997). Therefore, it is important to identify a safe concentration of thrombin to maintain post transplantation neuron-specific properties. The components of fibrin may be safe for *in vivo* use when used in optimal concentrations or proportions.

1.8. Definition of the problem

Cell based therapy to improve the quality of life in victims of SCI is a current concept which is at various stages of experimentation. Identification of suitable cell source for effective use is a major step in developing cell transplantation therapy. Autologous ASCs have several scientific, ethical and legislative advantages over ESCs. An important consideration for collection of autologous cells is that the tissue should be collected easily using a painless procedure for isolation of stem or progenitor cells. As explained in previous sections, peripheral blood may be an easy obtainable tissue for isolation of NPC. However, the PBMNC fraction in which NPCs are present is a heterogeneous population containing very low numbers of NPCs. Therefore, a suitable method may be standardized to isolate NPCs at

proliferating phenotype so that sufficient number of homogenous NPCs may be obtained for therapeutic application. Before attempting experimental transplantation, it is important to demonstrate that the isolated NPCs have the potential to differentiate to mature neurons. The lineage commitment/differentiation would follow a programmed pattern similar to biological processes. So studying the signaling process is important to prove that the mechanism is suitable for continued development to neurons even after transplantation. Also for post-transplantation survival and homing of NPCs, the niche conditions may be improved using a biocompatible and biomimetic matrix. In this background, a study hypothesis was developed in order to address few of the above stated problems associated with the use of circulating MAPC for transplantation to promote SCI regeneration.

1.9. Hypothesis

- An NPC population suitable for regeneration of SCI may be isolated from adult PBMNCs using a standardized, bioengineered and biomimetic selection niche which is compliant/gentle for supporting cellular proliferation and differentiation.
- Availability of a biosimilar signal within the bioengineered niche to direct cell differentiation may be established by analysis of a selected signaling pathway.
- The significance of a biocompatible and bioengineered niche for survival of NPCs may be identified using a proof-of-concept rat model of SCI.

1.10. Objectives

To prove the above hypothesis, a study was designed with 5 major objectives. Separate experimental strategies were employed for achieving each objective; therefore, the major objectives were subdivided as shown below.

1. To standardise the niche composition for selective isolation and lineage commitment of NPCs from PBMNCs using a matrix selection strategy
    - To promote neuronal induction of MAPC from PBMNCs
    - To study the influence of niche components on NPC growth
    - To look into the effect of reported differentiation molecules - proteoglycan, Chondroitin sulphate proteoglycan (CSPG) and chemical inducers, Retinoic acid (RA) and cyclic (cAMP)
    - To optimize the concentration of growth factors (GFs) - Hypothalamus Extract (HE) & Platelet Growth Factor (PGF) and glycosaminoglycan - Hyaluronic Acid (HA)
    - To establish occurrence of important constituents present in fibrin composite matrix
2. To study the potential of biomimetic niche and culture protocol on programming PBMNC-derived NPCs to different stages of differentiation
    - To evaluate the cells at various stages of differentiation for expression of cell-specific markers
    - To study the influence of potassium chloride (KCl) on morphological changes
    - To analyse the role of KCl on NPC proliferation and survival at definite periods
    - To demonstrate the effect of KCl on expression of few selected functional protein markers

3. To study the role of Wnt-3a signaling pathway elicited by the niche components on NPC to induce differentiation

    - To study the effect of Wnt-3a signals from niche, added Wnt-3a and Wnt-3a inhibitor on neural cell morphology and cell length
    - To delete individual components from the composed niche to identify the most crucial factor in lineage commitment of MAPC
    - To establish that the niche derived Wnt-3a signaling is active using transcriptional level studies
    - To establish the influence of niche derived Wnt-3a signaling on proliferation and expression of various neural markers

4. To isolate rat NPCs with proliferation & differentiation potential as well as to standardise niche composition for using both in testing the transplantation efficiency *in vivo*

    - To isolate NPC from rat PBMNCs using optimised niche
    - To establish the differentiation potential of rat NPCs to neurons on 2-dimensional (2D) fibrin matrix-coated tissue culture dishes
    - To identify optimum concentration of thrombin and fibrinogen concentrate to achieve optimum fiber thickness and porosity
    - To test cell survival on the composed 3-dimensional (3D) fibrin and qualify the matrix to be compatible for use as NPC delivery vehicle
    - To prove that the optimized 3D fibrin gel composition is compatible for growth of NPCs

5. To test the efficacy of the optimised niche to deliver and retain the transplanted rat NPCs at the injury site in an acute model of rat spinal cord injury

- To create a SCI model for transplantation studies
- To use the isolated rat NPCs as an autologous cell source for safe transplantation in SCI model of inbred rats without much immune response
- To analyse the efficiency of composed fibrin matrix to promote survival of transplanted NPC as compared to medium as delivery vehicle
- To examine the injury site for transplantation associated reparative changes after short duration
- To track transplantation efficiency by tagging cells with fluorescent dye together with immunochemical staining for specific markers

CHAPTER 2

2. LITERATURE REVIEW

This section summarises the published literature to comprehend the basic concepts, current approach and developments in the field of neural cell transplantation. The development of CNS and associated signaling mechanisms, pathophysiology of SCI and the major challenges in regeneration of the injury are reviewed in detail to justify the significance of the current study. The choice of adult stem cells and the importance of a biomimetic niche for their isolation and lineage commitment are described in the light of current literature The importance of ECM components for homing of transplanted cells and various challenges for ensuring appropriate homing, survival and differentiation to solve issues for improving transplantation success are also reviewed. Selection of models used to study SCI, modes of transplantation and evaluation methods also are reviewed.

2.1. Central Nervous System

The CNS is the processing center for the nervous system. It consists of the brain and spinal cord, both protected by three layers of connective tissue called the meninges. These layers are from outside duramater followed by arachnoid membrane and piamater. The network of hollow cavities in the brain called ventricles is continuous with the central canal of the spinal cord and is filled with cerebrospinal fluid which surrounds, cushions, nourishes as well as protects these organs from trauma.

2.1.1. Cell types

Neurons and glia/neuroglia are the two principal kinds of cells that exist in the nervous system.

Neurons are the basic unit that carries out the work of the nervous system which is conduction of nerve impulses from one body part to another. As many as 100 billion neurons are estimated to be present in our nervous system. They are highly polarized cells with distinct subcellular domains that in turn serve different functions. Morphologically, three major regions can be defined in a typical neuron (a) the cell body/soma/perikaryon (b) axon and (c) dendrites. The *cell body* is the site of synthesis of virtually all neuronal proteins and membranes. Axons are specialized for the conduction of a particular type of electric impulse called action potential away from the cell body towards the axon terminus (Bear, 2007). Most neurons have multiple *dendrites*, which extend outward from the cell body and are specialized to receive chemical signals from the axon termini of other neurons. Dendrites of the post-synaptic neurons convert the chemical signals obtained from the pre-synaptic neuron into small electric impulses and transmit them in the direction of the cell body. Neuronal cell bodies can also form synapses, receive signals and carry the action potential further (Carter, 2009).

Neuroglia cells are the supporting cells that make up 90% of the brain's cells. They act as connective tissue besides serving to support and protect the neurons. They are of different types and perform unique functions.

Astrocytes afford a connection between the vasculature and neurons there by assisting the transport of glucose and other substances out of the blood stream. Glucose is processed by astrocytes to produce lactate, which is the main energy source for neurons. Astrocytes also play a role in the propagation of action potentials by uptaking the neurotransmitters and regulating the extracellular potassium ion concentration. In addition, they perform immune functions, synthesise and release

neurotrophic factors and are involved in the formation of neural scars following injury. During the developmental process, radial glia, which differentiate into astrocytes provide a supporting matrix for neuronal migration as well as synaptogenesis (Kimelberg and Nedergaard, 2010).

Oligodendrocytes comprise numerous short processes, which wrap themselves around the CNS axons and are responsible for axonal regulation, generation and maintenance of myelin sheath. Myelin mediates rapid saltatory propagation of action potential between nodes of Ranvier, thereby facilitating neurotransmission. They also secrete neurotrophins that provide local trophic support for neurons. Peripheral axons are myelinated by Schwann cells and facilitate neuronal regeneration following injury (Bradl and Lassmann, 2010).

Ependymal cells are special cells that line the ventricles and the central canal of the spinal cord. These cells are believed to be involved in the directional movement of cerebrospinal fluid that facilitates transport of nutrients into the brain and the removal of toxic metabolites. During early development, ependymal cells are suggested to serve as an axonal guidance system (Del Bigio, 1995).

Microglia are the immune effector cells of the CNS that are present in abundance in the brain parenchyma and constitute ≈10-20% of the total population of adult glial cells. These are small round cells with numerous branching processes and possess little cytoplasm. Microglial processes through localised mobility directly contact neuronal cell bodies, astrocytes and blood vessels, thus monitoring the well-being of the brain; cleanse the extracellular fluid through its pinocytotic activity in order to maintain central homeostasis; respond to released neurotransmitters; transform into active phagocytic macrophages, which phagocytose damaged cells and debris at the

site of injury or pathogen invasion playing a neuroprotective role. During development microglia play a crucial role in removing inappropriate axons and promoting axonal migration and growth (Rock et al., 2004).

2.1.1.1 Classification of neurons

Based on the number of processes, the neurons are classified in to multipolar, bipolar and unipolar neurons. Brain and spinal cord neurons are generally *multipolar neurons. Bipolar neurons* are found in the retina of the eye, the inner ear, and the olfactory area. *Unipolar neurons* are found in the posterior root ganglia of the spinal nerves.

Based on the functions as well as the direction in which the neurons transmit impulses, the neurons are classified into sensory, motor and interneurons. *Sensory/afferent neurons* conduct impulses from the receptors in the skin, sense organs, and viscera to the brain and spinal cord. *Motor/efferent neurons* carries the impulses transmitted from the CNS to either skin, muscles, glands and organs of the body (Tortora, 2013). Interneurons relay signals between motor and sensory neurons. The CNS require peripheral nervous system (PNS) to transmit information about sensation from the body to the CNS and then send the information back to the body, which is mediated through the cranial and spinal nerves (Anon,).

2.1.2. Functions of CNS

The brain is responsible for controlling movements, thought, speech, behaviour, vision, perceivedness, language and balance. It also processes sensory information and controls autonomic functions including breathing, heart rate, digestion etc. The nerves of the spinal cord are grouped into bundles of nerve fibres that travel in two pathways to perform specific functions. *Ascending nerve tracts* carry sensory

information from the body to the brain. *Descending nerve tracts* send information pertaining to motor function from the brain to the rest of the body. They also help to maintain homeostasis by assisting in the regulation of autonomic functions such as heart rate, blood pressure, and internal temperature (Anon,). Reflexes and repetitive movements are controlled by spinal cord neuronal circuits that are stimulated by sensory information without input from the brain. The neurons and their dendrites are contained within the H-shaped grey matter region of the spinal cord. This is surrounded by white matter and contains axons covered with insulating myelin.

The axons that link the spinal cord to the muscles and the rest of the body are bundled into 31 pairs of spinal nerves, each pair with a sensory root and a motor root that make connections within the grey matter. These nerves must pass between the protective barrier of the spinal column to connect the spinal cord to the rest of the body. The location of the nerves in the spinal cord determines their function. *Cervical spinal nerves (C1 to C8)* control signals to the back of the head, the neck and shoulders, the arms and hands, and the diaphragm. *Thoracic spinal nerves (T1 to T12)* control signals to the chest muscles, some muscles of the back, and parts of the abdomen. *Lumbar spinal nerves (L1 to L5)* control signals to the lower parts of the abdomen and the back, the buttocks, some parts of the external genital organs, and parts of the leg. *Sacral spinal nerves (S1 to S5)* control signals to the thighs and lower parts of the legs, the feet, most of the external genital organs, and the area around the anus. The single *coccygeal nerve* carries sensory information from the skin of the lower back(Anon,). Locations of spinal cord segments do not correspond exactly to vertebral locations, but they are roughly equivalent.

2.2. Developmental Biology of CNS

There are 9 stages in the nervous system development. The process and the major signaling mechanisms involved during each process are described below.

2.2.1. Neural Induction

The early processes of animal development follow a conserved pattern. Blastulation process occurs in the embryo followed by gastrulation which results in the generation of a three-layered structure - the outer ectoderm which develops in to skin and neural tissue due to signals from mesoderm), the middle mesoderm (skeleton, cardiac) and the inner endoderm (digestion, respiratory). After implantation, the neural tube is formed from the ectoderm overlying the involuting mesoderm, which then rolls up to form the brain and spinal cord. The two strips of neural crest tissue running lengthwise above the neural tube forms the peripheral nervous system (PNS). The ectoderm transition to neurons rather than epidermis occurs through inactivation of bone morphogenic (BMP) pathway, which inactivates Smad or activates fibroblast growth factor (FGF) - mediated Sox expression to trigger the proneural gene transcription (Squire et al., 2008).

2.2.2. Polarity and segmentation

The nervous system is regionally specialized and polarised. SHH generated ventrally in the notochord and floor plate of the spinal cord induces motor neuron formation at high concentration and ventral interneurons at low concentrations. Bone Morphogenic Proteins produced in the roof plate/dorsal pole generates dorsal cell types. Retinoic acid arising from the adjacent somites is responsible for generating certain interneuronal and ventral neuronal populations. Wnts, RA and FGFs produced by the newly generated mesoderm induce posterior nesemiural gene

expression by down regulating anterior genes. The same molecular pathways may play a role in more than one steps at different times and places (Squire et al., 2008).

2.2.3. Genesis and Migration

Neurogenesis and gliogenesis during development occurs via proper migration of neurons and glia from their site of origin to the final position in an adult brain. This process is regulated by a cellular clock or the extracellular signaling either promoting or inhibiting progenitor cells to enter/exit the cell cycle. The mitotic figures are located in the innermost ventricular/ependymal zone (near the ventricle) and the postmitotic neurons in the marginal zone. Secreted signaling factors like FGFs, Transforming Growth Factor-alpha (TGF-α), Epidermal Growth Factor (EGF), Insulin-like Growth Factor (IGF), SHH and Wnt proteins stimulate or inhibit the progress of stem/progenitor cells through the cell cycle. Both neurons and glia are produced in a highly stereotypic ratio which is regulated by many growth factors. FGF2 and Neurotrophin3, through the increase in expression of proneural basic Helix Loop Helix genes such as NeuroD1, Neurogenin, and Mash1 promote progenitor cells isolated from brain to develop primarily as neurons. BMP, EGF and Ciliary Neurotrophic Factor (CNTF) promote glial development, partly through the ignal Transducer and Activator of Transcription (STAT) pathway and partly through inhibition of proneural genes via the hairy and enhancer of split-1 (Hes) -Notch pathway. Neuronal migration is regulated by reelin, astrotactin, integrin and neurogulin (Sanes et al., 2011).

2.2.4. Determination and Differentiation

For the progenitor cells to differentiate into mature neurons of right type at the right place, diffusible molecules, cell surface proteins and the ECM factors in the

environment supports. This influences the genes that developing neurons express which direct neuronal shape, axonal pathways, connectivity, and chemistry. The dorsally shifting expression of Nkx2.2 causes a change in gene expression in the progenitor cells driving the switch point from neurons to glia. Astrocyte progenitors are distributed at all levels, whereas oligodendrocyte progenitors come from the ventral neural tube. The internal clock that determines when an OPC stops dividing depends on the level of the p27Kip1 protein, a cell cycle inhibitor that builds up over time and finally drives the cells to exit the cell cycle (Sanes et al., 2011).

2.2.5. Growth Cones and Axon Pathfinding

The growth cone is the key decision-making component in the elaboration of axonal pathways which is controlled by cues in their outside environment that ultimately direct them toward their appropriate targets. Each class of axons navigate along a distinctive prescribed pathway to reach its target. A variety of repulsive and attractive cues are involved in the simultaneous and coordinated guidance mechanisms which include semaphorins, netrins, slits, ephrins, BMPs, hedgehog, Wnt families, hepatocyte growth factor (HGF), FGFs, glial-derived neurotrophic growth factor (GDNF) and neurogulins. Attractive and repulsive signals promotes the extension of the leading edge by increasing the local rate of actin polymerisation in preferred direction, by decreasing the rate of depolymerisation in other directions and modulating rate of myosin-mediated F-actin translocation (Squire et al., 2008).

2.2.6. Target selection & Synapse formation

The formation of a proper functioning nervous system depends on the development of precise connectivity between appropriate sets of neurons or neurons with peripheral targets such as muscles, tendons, skin etc., which is accomplished by

interstitial axon branching. During development, neurons often project to more targets than in the adult and generate their adult pattern of connections through a process of selective axon or collateral elimination. Transmembrane proteins interacting with cognate receptors on the opposing cell surface like neuroligin/β-neurexin, SynCAM, ephrinB; diffusible molecules like FGF22 and WNT-7a; protocadherins and neurexins may contribute to achieving remarkable synaptic specificity (Sanes et al., 2011).

2.2.7. Programmed Cell Death

This is the spatial and temporal species-specific loss of large numbers of individual cells during development which is dependent on both intrinsic as well as extrinsic signals arising from diverse kinds of cellular interactions. This establishes optimal levels of connectivity between neuronal populations, eliminates aberrant cells or connections, regulates the size of progenitor populations and serves transient functional or other needs of immature animals. Programmed Cell Death (PCD) occurs in all cell types from the time of proliferation until the establishment of synaptic connections. Growth factors through specific pathways activate survival or apoptotic signaling pathways. Upon binding of neurotrophins to Tropomyosin Receptor kinase (Trk), phospholipase C, Ras-MAP kinase and phosphoinositide-3 kinase (PI-3K) pathway were activated leading to nuclear translocation of cAMP response element-binding protein (CREB) and nuclear factor *kappa*-light-chain-enhancer of activated B cells (NFκB) ultimately regulating cell survival. The binding of neurotrophins or proneurotrophins to neurotrophin receptor p75 (p75NTR) activate Bcl-2-associated death promoter (BAD) via Jun N-terminal kinase (JNK) cascade which initiates apoptosis through caspase activation (Squire et al., 2008).

2.2.8. Synapse Elimination

Elimination of synaptic connections during development may be an adaptation that converts highly overlapping connections of redundant neurons into unique circuits. Because this conversion may be based on experiences, the synapses which have been used repeatedly tend to remain and those which haven't been used often are eliminated.

2.2.9. Dendritic Development

Ephrins, slits, robo, glutamatergic synaptic activity through N-Methyl-D-aspartate (NMDAR), neurotrophins and voltage gated Ca^{2+} channels promote dendritic growth and branching whereas notch inhibits the same. This is followed by myelination which speeds up the travel of impulses making it more efficient (Ming and Song, 2005).

2.3. Neural stem cells and homeostasis

Neural stem cells (NSCs) are the most primitive cells in the CNS which are very important in sustaining the development and homeostasis of nervous tissue, because neurons are specialized cells which are quite sensitive to environmental changes such as oxygen tension or excitoxicity. NSCs rest in a state of quiescence and give rise to other neural stem cells and/or transient-amplifying cells/ progenitors which progressively acquire a more restricted differentiation capacity into neurons, astrocytes, and oligodendrocytes. The dynamic equilibrium between self-renewal and differentiation is critical to both the maintenance of the stem cell pool and active neurogenesis, which is regulated by the niche. There is also evidence of endogenous NSC potential to respond to neurological injuries.

Germinative zones of the brain have been identified in the subgranular zone (SGZ) of the hippocampus, the olfactory bulb, the subventricular zone (SVZ) surrounding the ventricles and the subcallosal zone underlying the corpus callosum. Presence of active neurogenesis in the adult cerebellum and many parts lining the ventricle is also reported (Ma et al., 2009). In the spinal cord, the stem/progenitor cells were located primarily in the central canal region, most prominent in the dorsal part. Progenitor cells with a more limited proliferation potential are also present in the parenchyma (Hugnot, 2012).

2.4. NSC niche

The niche is the microenvironment that intimately supports and tightly regulates stem cell behaviors, including their maintenance, self-renewal, fate specification and development. While dormant NSCs might be present and can be derived from multiple regions of the adult brain, unique local niche structure seems to restrict active neurogenesis from adult NSCs. In the SVZ, the NSC niche spreads extensively from the lateral ventricle along the rostral migratory stream to the olfactory bulb (OB) to accommodate local generation of new neurons there. In the adult SGZ, the niche is less structurally apparent and largely confined within the SGZ hilus region. However, the cellular niche components and the extracellular niche signals that regulate behavior of adult NSCs and their development are similar. The central canal region of spinal cord is composed of several cell types, which are located either in direct contact with the lumen or in a subependymal position, evoking a pseudo-stratified epithelium. Ependymocytes and tanycyte are the primary cell types found around the central canal (Li and Xie, 2005). NSCs of both brain and spinal cord are closely associated with the vasculature and with basal lamina components. They

reside adjacent to their own neuronal progeny, resident mature astrocytes and microglia as well as endothelial and smooth muscle cells of blood vessels. Astrocytes were also found to regulate NSC self-renewal and differentiation. The presence or absence of mature neurons can influence NSC fate through a feedback loop. Additional factors such as oxygen tension regulate NSC activities. Different physiological stimuli such as learning, exposure to environmental enrichment, running and stress, can affect the rate of proliferation, differentiation and survival of new-born neurons. Besides these different cells and factors, the ECM is also an important component of the neurogenic microenvironment.

2.4.1. Extracellular matrix

Integrins are the key receptors involved in the ECM-stem cell interactions which aids in the adhesion, anchorage and homing of stem cells. Integrins represent transmembrane receptors that connect the extracellular environment to the intracellular cytoskeleton, thus mediating cell migration, proliferation, survival and differentiation. Both SVZ and SGZ niches express various ECM molecules, such as tenascin-C, netrins, laminin and various proteoglycans. In the SVZ, NSCs are in association with projections of the vascular basement membrane composed of laminin, collagen IV, fibrinogen, fibronectin, nidogen and proteoglycans. *Laminins & collagen IV p*rovide anchoring, migratory guidance and survival signals in addition to promoting the differentiation of neuronal progenitors and clustering of synaptic vesicles as nerve terminal forms. *Fibronectin and fibrinogen are* important for cell adhesion, neuronal migration and outgrowth during development as well as participates in remodelling of injured brain tissue. *Proteoglycans* & *reelin* regulate cell adhesion, neurite outgrowth, neuronal patterning, ECM assembly, cell migration

and serves as cofactors and regulators of growth. *Agrin* has the ability to induce and stabilize accumulations of postsynaptic acetylcholine receptors and presynaptic proteins. *Tenascins, lecticans & hyaluronic acid* promote long-term potentiation and depression, homeostatic regulation of synaptic strength and plasticity as well as learning and memory (Zimmermann and Dours-Zimmermann, 2008). Some ECM molecules, such as netrin-1, Slit-1 and Slit-2, are involved in providing the direction for migration of neurogenic precursors.

2.4.2. Growth factors

Several ECM components are able to strongly bind growth factors, regulating their local availability and establishing a biochemical gradient. On the one hand, the ECM can function as reservoir of growth factors, by making them insoluble, unavailable and non-bioactive. This action is shown by fibronectin, vitronectin, collagens and proteoglycans, which bind FGFs, HGFs, VEGFs, BMPs and TGF-β. On the other hand, proteins and proteoglycans of the ECM can function as distributors of growth factors following the action of enzymes, such as metalloproteinases, which induce the remodelling of ECM components and permit the release of factors that were otherwise in an insoluble state. Growth factors, including FGFs and neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotransmitters also significantly contribute to proliferation, survival and dendritic development of new born neurons in the adult brain (De Filippis and Binda, 2012). Wnt, SHH, BMP antagonists, membrane-associated Notch signaling, Phosphatase and tensin homologue (PTEN) /PI3K, leukemia inhibitory factor (LIF), transforming growth factor-alpha (TGF-α) and cytokines have also been shown to promote progenitor proliferation and maintenance. Signaling in the spinal cord niche involved Notch

(Jagged, Hes1), Wnt (Wnt7a, Fzd3), BMP (DAN, BMP6) and Hedgehog (SHH) pathways (Li and Xie, 2005).

2.4.3. Signaling in stem cells

Stem cells are defined by three essential features, as they are undifferentiated cells and able to give rise both to cells that retain their stemness by self-renewal and differentiated daughter cells. Embryonic and adult stem cells have different capabilities to produce differentiated cells, a property known as potency. Cells present in the early embryo until the blastocyst stage are pluripotent, since they produce all differentiated cell types present in the body, whereas fetal and adult stem cells are able to produce multiple cell lineages (multipotent) or a single differentiated cell lineage (unipotent). In *embryonic stem cells* which offer enormous potential for producing a variety of differentiated cells for cell therapy, drug discovery and toxicology screening, the interplay between the LIF and BMP is responsible for the self-renewal of ESCs (Friel et al., 2005). In *adult stem cells which* provide a source for replenishment of tissue over time in response to injury, damage or simply wear and tear, also possess the ability to both self-renew and differentiate down to specific lineages (Lowry and Richter, 2007) through the interplay between different pathways.

2.4.3.1. Notch Signaling

This is a highly conserved cell signaling system present in most multicellular organisms which promotes proliferative signaling during neurogenesis. Upon receipt of ligand, the truncated Notch protein formed in the cytoplasm enters the nucleus and activate transcriptional repressor genes thereby suppressing cell fate choice in lateral inhibition (Lowry and Richter, 2007). Notch signaling pathway components

including Notch1, Jagged1 and the downstream target Hes5 are expressed in the adult SEZ suggesting its role in adult neurogenesis as in the embryo. Conditional inactivation of Notch1 from adult-derived NSCs resulted in a complete loss of self-renewal and stem cell character without changing cell viability or lineage potential. Jagged1 expressed by ependymal cells and SEZ astrocytes might provide the maintenance signal to Notch expressing NSCs. In glial progenitors, Notch promotes astrocytic fate.

2.4.3.2. Transforming Growth Factor- β signaling pathway

This superfamily of signaling molecules is divided into TGF- β and BMP. Both ligand families bind to tyrosine kinase receptor to stimulate downstream effectors. The TGF -β and their receptors signal through SMADs 2&3; BMP signal through SMADs 1, 5&7 leading to activation of distinct transcriptional target genes (Lowry and Richter, 2007). In the neural progenitors of olfactory epithelium, TGF- β inhibition led to neurogenesis. The TGF- β pathway seems to be involved in neurogenesis in the olfactory bulb. The BMP pathway was found to drive NSCs to a glial fate and can also drive glial progenitors down astrocytic lineages. It inhibits oligodendrocyte fate. BMP can direct Neural Crest Stem Cells to undergo neurogenesis eventually creating neurons with characteristics of the autonomic nervous system.

2.4.3.3. Sonic hedgehog Signaling

Sonic hedgehog (SHH) is a ligand for a receptor called patched. This receptor acts as an inhibitor of another transmembrane protein called smoothened. The SHH signal actually inhibits patched which leaves smoothened free to stimulate a set of transcription factors which enter the nucleus and drive expression of many genes

implicated in developmental contexts, most frequently as a potent stimulator of proliferation. This signaling has been shown to be required for the maintenance of NSCs in both SGZ and SVZ of adult brain. The *in vivo* role of SHH in maintenance of NSCs and their role in neurogenesis was demonstrated. Blocking SHH using cyclopamine blocked proliferation. *In vitro,* the SHH pathway seems to play a critical role in neurosphere formation (Lowry and Richter, 2007).

2.4.3.4. Epidermal growth factor & fibroblast growth factor signaling

The extracellular factors like epidermal growth factor (EGF) and fibroblast growth factor (FGF) control various aspects of morphogenesis, patterning and cellular proliferation. Activation of the receptors results in autophosphorylation of key tyrosine residues that allows proteins to bind through their Src-2 domains leading to activation of downstream signaling cascades including RAS/ ERK (extracellular signal-regulated kinases), PI3K and JAK/ STAT pathways thereby acting in a coordinated manner to promote cell proliferation, growth and survival. EGF & FGF2 have been used classically to isolate and expand adult NSC-like cells. In most of the systems, it is primarily the distribution of these factors that controls the differential behavior of responding cells.

2.4.3.5. Platelet growth factor signaling

Platelet growth factor (PGF) in combination with FGF2 is shown to be a mitogen for spherogenic NSCs isolated from adult mouse brain. PGF-receptors on SEZ cells are phosphorylated *in vivo,* suggesting that signaling is active in adult neurogenic forebrain. Similar to EGF, PGF infusion into the lateral ventricles of adult mice resulted in activation of SEZ astrocytes, hyperplasia and gliogenesis at the expense of neurogenesis.

2.4.3.6. Vascular Endothelial Growth Factor signaling

Vascular Endothelial Growth Factor (VEGF) is essential for angiogenesis and haematopoiesis through its cognate receptors Flt1 and Flk. VEGF is produced by endothelial cells and in the brain by the choroid plexus. VEGF enhances, and blocking Flk1 activity reduces NSC expansion *in vitro*, and infusion of VEGF into the lateral ventricles of rats increases neurogenesis potentially by acting as a trophic survival factor for neural progenitors. The survival effect of VEGF on NSCs is also seen during embryonic development and may reflect the close interplay between angiogenesis and neurogenesis.

2.4.3.7. Wingless (Wnt) signaling

It is a network of proteins known for their roles in normal physiological processes in addition to embryogenesis and cancer. Wingless (Wnt) serves as a ligand for a serpentine receptor with seven transmembrane domains called Frizzled. Activation of this receptor leads to cytosolic stabilization of its critical intracellular mediator, beta-catenin. Cytoplasmic beta-catenin is normally degraded by a complex machinery involving dishevelled (Dsh), glycogen synthase kinase-3 β (Gsk3-β), axin and adenomatosis polyposis coli (APC), which collaborate to phosphorylate beta-catenin, targeting it for ubiquitination and subsequent degradation. Upon receipt of Wnt, the stabilised β-catenin accumulates in cytoplasm, translocates to nucleus, associates with transcription factors T-cell factor/lymphoid enhancing factor Lef/Tcf or Sry-related HMG box (Sox) and activates or suppresses target gene expression.

Wnt1 and Wnt-3a were demonstrated to activate not only the canonical Wnt signaling pathway but also the non-canonical based on the cell or tissue type. Wnt signals are transduced to the canonical and non-canonical pathways based on the

expression profile of various Wnt receptors, co-receptors, and the activity of cytoplasmic Wnt signaling regulators (Katoh and Katoh, 2007).

In the CNS, Wnt drives proliferation in neural precursors through beta-catenin mediated canonical signaling. Elevated expression of Wnt-3a in NSC residing areas of brain led to increased neurogenesis, which was demonstrated to be due to the proliferation of neuroblast pool which ultimately generated differentiated neurons. Blocking of Wnt signaling cascade in adult brain blocked neurogenesis and indicated about the role of Wnt in cell fate driving. In contrary to the non-responsiveness of NSCs to the Wnt signal, in more lineage restricted neural cells there is evidence that Wnt plays a role in promoting the differentiation down various lineages (Lowry and Richter, 2007). Even though specific pathways have roles in either maintenance or differentiation of stem cells, it is ultimately the interplay between multiple signaling pathways from the ECM and intrinsic regulator that controls the fate of adult NSCs by affecting their proliferation, self-renewal, choice of lineage differentiation and survival.

2.5. *In vitro* ECM based differentiation of stem cells to neurons

Several ECM molecules play regulatory functions for different types of stem cells. Based on its molecular composition, the ECM can be deposited and finely tuned for providing the most appropriate niche for stem cells in the various tissues either to self-renew or to differentiate. Engineered biomaterials will be able to mimic the *in vivo* characteristics of a niche. In a report, the *in vitro* culture of ESC derived NPC on a collagen based scaffold produced cells that expressed more than 90% of differentiated cells expressing many neuron-specific antigens including β-III tubulin, MAP-2, synaptophysin and neuro-filament protein (Baharvand et al., 2007). In

another study of culturing human ESC-derived embryoid bodies on to Poly-D-Lysine (PDL), PDL/fibronectin, PDL/laminin, type I collagen and Matrigel coated dishes in neural differentiation medium, all the five substrates were found to instruct neural progenitors at differing degrees. This followed by neuronal differentiation. Neural progenitor and neuronal generation as well as neurite outgrowth were significantly greater on laminin and laminin-rich Matrigel substrates as compared to others. Glia did not appear until 4 weeks (Ma et al., 2008). Another study based on biomimetic poly-L-lysine/hyaluronic acid film enhanced neurite outgrowth length, regulation of neuron differentiation and the formation of a network upon differentiation of stem/progenitor cell which may provide a versatile platform that could be useful for surface modification for applications in neural engineering (Lee et al., 2015).

When adult enteric neuronal progenitor cells were cultured on different substrates like Poly-lysine, laminin, collagen I, collagen IV and heparin sulfate, laminin-collagen IV combination with heparin addition resulted in improved neuronal growth (Raghavan et al., 2013). Another neural extracellular matrix-based on basement membrane extract and hyaluronan supported *in vitro* hippocampal neuron culture and dopaminergic differentiation of neural stem cells (García-Parra et al., 2013). Collagen 3Dl gel matrices prepared in combination with adhesive proteins such as fibronectin and laminin provided significant cues to the differentiation of mesenchymal stem cells derived from rat bone marrow into neuronal lineage even without the use of chemical differentiation factors (Lee et al., 2011a).

The conversion of stem cells to neural/glial lineage progenitors and their differentiation to mature cells may be tracked using specific markers of each stage of development.

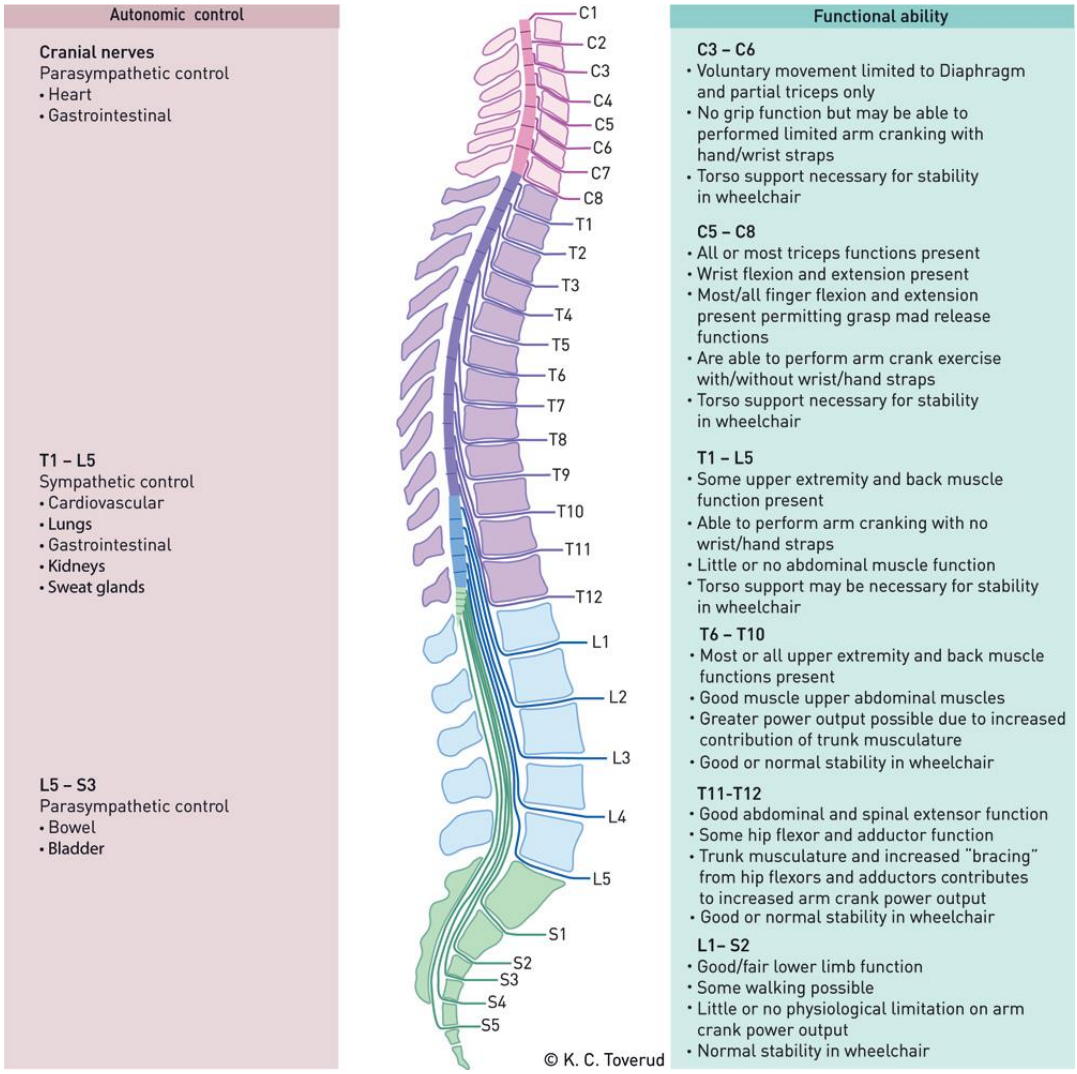
2.5.1. Important Neural lineage markers

The usual markers for NSCs include Nestin and SOX-2. These are intracellular markers. Cell surface markers for identifying NSCs include ABCG2, FGFR4, and Frizzled-9. Nestin, NCAM and Musashi-1 are the commonly expressed neural progenitor markers. The differentiation markers include Doublecortin (DCX), β-III tubulin (TuJ1), Microtubule-associated protein 2 (MAP-2), Neuron specific enolase (NSE). DCX is a microtubule-associated protein that is widely expressed in the soma and leading processes of migrating neurons and in the axons of differentiation neurons. Its expression is downregulated with maturation. TuJ1 is present in newly generated immature postmitotic neurons and differentiated neurons and in some mitotically active neuronal precursors. MAP-2 is a cytoskeletal protein. Its expression is weak in neuronal precursors but it increases during differentiation and is confined to neurons and reactive astrocytes. NSE is a cytosolic protein that is expressed in mature neurons. NSE levels increase along the neuron development reaching higher level in later stages. It can be expressed in glial cells during oligodendrocyte differentiation with the same levels that have been found in neuron culture, but are repressed when cells become mature. Synaptophysin (Syn) is involved in vesicle formation during synapse development. There are also markers available to label specifically the type of neurons including cholinergic, dopaminergic, serotonergic, Gamma-amino butyric acid (GABA) ergic or glutamatergic neurons. Tyrosine hydroxylase (TH) is a protein expressed by dopaminergic neurons (Anon, 2015a).

2.6. Spinal cord Injury

Injury to the CNS including the brain and spinal cord are major health problems both nationally and internationally. The complexity of the CNS and variability in clinical presentation make efforts to repair damaged CNS tissue and restore function particularly challenging. Among the various diseases occurring to the CNS, Spinal cord Injury (SCI), Traumatic brain Injury (TBI) and stroke are most common. Spinal cord injury is one of the devastating condition that result in either temporary or permanent changes in the normal motor, sensory or autonomic functions of the body. The effects of SCI varies depending on the type, severity and level of injury and may include one or a combination of paralysis (Quadriplegia or Paraplegia), loss of sensation, loss of reflex function, loss of autonomic activity, dysfunction of bowels and bladder, sexual dysfunction, muscle spasms and chronic pain. Among the causes for SCI, motor vehicle accidents stand first followed by falls, violence and sports incidents. It is estimated that about 4.5 million people worldwide suffer from SCI (WHO) of which 1.5 million people are from India. Among the injured, 82% are males in the age group of 16-30 years. About 40% of the patients suffer from quadriplegia while 60% from paraplegia. These statistics, lack of effective pharmacological therapies combined with negative health/sociological implications and high economic impact prompt the demand for novel and clinically effective treatments. Spinal cord damage occurs through cutting/transection either complete or incomplete; pinching of the SC with or without vertebral displacement; stretching; compression fractures of vertebrae; displacement of vertebrae; bruise/contusion; overstretching and other damage to ligaments and muscles involved in the spinal cord. The spinal cord is divided in to cervical, thoracic, lumbar, sacral and coccygeal

sections. The level of injury determines the extent of paralysis and loss of sensation (Anon,).The functional disability that occurs with changes in the level of injury is briefed in the picture below.



2.6.1. Classification

Depending on the severity of the injury, the patient may have a *complete injury*, losing all conscious motor and sensory function below the injury or may have an *incomplete injury*, retaining some motor or sensory function(Anon,). Many clinicians use the American spinal cord injury association (ASIA) Impairment Scale to grade

the severity of neurological loss. A = Complete: No motor or sensory function is preserved in the S4-S5 sacral segments; B = Incomplete: Sensory function but not motor function is preserved below the neurological level and includes the S4-S5 sacral segments; C = Incomplete: Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3; D = Incomplete: Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more; E = Normal: Motor and sensory function are normal (Kirshblum et al., 2011).

Trauma to the spinal cord can affect all the body systems. The resulting dysfunction depends on the position of the damaged spinal cord. *Cervical cord injuries* cause paralysis of all the extremities and the body trunk, bowel and bladder dysfunction and respiratory failure. *Thoracic cord injuries* cause paralysis of the lower extremities, bladder and rectum, pain to the chest or back and abdominal distention. *Lumbar cord injuries* cause paralysis of the lower extremities. In the phase of the spinal shock, the vasomotor and visceral motor innervations are disrupted resulting in tachycardiac/bradycardiac arrhythmia but also autonomic dysreflexia. In addition, atony of the efferent urine ducts, stomach and intestine; endocrine disruptions like hyperglycemia and derailment of electrolyte metabolism as well as malfunction of body temperature control occurs (Teasell et al., 2000).

2.6.2 Pathophysiology

This comprises the primary and secondary phases of injury. The neurological damage that is incurred at the time of mechanical trauma is called the primary injury, which causes cell necrosis. This provokes a cascade of cellular and biochemical reactions that lead to further damage called secondary injury. Vascular changes at the site of injury are the most important events and include haemorrhage, vasospasm, thrombosis, loss of autoregulation and increased permeability. These, along with edema lead to hypoperfusion, ischemia and necrosis. Other mechanisms include: free radical formation and lipid peroxidation; accumulation of excitatory neurotransmitters and neural damage due to excitotoxicity; intracellular imbalance of sodium, potassium, calcium and magnesium and subsequent increased intracellular calcium level; increased levels of opioids; depletion of energy metabolites leading to anaerobic metabolism at the site of injury and increasing of LDH activity and finally provocation of an inflammatory response and recruitment and activation of inflammatory cells associated with secretion of cytokines, which contribute to further tissue damage and activation of calpains and caspases leading to apoptosis. Primary and secondary injuries lead to the cell loss in the spinal cord. In penetrating injuries, this leads to scarring and tethering of the cord. Demyelination occurs following the loss of oligodendrocytes, which causes conduction deficits. In contusion injuries, a cystic cavity surrounded by an astrocytic scar is formed following this tissue loss. Where the injury extends to piamater, collagen will also contribute in the formation of the scar tissue (Samadikuchaksaraei, 2007).

2.6.3 Phases of injury

The phases of SCI can be divided into the acute-phase (2 hours–2 days), the sub-acute phase (days–weeks), and the chronic phase (months–years). The patho-physiological changes that occur within these different phases are distinct. During the *acute phase* many mutually dependent events such as edema, ischemia, haemorrhage, reactive oxygen species (ROS) production and lipid peroxidation, glutamate-mediated excitotoxicity, ionic dysregulation, blood-spinal-cord barrier permeability, inflammation, demyelination, neuronal cell death, and neurogenic shock occurs. *Sub-acute phase* is the time when macrophage infiltration, microglial activity, astrocyte activity, scar formation and initiation of neovascularization occurs. During *chronic phase*, Wallerian degeneration, glial scar maturation, cyst and syrinx formation, cavity formation, and schwannosis are the major occurrences (Li and Lepski, 2013).

2.6.4 Current practices for SCI treatment

Current treatment strategies involve prevention of further damage by stabilization of the vertebrae, preventing inflammation by administration of steroids including methyl prednisolone, emotional therapy, physiotherapy and administration of painkillers. However, due to the lack of effective pharmacological therapies, physical and financial burdens produced to the patients, modern medicine is exploring alternative approaches to improve neuronal regeneration with the major therapeutic goal aiming to restore at least minimum functional abilities of the patient.

2.7. Experimental strategies to promote spinal cord regeneration

After a SCI, several endogenous regenerative events occur indicating that the spinal cord attempts to repair itself. Schwann cells which are the myelinating as well as

regeneration-promoting cells in the PNS migrate from spinal roots into the damaged tissue and myelinate spinal cord axons. The expression of regeneration-associated genes is increased in damaged neurons. An increased proliferation of local ASCs and progenitor cells occurs. However, growth inhibitors present on oligodendrocyte myelin debris and scar tissue cells prevent axonal growth. In addition, the new born stem/ progenitor cells do not integrate functionally into the injured spinal cord tissue, thus failing to repair the spinal cord. Many efforts to improve neuronal regeneration aiming at different issues are ongoing and are described in detail in the coming sections.

2.7.1. Neuroprotection

This involves protection of both neurons and glia. At a very early stage (<6 h), a neuro protective surgical intervention to decompress the swollen, edematous lesioned spinal cord by laminectomy might be successful (Chen et al., 1998). Administration of methyl prednisolone at high dose during the early acute phase (<8 h) helps reduce formation of cytotoxic edema, inflammation, release of glutamate and free radicals. However, it is said to have various side effects (Short et al., 2000). Cyclooxygenase inhibitors (Hurley et al., 2002), asialoerythropoetin (Gorio et al., 2002), antibody blockade of the CD95 (FAS) ligand were discovered to have anti inflammatory-neuroprotective effects. Systemic application of minocycline reduced the gliotic response and diminished apoptotis of oligodendrocytes.

2.7.2. Neurorestoration

This aims at improvement of remyelination and conduction abilities of neurons. Pharmaceuticals that improve nerve conduction efficacy by switching from non-saltatory to saltatory conduction along the demyelinated axon areas like potassium

(K+) blocker 4-aminopyridin are used. An intact myelin sheath is required for regular axon conduction which was thought to be achieved by identifying compounds that stimulate the oligodendrocytes to remyelinate the damaged or missing myelin sheaths of the surviving axons. Other therapeutic option are the promotion of remyelination through the transplantation of macrophages (Brüstle et al., 1999) and transplantation of precursor and glial cells (Bunge, 2002).

2.7.3. Neuroregeneration

This involves antagonisation of inhibitory factors at the inhibitory environment that halts the axonal growth and/or administration of axonal growth or neurotrophic factors. The inhibitors include myelin inhibitors, glial scar inhibitors as well as many guidance molecules that are upregulated at the lesion site. Inactivation of Rho relieves the growth arrest and induces axonal growth. Increasing the intracellular cAMP level by administering phosphodiesterase inhibitors or growth stimulating/neurotrophic factors triggers an intracellular signal that leads to axonal sprouting and neuronal survival. Deglycosylation of CSPG through chondroitinase ABC and delaying the synthesis of the basal lamina which functions as a matrix for sticky inhibitors by administration of iron chelators that inhibit prolyl-4-hydroxylase has been tried out (Schwab et al., 2006). Immunization with a homogenate of CNS tissue that contains all of the inhibitors of the myelin and the scar may lead to antibody production against a multitude of inhibitory proteins (Caroni and Schwab, 1988).

2.7.4. Specific regeneration

This is achieved by reconnection with deafferent targets through the help of axon guidance molecules (Wizenmann et al., 1993).

2.7.5. Neuroreconstruction

This involves replacing the perished neuronal cells by cell or tissue transplantation. The use of tissue transplants is limited due to various reasons. In cell transplantation, cells/ stem cells/ progenitor cells may be injected directly at the injury site or grown on to biological or synthetic scaffolds with or without growth factors and transplanted at the affected area. Stem/ progenitor cells are preferred because they are plastic and have good proliferative potential.

2.8. Prospects of cell/stem cells for neural regeneration

There are two approaches in stem cell biology to promoting SCI regeneration. One involves activating the endogenously available NSCs to promote regeneration and the other involves cell transplantation using exogenously sourced cells/stem cells/ predifferentiated cells.

2.8.1. Endogenous stem cells

The adult brain and spinal cord contain stem cells that are still able to divide after completion of CNS development which were initially considered essential solely to learning and memory. This endogenous stem cell pool could be modulated by pharmaceuticals and they react in response to different types of stressors. As recruitment signal, lesional expression of the inflammatory stromal cell derived factor-1alpha (SDF-1α) binds to the cognate receptor CXCR4 expressed by neural stem cells which triggers their proliferation directed migration towards injury site demonstrating the potential of endogenous stem cells to contribute to cell renewal (Chen et al., 2004). Within the lesion, these stem cells are able to proliferate and differentiate into fully developed, myelinating oligodendrocytes at least in models of acute demyelination (Gensert and Goldman, 1997). However, the sufficiency of stem

cell proliferation, its ability for fate specification and risks contributing to aberrant network organization leading to convulsion susceptibility needs to be considered.

2.8.2. Embryonic stem cells

ESCs are pluripotent cells that are derived from the inner cell mass of the early embryo. These cells have the ability to replicate indefinitely and differentiate into all three primary germ layers (generating all cell types in the body). Since highly plastic, with appropriate combination of growth factors ESCs could be used to obtain neurons and glial cells. Human ESC were directed to differentiate to neural progenitors by first inducing embryoid body formation that were subsequently plated onto appropriate substrates in defined medium containing mitogens (Carpenter et al., 2001). ESCs were transformed into electrophysiologically active motor neurons that express homeobox (HB9), Hox-C8, choline acetyl transferase and vesicular acetylcholine transporter using RA and SHH (Li et al., 2005) for neural regeneration application. Neural rosettes derived from ESCs upon culture in Murine stromal (MS) 5 stromal layer in presence of SHH and RA, efficiently differentiated to spinal motor neurons with appropriate *in vitro* morphological, physiological, and biochemical properties (Lee et al., 2007). ESC-derived neuroepithelial cells can be made to differentiate predominantly into dopaminergic neurons using FGF-8 and SHH. The same cells were made to generate spinal cord motor neurons in response to caudalizing signals such as retinoic acid. The period during which these cells respond to the directive cues is critical and if this period is passed, the cells tend to follow their intrinsic program of development (Guillaume and Zhang, 2008). Even though several research groups tried to compensate at least partially for the loss of cells due to a lesion through the transplantation of ESCs, only a few studies could indeed show

a functional improvement (Bjorklund et al., 2002). Oligodendroglial differentiation of ESCs upon transplantation into the injured rat spinal cord was observed more than neuronal. The rats showed enhanced mobility after transplantation. However, this study could not answer if the positive structural reorganisation was due to the surviving NPCs or due to the factors secreted by ESCs. Later, the electron microscopic evidence of synapse formation between the differentiated stem cells that were transplanted and the mature host neurons following SCI was demonstrated (Cummings et al., 2005). However, ESCs have several challenges associated with it like differentiation in to multiple lineages, which can cause post-transplantation issues, immune rejection, tumorigenicity as well as legal and ethical problems.

2.8.3. Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes like *Oct4, Sox2, Klf4* & *c-Myc* important for maintaining the defined properties of ESCs like proliferative and differentiation capacity. Induced PSCs are expected to be a solution to problems identified with ESCs, as these cells can be established from the somatic cells of patient itself. However, these cells may be associated with a greater risk of tumorigenesis because foreign genes are introduced into the chromosome and there is possibility that the reprograming is not necessarily complete. Induced PSCs were found to be capable of generating electrophysiologically functional neurons, astrocytes and oligodendrocytes *in vitro* (Miura et al., 2009). However, their safety after transplantation varies greatly depending on the somatic cells from which the iPSCs are derived. Ability of iPSCs to differentiate into dopaminergic neurons through embryoid body generation followed

by selection of nestin-positive cells and treatment with SHH and FGF-8 was demonstrated (Wenker et al., 2015). However, only 30% of the cells were tyrosine hydroxylase positive. Other differentiation protocols based on co-culture systems using meningeal cells; glial cells or stromal cells PA6 or MS5 cells was also found to be a better, simpler and faster method with 65% efficiency. Differentiation to other subtypes including glutamatergic, GABAergic and serotoninergic neurons was also reported. Preclinical studies have demonstrated promising therapeutic effects upon transplantation of human iPSC-derived NSCs into pre-clinical models of SCI. However, the complications involved in the process of cell transformation, reduced reprogramming efficiency and safety issues had to be foreseen and resolved before therapeutic interventions.

Considering the limitations of ESCs and iPSCs, ASCs are a promising and safe option for regenerative medicine. An ASC cell is an unspecialised cell that is capable of long-term renewal and differentiation into specialised cell types. In humans, the key functions of ASCs are to maintain and repair the specific tissues where they reside (Anon,). The use of ASCs is more widely accepted because it does not require destruction of an embryo. It also does not have the same immunological challenges as ESCs because they are harvested from the patient and therefore less likely for the body to reject. The limited proliferation potential of ASCs also reduces the chances for tumor formation upon transplantation. These properties make it a better option for patient-specific regenerative medicine. They are present in almost all tissues of the body.

2.8.4. Neuronal stem cells

Neuronal stem cells (NSC) are adult self-renewing, multipotent cells that generate the main phenotype of the nervous system (Anon, 2015b) *in vitro* and *in vivo* (Reubinoff et al., 2001). These cells can form multipotent neurospheres when cultured *in vitro* which can differentiate into specified neurons, glial cells, and oligodendrocytes. EGF and FGF are two vital growth factors that promotes neural progenitor and stem cell growth *in vitro* and *in vivo* (Lee et al., 2009)(Türeyen et al., 2005). The EGF and FGF receptors are widely expressed in the cytoplasm and nucleus of neural stem/progenitor cells. Neural colony forming cell assay discriminates NSCs from various progenitor cells(Louis et al., 2008). Pluripotent NSCs could be isolated from both embryonic and adult mammalian CNS (Reubinoff et al., 2001) (McKay, 1997) and the transplanted stem cells were able to specifically adapt to the local CNS environment and differentiate into the corresponding neuronal or glial subpopulations. Cell suspensions isolated from the neonatal brain of mice produced NSCs upon culture in DMEM-F12 medium containing N2 supplement, 10 ng/mL bFGF and 20 ng/ml EGF. NSCs produced β-III tubulin^{+} neurons, glial fibrillary acidic protein/GFAP^{+} astrocytes and O4^{+} oligodendrocytes upon *in vitro* differentiation as well as *in vivo* grafting (Mokrý et al., 2005). However, the underlying molecular bases of stem cell navigation in the CNS-parenchyma and of the interaction with the intact cells are not completely understood. The transplanted stem cells were able to contribute towards neurogenesis and replace apoptotic cortical neurons (Park et al., 1999). Though, there is a need to establish uniform evaluation standards that clearly define if and which of the surviving stem cells

differentiate and becomes functional. There is also practical difficulty with the isolation of these cells.

2.8.5. Schwann cells and Olfactory Ensheathing cells

These cells are easily obtained and cultured *in vitro* making them attractive sources for translational studies. Schwann cells could be harvested autologously from peripheral nerves in a manner similar to how nerves are harvested for isograft use for repairing large gaps produced by PNS injury (Pannucci et al., 2007). Schwann cells stimulate axonal fibre growth through the expression of growth factors (NT-3, NGF, BDNF, CNTF, and FGF), cell adhesion molecules, surface integrins and ECM components. Schwann cells produce the myelin sheaths that surround the neurons of the PNS (Oudega and Xu, 2006). Schwann cells were studied for their potential to promote axonal regeneration and myelination after SCI (Blakemore, 1977). Many studies report that there is no sensory improvement nor improvement in motor functions.

Olfactory Ensheathing Cells (OECs) are a kind of glial cells that direct the differentiation of progenitor cells present in the olfactory system towards OECS during replacement (Ruitenberg et al., 2006). They could be obtained for autologous transplantation through nasal biopsies which can be expanded in cell culture for several passages until use (Féron et al., 2005). These methods for harvesting and culture allow for transplantation without the issue of rejection. However, it takes at least 2 to 4 weeks of culture to generate sufficient cells for transplantation. Several groups doubt on the functional improvements induced by OEC grafts as well as the therapeutic potential of OECs after SCI.

2.8.6. Macrophages and Dendritic cells

Post-injury recovery in most tissues requires an effective involvement of macrophages. However, in the adult mammalian CNS this was found restricted because of its immune-privileged status due to the blood-brain barrier. Initial comparisons of inflammatory responses associated with PNS injury *versus* CNS trauma suggested that the lack of regeneration in CNS could be attributed to a compromised recruitment of macrophages (Hirschberg et al. 1995). Implantation of stimulated macrophages into transected rat spinal cord promoted tissue repair and partial recovery of motor function which was manifested behaviourally as well as electrophysiologically. However, controversy exists regarding the use of activated macrophages to boost post-injury inflammatory responses (Xu and Onifer, 2009). Transplantation of dendritic cells into the injured spinal cord of mice led to better functional recovery as compared to controls. The implanted dendritic cells induced proliferation of endogenous neural stem cells and led to neurogenesis. This could be due to the action of secreted neurotrophic factors such as neurotrophin- 3, cell-attached plasma membrane molecules, and possible activation of microglia/macrophages by implanted dendritic cells and therefore limited (Samadikuchaksaraei, 2007).

2.8.7. Bone marrow stromal cells / Mesenchymal Stem Cells

This is a kind of self-renewing and multipotent stem cell, obtained from bone marrow by simple iliac crest puncture. They are biologically safe and used in patients suffering from haematological cancer. Bone marrow stromal cells (BMSCs)/ Mesenchymal Stem Cells (MSCs) are plastic-adherent and must be able to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro.* They must express

Cluster of Differentiation (CD) 105, CD73, and CD90, and lack the expression of CD45, CD34, CD14, or CD11b, CD79a, or CD19 and HLA-DR surface molecules. The isolation of a population of multipotent stem cells from human bone marrow and demonstration of their spontaneous neuronal differentiation along with isolation of subtypes of non-hematopoietic MSCs capable of neuronal differentiation have paved the way for their clinical use in neurorestorative approaches. In order to overcome the potential problems associated with direct transplantation of undifferentiated MSCs, researchers have tested several modifications of transplantation strategies such as pre-transplantation neural differentiation, neurotrophic gene transduction etc. Rodent MSCs were able to efficiently differentiate into neural precursors by culturing with bFGF, EGF and heparin (Lepski et al., 2010). bFGF gene modified MSCs might be effective in promoting axon regeneration and functional recovery after SCI and same with the neurotrophin (NT)-3 modified groups. Functional motor neuron (MN)-like cells were derived from genetically engineered human MSCs (Park et al., 2012). They transduced motor neuron-associated transcription factor gene into the human MSC, then treated the engineered MSCs expressing Olig2 and Hb9 with optimal MN induction medium. By using an *ex vivo* model of SCI, they showed that these reprogrammed MSCs exhibited characteristics of MN-like lineage and are potentially therapeutic for autologous cell replacements. Neurally modified BMSCs when injected rostrally and caudally to the T8 lesion immediately after injury (Alexanian et al., 2011), locomotor function improved significantly and the volume of lesion cavity and white matter loss were significantly reduced in a twelve weeks period. However, the improvement of thermal sensitivity was not observed. Similarly neurally differentiated rat MSCs were transplanted and recovery of motor

function was reported nine weeks after transplantation (Cho et al., 2009). Autologous neural differentiated and undifferentiated MSCs were co-transplanted into the center of lesion cavity of a contusion thoracic SCI model. Five weeks after, the BBB scores were reported to be significantly higher in the pre-differentiated group as compared to undifferentiated group (Pedram et al., 2010).

Although bone marrow is the main source of MSCs, researchers have been looking for other sources because bone marrow derived cells are highly vulnerable to viral infection; have significantly increased cell apoptosis and loss of differentiation capability with age as well as due to the harvesting associated discomforts. Alternative sources of MSCs like adipose tissue, amniotic fluid, placenta, umbilical cord blood (UCB) and peripheral blood have been identified.

2.8.8. Umbilical Cord blood stem cells

Umbilical cord blood stem cells (UCBSCs) are easy to collect and have the advantage of storing in advance making it rapidly available when needed. The MSCs from UCB is more primitive than MSCs collected from other sources. Therefore they have superior proliferation potential and are less likely to induce graft-versus-host reactivity due to their immaturity (Park et al., 2006) (Lee et al., 2011b). In most studies, assessing the long-term effects of treatments is technically difficult due to associated risks of weight loss, urinary infection, and sepsis in injured animals. UCB also has the risk of transmitting infections with some level of immune rejection and ethical-legal issues.

2.8.9. Peripheral blood stem/ progenitor cells

The peripheral blood contains stem cells released from bone marrow and is the best substitute for BM-derived stem cells for *in vitro* differentiation as well as *in vivo*

studies. The circulating blood has many advantages as compared to bone marrow. The collection of blood is easier and non-invasive for the donor. It can also be collected from patient's own body serving as an autologous source and thereby not likely to induce Graft versus host disease. There are various studies reporting the use of PBMNCs from blood for the derivation of endothelial cells, smooth muscle cells and even keratinocytes under specific culture conditions. PBMNCs derived from peripheral blood was found to have the potential to differentiate to KPCs when appropriate growth conditions were provided (Nair and Krishnan, 2013). A natural clot based scaffold, fibrin along with fibronectin, gelatin and growth factors has been used to induce endothelial and smooth muscle progenitor cells from peripheral blood mononuclear cells to differentiate into endothelial cells and smooth muscle cells, respectively. The adhesive proteins used for the fabrication of endothelial progenitor cells matrix and smooth muscle progenitor cells matrix were the same, but specific differentiation was brought about by modulating the growth factor composition in the matrix and in the culture medium. (Sreerekha et al., 2006). Administration of human peripheral blood derived CD133+ cells accelerated functional recovery in a rat SCI model (Sasaki et al., 2009). After cell transplantation in injured spinal cord, intrinsic angiogenesis and axonal regeneration were found enhanced and cavity formation reduced histologically, with significant functional recovery. However, the use of blood cells as such poses lots of risk since it is a heterogeneous population and has the tendency to differentiate in to other lineages. Therefore, it may be crucial to adopt methods to selectively culture cells that have the ability to proliferate and differentiate into nervous system cells.

2.9. Milieu/niche/scaffold for cell transplantation

Cell transplantation by themselves has many issues. To maximize stem cell-based therapeutic efficiency, certain critical concerns need to be addressed. One of the major ones is that the exposure of cells to hostile conditions such as oxidative stress can result in considerably low survival rate of transplanted cells and affect the regenerative outcome (Bokara et al., 2014). The lack of active adhesion sites at the injured site is a considerable problem that can result in very low rate of sustained engraftment of transplanted cells. The lesion site may not have sufficient molecular signals to promote cell survival and differentiation. The untoward effect of inflammatory micro and macroenvironment may result in undesirable post-transplant NPC differentiation which in turn may limit formation of new tissue and its integration with the host tissue in the proximity of the injury site (Johnson et al., 2010a). A possible method to reduce the problems is to deliver cells to the injury site using cells implanted into scaffolds. Also, inflammation, free radical release and lack of ECM at the lesion site affects organizing of wound healing cells and survival of existing cells which interferes with the regenerative process. Scaffolds provide a bridge through which the regenerating axons can be properly guided from one end of the injury to the other end. Use of scaffolds provides a more hospitable environment for cell survival as well as trophic support for cells. Scaffolds can also be modified to contain cues to promote cell survival and progenitor cell differentiation (Samadikuchaksaraei, 2007). Among the various biomaterials available, hydrogels appear as an excellent option because their physical properties closely mimic the soft tissue environment and architecture of the CNS. Also, their chemical composition can be adapted to integrate ECM molecules as well as other adhesive proteins

providing support and guidance for axonal regeneration. The following substances have been applied as support or membrane for transplanted cells: natural biological hydrogels like collagen, alginate, matrigel, fibrin, fibronectin and agarose; synthetic hydrogels such as poly [N-(2-hydroxypropyl) methacrylamide] (PHPMA) and poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA-MMA); (Assunção-Silva et al., 2015). Zurita et al. developed a biologic scaffold system from blood plasma, called platelet rich plasma scaffolds which demonstrated optimized capabilities of survival and neural differentiation from hMSCs after the administration of BDNF. In another study, a combination of matrigel and neural-induced adipose-derived MSCs showed better functional recovery in dogs 8 weeks after administration. With the combinatory use of attractive substances (alpha FGF and fibrin glue) sprouting of axons was also achieved (Schwab et al., 2006). The problems of surgical interventions along with renewed trauma and scar formation associated with the use of scaffolds may be avoided using injectable hydrogels.

2.9.1. Fibrin based niche for transplantation

Fibrin polymer is formed from fibrinogen aggregates following thrombin cleavage. Fibrin plays an important role in healing and tissue repair in adults and functions as bridging molecule for many types of cell-cell interactions. At the site of a natural injury, many cells can be seen directly binding to the fibrin via their surface receptors which helps in the localization of these cells to the site of injury and carrying out their specialized function (Laurens et al., 2006). In general, matrices of fibrin tend to have greater intrinsic strength and support for cell ingrowth from surrounding tissues than other protein scaffolds. The malleability and easy application of fibrin as injectable liquid form or gel form or lyophilised scaffold form, coupled with its

availability in a clinical form enhances its application for tissue engineering and regenerative medicine applications. It is also possible to adjust fibrin matrix micromorphology by varying the fibrinogen or thrombin concentration. In the treatment of SCI, the fibrin enriched with acidic FGF had positive impact on axonal growth. Fibrin gel which was engineered to release neurotrophin-3 caused vigorous cellular infiltration of the fibrin and diminished formation of the glial scar at the site of cord injury (Johnson et al., 2010b). Fibrin scaffolds have also been evaluated as a method for differentiating and delivering mouse ESCs. Previous *in vivo* work has shown that controlled growth factor release from such scaffolds provides beneficial short-term effects as a treatment for SCI. The effect of different doses and combinations of growth factors on the differentiation of mouse ESCs seeded inside of 3D fibrin scaffolds was also evaluated (Willerth and Sakiyama-Elbert, 2008).

Fibrin contains traces of fibronectin (FN), which is a glycoprotein found in many extracellular matrices and in plasma. The fibrin we used was also found to have FN in it. FN is also involved in cell attachment and migration due to its interaction with cell surface receptors (Ahmed et al., 2003). This FN helps in the formation of single direction oriented pores. FN mats (King et al., 2003) when implanted in hemisected rats spinal cords, well integrated with the spinal cord with orientated growth of different sub-types of neurons including GABAergic, cholinergic, glutamatergic, noradrenergic and Calcitonin gene-related peptide $(CGRP)^{+}$ neurons was observed including axonal myelination. However, there was only little axonal outgrowth from the mats into the host spinal cord which was attributed to the astrocytosis and glial scar formation around the implant (King et al., 2004). Combination of FN and Fibrinogen has been used as contact guidance substrates, particularly for repair of

peripheral nerve lesions. Fibrin and Schwann cells along with Brain Derived Neurotrophic Factor (BDNF) and neurotrophin-3 led to significant improvement of hind limb function in treated animals (Blits et al., 2003).

2.10. Animal models in SCI

Animal models of SCI are designed to simulate different types of human SCI. Neuroprotective strategies often employ contusion or compression injuries. Pro-regenerative strategies involve transection of all tracts (complete) or some tracts (e.g., dorsal column for sensory axons ascending in the fasciculi gracilis and cuneatus) as these allow one to distinguish regenerating axons from axons that were spared or that sprouted a collateral. Cervical injuries are typically chosen to test therapies for improvement of forelimb movement. Thoracic injuries are selected to evaluate therapies for improvement of hind limb movement. Different surgical methods are used to create SCI in preclinical settings.

2.10.1. Types of Injury

2.10.1.1. Contusion

This is a bruise kind of injury which leaves the glial limitans and piamater intact. Fluid-filled cavities or cysts evolve from the hemorrhage into spinal cord parenchyma where tissue has degenerated. This is more representative of what occurs in most human injuries. Pattern of injury maturation appears to be reasonably well simulated in spinal cords of animals after a blunt injury, thereby providing a setting for evaluating neuroprotective strategies in acute phase of injury (McDonough and Martínez-Cerdeño, 2012). This kind of injury studies are carried out to examine the consequences of common types of human injury and to test therapies aimed at minimizing secondary tissue injury. Blunt injury models produce

a lesion in which neuronal tissue remains intact along a peripheral rim, the quantity of which is correlated with residual locomotor function.

One of the disadvantages of this model is that due to incomplete nature of injury and the complexity of the tracts, it is very difficult to verify anatomic axonal regeneration in these models. Experimental animals are under anesthesia during the injury and not subjected to other drugs as traumatized patients which creates a different pharmacologic environment for the neuroprotective agent in question. The commonly used impactors to create SCI contusion models are New York University Impactor and Ohio State University impactors where animals are subjected to predefined forces which creates the lesion (Kwon et al., 2002).

In a T9 contusion model in female Lewis rats, bone marrow stromal cells from male rats labelled with Green Fluorescent Protein were injected into the lesion as well as rostral and caudal to it at $3x10^5$ cells after acute and sub-acute injuries. Expression of some neuronal markers *in vitro* was observed but no action potentials or voltage-gated sodium/potassium currents (whole-cell recordings).Cell counts at 5weeks post-injury revealed significantly more BMSCs as compared to 1week post injury than in those treated immediately after injury (Hofstetter et al., 2002).

2.10.1.2. Clip Compression

This model simulates ongoing cord compression secondary to residual spinal column displacement. Here the spinal cord of the experimental model is compressed for variable durations (3s, 1min, 5min) between the arms of a modified aneurysmal clip. This model is important for illustrating the relation between severity of neurologic injury and duration of compression. It is possible to produce consistent injuries with this technique. Different severities are produced by adjustment of closing force of

clip, duration of compression or both. Compressive force is applied to both the volar and dorsal aspects of cord, making it somewhat more representative of human injury (McDonough and Martínez-Cerdeño, 2012). Female nude mice with T8 weight compression sub-acute injury was transplanted with human NSC number of labelled NPCs that expressed GFAP and β-III tubulin was found at the spinal cord lesion site when injected on day 7 than on day 3 or 10. A trend of a better BBB score at 21 days was observed.

2.10.1.3. Complete transection

This model is favored for the study of regeneration. This ensures absolute completeness of injury. It is easier to evaluate the effectiveness of interventions with regard to both axonal regeneration and functional recovery. With ensured completeness of lesion, anterogradely labelled axons observed distal to the lesion have indeed regenerated from above and are responsible for the functional recovery of the animal. If functional recovery is observed after the application of a particular therapy to a fully (or partially) transected spinal cord, the fact that this recovery is due to axonal regeneration of descending systems and not to native spinal cord circuitry is supported when the function is lost after a retransection of the cord (Kwon et al., 2002). In a complete transection model of male Wistar rat at T8, a 5mm tube of ultrafiltration membrane filled with matrigel along with BMSC -BDNF was grafted. Significant increases in the number of Growth Associated Protein 43+, TH+ and CGRP+ axons in various portions of the grafts in animals treated with BMSC-BDNF, less with BMSC-LacZ was observed. Average BBB scores at 6week post implantation were 6.1, 6.0, and 3.6 for BMSC-BDNF, BMSC-LacZ, and control respectively (Koda et al., 2007).

2.10.1.4. Partial section: dorsal & lateral hemisection

Here attempt is made to cut tracts of the spinal cord selectively. Depending on the severity of the lesion, the resulting neurologic deficit can be relatively mild, making the postoperative animal care easy. This allows comparison of regenerative response in a particular tract with its uninjured partner on the contralateral side. Lateral position makes it relatively easy to cut in a unilateral fashion while leaving the contralateral tract uninjured. In the case of dorsal hemisection, lesion transects rubrospinal and corticospinal tracts bilaterally. Here it is more difficult to define the extent of injury. This raises the possibility that axons of the particular tract in question might have escaped injury. If a tracer is applied distally to the site of partial injury, its histologic appearance proximally in the cell body of a neuron implies that this neuron's axon was not cut during the injury (Kwon et al., 2002). Conversely, the absence of tracer confirms the injury's completeness. The spinal cord may rotate locally in the process of making a lateral hemisection lesion, so that orientation of the section with respect to vertical axis may be difficult to maintain. In a C4 dorsal column transection female Fischer rat model transplanted with autologous rat GFP-labelled BMSCs ($2x10^5$ cells), dibutyryl-cAMP and NT-3, grafts survived and integrated well with host tissue and rostral injection resulted in minimal parenchymal disruption. BMSCs remained within the lesion site or migrated only short distances from the lesion. Growth of sensory axons into the BMSC graft was significantly augmented by either cAMP or NT-3 treatment as compared to control. Sensory axons extend beyond the lesion-graft boundary only in subjects that received rostral NT-3 injections. That growth was significantly enhanced with the addition of cAMP treatment (Lu et al., 2004).

2.10.2. Delivery methods for cellular therapy

Once a decision has been made regarding what type of cells and what animal models to use, the next consideration is how to deliver the cells to the injury site. The different delivery methods include directly injecting the cells into and around the injury site. Other minimally invasive injection methods have been studied, such as intravenous injection, infusion into CSF and lumbar puncture (Ohta et al., 2004) (Bakshi et al., 2006)(Khalatbary and Tiraihi, 2007)(Vaquero et al., 2006). An alternative method of delivery involves seeding cells into scaffolds and then implanting the scaffolds into the injury site. Many studies inject cells rostrally or caudally from the injury site to achieve better levels of survival. Direct injection into the injury site often produces poor results in terms of cell survival due to the harsh environment in the lesion. Systemic administration of BMSCs by injection into the tail veins of rats has been studied as a minimally invasive injection technique. Although cells delivered using this method were found at the injury site, direct injection of BMSCs into the injury site produced superior functional recovery. Other injection methods include using lumbar puncture to deliver cells and injecting the cells into the CSF containing cavities found in the brain (Shi et al., 2007). When BMSCs were injected at L4–5 using a lumbar puncture method, best results including a reduction in cyst volume and injury size was obtained. Additionally, this method allows for multiple injections, which could be useful for treatment of chronic injuries. When cells were injected into the fourth ventricle of the brain, which consists of a cavity containing CSF, an improvement in BBB score up to 35 days observed. However, more research needs to be done to fully characterize the efficacy of these strategies compared to the direct injection method.

2.10.3. Evaluation methods

There are several tools to assess the functional recovery as well as morphological recovery in rat SCI models. The most widely used one is behaviour or functional analysis which include BBB scoring, inclined plane test etc. Others include electrophysiology and histology (Anon,).

2.10.3.1. Behaviour / Functional analyses

Many of the current models of SCI utilize the Basso, Beattie, and Bresnahan open-field locomotor rating scale to assess functional outcome. The BBB scale has a range from zero (no hind limb movements) to 21 (normal coordinate gait), using paw placement, joint movement, and truncal stability as important factors in determining the level of functional recovery. Scores in the 0 to 7 range (early stage) focus primarily on hip, knee, and ankle joint movement, 8 to 13 range (intermediate stage) keys in on paw placement and coordination and scores of 14 to 21(late stage) rely heavily on trunk stability, tail position and paw placement.

Tarlov and Klinger developed a simple open-field behaviour test for SCI to assess the locomotor ability, the modified version of which consists of six levels of motor movement (from 0 for no hind limb movement to 5 for normal walking). Inclined plane is a widely used test to evaluate functional outcome in rats after SCI. The device consists of a hinged board raised and lowered to different angles. The assigned score is the maximum angle of the plane that the rat can maintain for 5 seconds without sliding off onto a padded surface. Other analyses of locomotion include footprint analysis and the Cat walk-assisted gait analysis.

2.10.3.2. Electrophysiology

This is a valuable tool for investigation the neural deficits and functional recovery in animals. Terminal electrophysiology procedures are useful in experimental animals, which is much easier to reproduce after SCI without anaesthesia. The transcranial magnetic motor evoked potential (tcMMEP) procedure involves non-invasive magnetic stimulation at the unanaesthetized rat's skull and recording of evoked potentials with EMG electrodes temporarily inserted into hind limb muscles. While the tcMMEP procedure assesses supraspinal axon conduction, magnetically evoked interenlargement (MIER) and somatosensory evoked potential (SSEP) procedures are effective for the evaluation of propriospinal and sensory spinal axon conduction, respectively. The MIER procedure involves non-invasive magnetic stimulation at the rat’s hip or knee and the recording of evoked potentials with EMG electrodes temporarily inserted into forelimb and masseter muscles (Beaumont et al. 2006). The SSEP procedure involves electrical stimulation of the paws with electrodes temporarily inserted into them, and the recording of evoked potentials from electrodes previously implanted in the cranium over the somatosensory cortex.

2.10.3.3. Histology

Anatomic assessment is carried out to look at the morphology of axons and assess the degree of tissue sparing, injury and recovery. This involves use of histopathologic techniques and relies on histology, immunohistochemistry (IHC) and axonal tracers. Electron microscopy may also be used to look at the morphology of spinal cord at very high resolution. Histology used specific stains to assess the tissue architecture due to damage or recovery. IHC uses specific antibodies targeted against unique proteins found in certain axonal populations allowing visualization of specific

type of axons/neurons in histologic sections of spinal cord. Axonal tracers are molecules that can be picked up by neurons or axons and transported in either an anterograde or retrograde fashion. Anterograde tracers are applied to the cell bodies of neurons and then transported to the axons, where they can be visualized. Such tracers are therefore useful for identifying axons that are injured or regenerating at the injury site. Examples include biotinylated dextran amine, enzyme horseradish peroxidase conjugated to wheat germ agglutinin (WGA), and the cholera toxin B subunit. A tracer used retrogradely is applied to the axons and depending on the characteristics of the tracer, is taken up by cut or intact axons or by their terminal endings and transported back to the cell body. A variety of fluorochromes such as Fluoro-Gold, Fast-Blue, and Nuclear Yellow are commonly used retrograde tracers. Differential absorption and emission characteristics of these fluorochromes allow them to be used together, then distinguished on histologic sections with various riddles and wavelengths of light. The previously mentioned anterograde tracers (HRP-WGA, Cholera Toxin-B, and Biotinylated Dextran Amine) also can serve as retrograde tracers. The demerits with histology are difficulty to measure tissue preservation in a thoroughly meaningful manner; weakness; assessments cannot be made in real time; cannot be performed with living animals; difficulty comes in dealing with the measurement of partially damaged areas and evaluating the degree and functional significance of damage (Onifer et al., 2007).

CHAPTER 3

3. MATERIALS AND METHODS

In this study, effort was made to prove the concept of using autologous adult progenitor cells for SCI regeneration. The concept of NPC isolation from human blood and their ability for differentiation was proven using a fibrin-based matrix. Involvement of a bio-similar signaling mechanism for the differentiation process was demonstrated. The concept of NPC isolation and differentiation using the optimised fibrin niche was proven to be applicable for isolation of NPC from rat blood also with an objective to use it as an autologous source for cell transplantation in rat SCI model. The fibrin composition for *in vivo* NPC delivery was optimised *in vitro* to obtain optimal NLC growth. A SCI model was created and cell transplantation to test the ability of optimised fibrin composition to improve cell survival and retention at the injury site was carried out. The materials used for the experiments and tbe details of the standardized methods are as described below.

3.1. Preparation of biomimetic culture matrix

3.1.1. Preparation of fibrinogen concentrate and thrombin

Cryoprecipitated fibrinogen (Fib) prepared in-house was from pooled human plasma and meets the specified criteria as per European Pharmacopoiea and is routinely used for surgical soft tissue sealing. The concentration of the lyophilized fibrinogen concentrate is 100±20 mg when reconstituted in 1 ml

deionised water. The component was diluted to get 2mg/ml for preparing 2D cell culture matrix and 20mg/ml for 3D cell culture studies.

Thrombin prepared for clinical use from cryo-poor human plasma by diethyl amino ethyl (DEAE) cellulose ion exchange chromatography was used. The 250IU of lyophilised thrombin was reconstituted with 35mM calcium chloride to obtain the required concentration for use. Thrombin adsorbed to the tissue culture polystyrene surface from a working solution of 2IU was used for 2D cell culture and 1IU for making 3D clot for *in vitro* and *in vivo* experiments.

3.1.2. Preparation of Growth factors and glycosaminoglycans

Bovine hypothalamus extract (HE) was prepared as per the protocol of Maciag et al (1979). The extract is known to contain different GFs and cytokines and the important ones that are applicable for neural growth are basic fibroblast growth factor (bFGF), brain derived neurotrophic factor (BDNF) and other nerve growth factors. Briefly, bovine calf brain obtained from the slaughter-house in ice was dissected, hypothalamus was isolated, minced into small pieces, homogenised, stirred for 2h in ice-cold saline and centrifuged at 13000 x g, 4^{o}C for 15min. The supernatant was collected and mixed properly with 0.5 w/v percentage streptomycin sulphate (Sigma, USA). The mixture was then incubated overnight at 4^{o}C to remove the lipid content with subsequent centrifugation at 13000 x g, 4^{o}C for 15min. The supernatant was collected and dialyzed against 0.1M sodium chloride (NaCl) for 24h at 4^{o}C. The dialyzed solution was sterile filtered through a 0.22µm filter (Millex GP, Millipore, Ireland), assayed for Lowry's protein, lyophilised (Modulyo 4K Freeze dryer, Edwards, UK) and stored at -80^{o}C

(SANYO ultralow, Japan) for use in cell culture studies. Based on Lowry's protein concentration of HE cocktail, the quantities used in the matrix and medium was adjusted.

Platelet growth factor (PGF) was prepared as per the procedures published earlier (Resmi and Krishnan, 2002). Freshly collected platelet rich plasma (PRP) obtained from blood bank was added with 10% excess acid citrate dextrose (ACD) solution, centrifuged at 750*g* and cells were washed 2 more times with ACD-Tyrode's buffer. $CaCl_2$ was added to get 2mM final concentration, and challenged with 1IU/ml thrombin for 5min. EDTA (5mM final concentration) was used to stop further activation. Activated platelets were then centrifuged at 36000*g* for 1h at 4 ^oC and the released proteins were collected in the supernatant and dialysed against Ca^{2+} -Mg^{2+}- free Hank's Balanced Salt Solution (HBSS). It is then sterile filtered (0.22µm) and aliquots were stored at -40 ^oC until use. The concentration of PGF cocktail was determined by Lowry's assay and the concentration in the matrix and medium adjusted as per requirement.

Hyaluronic acid (HA) was prepared from human umbilical cord based on the published protocol (KAYE and STACEY, 1951)(Anilkumar et al., 2011) was used. Briefly, the umbilical cord was ground to form a solution. The pH was lowered to 2, pepsin was added and after overnight stirring at RT, pH was raised to 7.4 and trypsin was added and incubated overnight. Then the precipitate obtained by ethanol precipitation was dialysed, lyophilised and stored. Chemical analysis of HA was done by Fourier transform infrared spectroscopy and it was proved to be

a pure preparation with characteristic spectral peaks. The lyophilised powder was reconstituted in water to get a stock solution of 1mg/ml.

3.1.3. Preparation of matrix coated surfaces for cell culture

An established protocol (Prasad Chennazhy and Krishnan, 2005) was used to coat the tissue culture polystyrene (TCPS; NUNC, Roskilder, Denmark) dishes with a fibrin composite (FC) matrix, with modifications, for human as well as rat NPC isolation. Briefly, the composite comprising 2mgml^{-1} fibrinogen, 0.2% gelatin (Gel), 100 μgml^{-1} HE, 10 μgml^{-1} PGF and 100 μgml^{-1} HA was layered on the thrombin-adsorbed tissue culture polystyrene (TCPS) surface with a distribution of 8 μl cm^{-2} area. The fibrin clot formed was allowed to stabilize by keeping it at 37°C for 30 min. The dishes lyophilized (Edwards Modulyo, UK) under sterile conditions were either used immediately for cell culture or stored at 4 °C for later use. To study the effect of Gel and HA, the TCPS plates were treated for one hour with each individual component prepared in Hank's Balanced Salt Solution (HBSS). The non-adsorbed molecules were aspirated out and the isolated PBMNCs were seeded and cultured using the protocol followed for culture on a fibrin matrix. To optimize the concentration of each component, varying concentrations of that particular component was included in the composite, while keeping the concentration of other components constant.

For signaling studies, matrix without growth factor was prepared. Briefly, the TCPS was first treated with 2IU thrombin as described earlier. After removing the excess thrombin, 2mg/ml fibrinogen was layered over the thrombin-adsorbed TCPS to obtain a thin fibrin clot.

3.1.4. Preparation of 3-Dimensional fibrin matrix

For constructing 3-Dimensional (3D) fibrin for cell delivery, 20mg/ml fibrinogen was mixed with varying concentrations of thrombin such as 1IU, 5IU, 10IU and 15IU. The components from dual syringes were delivered into culture wells using fibrin sealant applicator with Y-connector. Once the mixed components formed a clot, it was analysed for porosity and fiber thickness after fixing with glutaraldehyde. The fixed fibrin clot was washed and analysed in low vacuum mode using Environmental Scanning Electron Microscopy (E-SEM) from FEI Quanta 200, USA.

3.2. Isolation and culture of cells

NPC isolation and differentiation from human/rat PBMNCs were carried out using cell-specific matrix in complete neural induction media (CNIM). The matrix and media together constitute the niche. The niche composition was standardised by changing the composition and concentration of both matrix and media constituents. For signaling studies, a modified protocol to exclude the interference of other components from the niche was employed.

3.2.1. Isolation and culture of human PBMNCs

The buffy coat (discards from the blood bank obtained with Institutional Ethics Committee (IEC) approval (SCT/IEC/FEB-2013, dated 25.02.2013)) was centrifuged at 750*g* (Biofuge stratos, Heraeus instruments, Germany) for 10min at 25°C, for settling down the Red Blood Cells (RBCs). After discarding the plasma, leukocytes and platelets which settles above the RBC bed was collected, diluted with HBSS (pH 7.4), layered over equal volume of Histopaque-1077 (Sigma

Aldrich, USA) and centrifuged at 400*g* for 30min at 25°C to separate the PBMNCs (Hofman et al., 1982). The white blood cell layer settled at the plasma-Histopaque interface was aspirated, washed with serum free medium (SFM) at 150*g*, 4°C, suspended in complete neural induction medium (CNIM) and cultured.

The CNIM comprised MCDB 131, 20% FBS, 10 $\mu g ml^{-1}$ HE, 1 $\mu g ml^{-1}$ PGF, 10 IU ml^{-1} heparin sulphate, 0.44 mg ml^{-1} glutamine, 25mM KCl and 1 x antibiotics (Gibco BRL (now part of Invitrogen Corporation, Grand Island NY, USA) and Sigma Aldrich). This was the composition and concentration of the final optimised CNIM. To obtain this composition, inclusion of different components at varying concentrations were used until best and consistent neural properties were seen in NPC culture. The components that were found to be hostile for the cells were excluded and the others were included.

For isolation of progenitors from peripheral blood, a method described by Asahara et al for the endothelial progenitor cell was modified and employed (Asahara et al., 1999). Briefly, the PBMNCs in the CNIM were seeded on a bare TCPS dish and incubated for one day (24h) under standard culture conditions. The medium and the floating cells were then removed and the partially attached cells collected in the CNIM were seeded and cultured on the fibrin matrix coated dishes. The medium was changed on the first two consecutive days and thereafter on alternate days. Excess potassium chloride (25mM KCl final concentration) was added to the cells on the fifth day (120h) of culture and was replenished with every medium change, which is denoted as KCl^{+} cultures (test). Culture without added KCl is denoted KCl^{-}cultures (control). The cell morphology was observed

periodically using a phase contrast microscope (DMIRB, Leica Microsystems, Wetzlar, Germany).

3.2.2. Isolation and culture of rat PBMNCs

The mononuclear cells from heparinised rat blood obtained by cardiac puncture were isolated using Histopaque-1083 density gradient centrifugation, with animal ethics committee approval (SCT/IAEC/B1342012X). The isolated cells were washed using SFM, suspended in CNIM and cultured as per the method described earlier in #3.2.1. Inbred strains of female Wistar rats were used blood donors.

3.2.3. Isolation and culture of PBMNCs for signaling studies

The PBMNC isolation was done as per #3.2.1. The cell culture protocol was adopted from Wexler with slight modifications (Wexler et al., 2009). Briefly, the PBMNCs were seeded in MCDB 131 media containing 1% FBS, 10 IU ml^{-1} heparin sulphate, and 0.44 mg ml^{-1} glutamine in the uncoated dish for 24h. The partially attached cells were then flushed out in 1% FBS containing media and seeded on to the fibrin-coated dishes for 48h. Cells were then transferred to SFM (MCDB) containing 20ng/ml bFGF (R&D Systems, Minneapolis, MN, USA) for another 48h to promote proliferation. The growth factor was then removed to allow the cells to differentiate for the next 48h, after which the cells were cultured in KCl^{+} medium for another 48h. This culture system is referred to as the standard niche (Std niche). To study the effect of added Wnt-3a on neuronal differentiation, commercially obtained Wnt-3a protein (R &D systems) at a concentration of 150ng/ml was added to the medium at the time of transferring the cells to the fibrin-coated dishes. This culture with added Wnt-3a is represented as Wnt+ in

this study. After 96h of treatment, Wnt-3a was withdrawn from the culture. The cell morphology was observed periodically using a phase contrast microscope (DMIRB, Leica Microsystems, Wetzlar, Germany). To establish the role of Wnt signaling on cellular growth parameters such as NPC attachment, morphology, proliferation and differentiation, Wnt inhibitor (PNU 74654, Tocris Bioscience) at an optimised concentration of 25ng/ml was used. To study the effect of individual/combined matrix components on Wnt signaling, antibodies raised against matrix components fibronectin (FN), fibrinogen (Fib), laminin (La) were used at 150µg/cm^2 individually or in combination for blocking the signals elicited from these matrix proteins, and the fate of cells was studied. The dose response of each molecule was analyzed and the most effective concentration was chosen for further studies.

3.2.4. In vitro niche optimization for in vivo application

To identify the influence of thrombin on cell growth (already committed NPCs), PBMNCs were cultured on the TCPS-fibrin niche and on the 5th day of culture, graded concentrations of thrombin in $CaCl_2$ ranging from 1IU to 15 IU were added into separate cultures and the cells were allowed to grow for 10 more days. The parameter analysed for assessing effect of thrombin on cells was morphology under phase contrast microscope (DMIRB, Leica Microsystems, Wetzlar, Germany).

To study the influence of thrombin concentration on cells, PBMNCs from TCPS-fibrin niche were flushed out on 5th day of culture and labelled with PKH26 as described in #3.4.2. The fluorescent-tagged cells were seeded on to the 3-D fibrin

gel in TCPS and cultured under standard differentiation protocol for 72h. Cell morphology was analyzed using fluorescence microscopy (DMIRB, Leica Microsystems, Wetzlar, Germany).

For studying the effect of thrombin concentration on cell growth, the samples on fibrin matrix were fixed using 2.5% glutaraldehyde, washed and visualized in low vacuum (LV) mode using E-SEM (FEI Quanta 200, USA).

3.3. Identification of active components in Matrix

3.3.1. Western blotting

The presence of brain derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) were analysed in PGF and HE cocktails by Western blot analysis. For protein separation by SDS-PAGE, 20µg of HE and 20µg of PGF cocktails per lane were loaded. The proteins were then transferred to the polyvinylidene difluoride (PVDF) membrane using the semidry method at 0.8 mA cm^{-2} current using Pharmacia LKB Multiphor II. To develop the specific bands, the membranes were blocked in 1% BSA for 1 h and incubated for 2 h in 1:500 dilutions of primary antibodies against FGF (18 kDa), BDNF (28 kDa) and VEGF (44 kDa) (Abcam, Cambridge, UK). The blots were incubated for 1h in corresponding HRP-conjugated secondary antibodies at a dilution of 1:1000 and the bands were visualized by adding PBS containing 0.05% diaminobenzidine, 0.1% hydrogen peroxide and 0.04% nickel chloride (pH 7.5). Once the best contrast was achieved, the reaction was stopped and imaging was carried out using gel documentation system Alpha Imager, (Protein Simple, USA). For signaling studies the antibody concentrations used were similar to # 3.4.2.

3.3.2. Immunodiffusion

To demonstrate the presence of adhesive proteins fibrin (Fib), fibronectin (FN) and laminin (La) in the matrix composite, Ouchterlony's double immunodiffusion method (Bailey 1996) was performed. Affinity purified polyclonal rabbit antibodies raised against pure antigens (obtained from Sigma Chemicals, USA) were used. For each reaction, the specific antibody (10μg) was added in the middle well and the serially diluted fibrinogen concentrate was added in the peripheral wells cut in 0.8% agarose gel (Sigma-Aldrich Chemicals, St. Louis, MO, USA). After incubation at 2-8 °C for 72 h, the gels were immersed in normal saline for 24 h to remove unreacted proteins, the wells were filled with molten gel and the slide was overturned on several layers of filter paper and kept until completely dried. The precipitin lines were developed with the standard Coomassie blue staining method and imaged using Alpha Imager.

3.4. Characterisation of human/rat NPCs and NLCs

After isolation and differentiation of NPCs on the culture matrix, the cells were characterised. Morphological analysis using phase contrast microscopy was used as the first level of evidence for transformation of cells. The morphology was continuously monitored during the culture period and was documented before further characterisation.

3.4.1. Estimation of cell length

Phase contrast/fluorescent images of cells in culture were captured using Leica Application Suite (LAS, Leica, Germany). At least 100 cells per donor in the less dense area were marked in each culture type at predefined periods and cell length

was quantified using ImageJ software (ImageJ, National Institutes of Health, USA; http://imagej.nih.gov/ij; Java 1.8.0_25). A minimum of 3 donors were used for all parameters reported and the average and S.D. was calculated.

After induction of differentiation, cell length was measured on 0^{th}, 8^{th}, 16^{th} and 24^{th} days. For signaling studies, cell length was measured on 8^{th} day of culture on the Std niche, Wnt+ and Inh+ cultures. For studying the *in vitro* effect of thrombin on cells, the length of 8^{th} day cells were harvested and seeded on 5^{th} day of culture on to 3D-fibrin matrix was measured.

3.4.2. Immunostaining for progenitor/differentiated cells

3.4.2.1 Reagents

The antibodies against Nestin, Syn and O4 were from R&D Systems, Minneapolis, MN, USA; the antibodies against MAP-2 were from Santa Cruz, Heidelberg, Germany; the antibodies against β-III tub were from Epitomics, an Abcam company, Burlingame, CA, USA; the antibodies against TH were from Abcam, Cambridge Science Park, Cambridge, UK; the antibodies against GFAP were from BD Bioscience and the antibodies against Annexin V were from Invitrogen, USA. The secondary antibodies used were from BD Biosciences (USA). Actin staining was done using Texas red-Phalloidin (Molecular Probes, now part of Invitrogen Corporation, USA). The nuclear stain used was 4, 6, diamidino-2-phenylindole (DAPI from Invitrogen, USA).

3.4.2.2. Human PBMNC

For the detection of specific molecules using fluorescence microscopy, PBMNCs were cultured on FC coated chamber slides (BD Biosciences, San Jose, CA, USA).

At predefined culture periods, the cells were fixed with 3.7% formaldehyde for 20 min, permeated using 0.1%Triton X-100 in PBS for 5 min and blocked with 0.5% BSA in PBST for 30 min. The cells were then incubated overnight with primary antibodies against the progenitor marker nestin (1:100), the intermediate neuronal marker β-III tub (1:100), mature neuronal marker MAP-2 (1:100), functional neuronal markers Syn (1:100) and TH (1:100), astrocyte marker glial fibrillary acidic protein (GFAP-1:100) and oligodendroglial marker O4 (1:100). Briefly, the cells were washed in PBS, fixed with 3.7% formaldehyde, permeated by 0.1% triton-X-100, washed and stained with actin (1: 500) for 1h. The source of antibodies are described in #***3.3.2.1.*** The images were acquired using a fluorescence microscope from Leica Microsystems (DMIRB, Germany) or confocal microscope (LSM Meta 510, Zeiss, Germany)

3.4.2.3. Rat PBMNCs

The cells were incubated overnight with primary antibodies against β-III tub (1:100), Ki 67 (1:100), MAP-2 (1:100), and Syn (1:100) after fixation, permeabilisation and blocking. The sources of primary antibodies are described in #2.4.2. The conjugated secondary antibodies were used at 1:200 dilutions for 1h. The nuclear stain used was DAPI. The images were acquired using a fluorescence microscope from Leica Microsystems (DMIRB, Leica Microsystems, Wetzlar, Germany).

3.4.3. Analysis using flow cytometry

3.4.3.1. Human PBMNCs

To quantify the antigen expression using flow cytometry, cells cultured for specific time intervals on matrix coated 6-well TCPS (10.75 cm^2 area) were harvested using the standard trypsin digestion method. The cells were then fixed in 3.7% formaldehyde, permeated using 0.1% triton X-100 for 5 min, blocked with 0.5% BSA for 30 min and incubated with primary antibodies against nestin (1:100), β-III tub (1:100), MAP-2 (1:100), Syn (1:100), TH (1:100), GFAP (1:100) and O4 (1:100). Proliferating cells were detected and quantified using Ki67 (1:500), and apoptotic cells by Annexin V FITC (1:500). The source of antibodies are described in #***3.3.2.1.***

Dot plots of forward scatter versus side scatter were used to select the desired cell populations and for excluding debris and clumps. The of unstained control was used for each separate experiment to acquire histogram of fluorescence against SSC was acquired using BD FACS Aria equipped with BD FACS Diva software (BD Biosciences, San Jose, CA, USA). The isotypic control was used in each case for gating the negative population for each fluorochrome. When dual staining was done, single stained cells were used for compensation to eliminate overlap of emission spectrum. Fluorescence intensity from 10,000 events was recorded using cells from at least three donors, for all markers studied at all periods. The percentage of cells expressing the specific markers was estimated using Diva software (BD, USA) followed by FlowJo software (Tree Star Inc., USA).

3.4.3.2. Rat PBMNCs

After initial fixation, permeabilisation and blocking, the cells were incubated with primary antibodies against nestin (1: 100), β-III tub (1:100), Ki 67 (1:100), MAP-2 (1:100) and Syn (1:100) and corresponding secondary antibodies as described in #3.3.2.2. A histogram of fluorescence against SSC was acquired using BD FACS Aria equipped with BD FACS Diva software and the percentage of cells expressing the specific markers was estimated using Diva software followed by Flow-jo.

3.4.4. Quantitative Real-time polymerase chain reaction

During specified periods of the human PMBNC culture, up/down regulation of neural marker genes was estimated using a quantitative Real-time polymerase chain reaction (qRT-PCR). The total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions and quantified using a Qubit RNA assay kit on a Qubit 2.0 fluorometer (Invitrogen, USA). 1µg of RNA was converted to cDNA using the Superscript III reverse transcriptase enzyme (Invitrogen). Real time PCR was carried out in a total volume of 25µl containing 250 ng cDNA, 100 pmol each of respective forward and reverse primers and 12.5 µl of SYBR-Green I master mix No ROX (Eurogentec, San Diego, CA, USA). Forty cycles of reaction were performed using the Chromo 4 system (MJ Research, now part of Bio-Rad, USA). The housekeeping control used was the GAPDH gene. Melt curve analysis confirmed the specificity of each reaction. Fold change was calculated after normalisation with GAPDH expression on each day of analysis using the formula $2^{-\Delta\Delta Ct}$. The molecular size of the products

obtained was verified using agarose gel electrophoresis (AGE). The human primer sequences are given in the table 1.

Genes	Amplicon size	Primer sequence (5'-3')
GAPDH	210bp	F-GCTTGTCATCAATGGAAATCCC R-TCCACACCCATGACGAACATG
Nes	200bp	F-GCCCTGACCACTCCAGTTTA R-GGAGTCCTGGATTTCCTTCC
β-III tub	148bp	F-GCTCAGGGGCCTTTGGACATCTCTT R-TTTTCACACTCCTTCCGCACCACATC
TH	218bp	F-GGTTCCCAAGAAAAGTGTCAG R- GGTGTAGACCTCCTTCCAG
Syn	184bp	F-CCAACAAGACCGAGAGTG R-ATGGAGTAGAGGAAGGCAAA
OSP	283bp	F-ACTGCTGCTGACTGTTCTTC R-GTAGAAACGGTTTTCACCAA
NSE	188bp	F-CTGATGCTGGAGTTGGATGG R-CCATTGATCACGTTGAAGGC
GFAP	70bp	F-ACATCGAGATCGCCACCTACA R-GTCTGCACGGGAATGGTGAT
Axin 2	202bp	F-AGTCAGCAGAGGGACAGGAA R-AGCTCTGAGCCTTCAGCATC
LEF	187bp	F-GACGAGATGATCCCCTTCAA R-AGGGCTCCTGAGAGGTTTGT
Cyc D1	241bp	F-ACCTGGATGCTGGAGGTCT R-GCTCCATTTGCAGCAGCTC
PCNA	115bp	F-AGTGGAGAACTTGGAAATGGAA R-GAGACAGTGGAGTGGCTTTTGT

Table 1: Primers sequences (human) used for qRT gene expression analyses

3.4.5. Reverse-transcriptase polymerase chain reaction

To track differentiation of rat PBMNC derived NPC, expression of neural genes at defined periods of PBMNC culture was analysed using RT-PCR. One microgram of Trizol extracted RNA was converted to cDNA using the Superscript III reverse

transcriptase enzyme. Two fifty nanogram template cDNA (5µl) was mixed with 100pmol forward primer, 100pmol of reverse primer and 0.5µl of Taq polymerase (5 IU/µl, Invitrogen, USA) to form a total volume of 25µl. Reverse transcriptase (RT) -PCR was carried out for forty cycles of reaction using the Master cycler (Eppendorf Hamburg, Germany). GAPDH was the housekeeping gene used. The molecular size of products obtained was verified using agarose gel electrophoresis (AGE). The rat primer sequences are included in the table 2.

Genes	Amplicon size	Primer sequence (5'-3')
GAPDH	210bp	F- GGCACAGTCAAGGCTGAGAATG R- ATGGTGGTGAAGACGCCAGTA
Nes	200bp	F- GGATGGGGACGAGGATCAAG R- TTCCCCTGAGGACCAGGAAT
β-III tub	148bp	F- GGGAGATCGTGCACATCCAG R- CCGAGTCCCCCACATAGTTG
Syn	218bp	F- GTCAAAGGGGGCACTACCAA R- ACGGCTGTAGCCAGAAAGTC

Table 2: Primers sequences (rat) used for gene expression analyses

3.5. *In vivo* evaluation

3.5.1. Allocation of animals

All experiments conformed to the guidelines issued by the CPCSEA committee and approved by the Institutional Animal Ethics Committee (IAEC). Sixty adult female inbred strains of Wistar rats (body weight: 250-300 g each) obtained from Division of Laboratory Animal Sciences, SCTIMST, India were used for the experiment. Few of the approved animals were used exclusively as blood donors. The animals were kept under standardized animal care conditions and were given

free access to food and water throughout the study. Experimental animals were randomly assigned to one of the following four groups before operation with at least 6 animals included in each group (i) Test [denoted as 'T' corresponds to cells transplanted in fibrin]; (ii) Control [denoted as 'C' corresponds to cells transplanted in medium]. Additional groups of control in which (iii) only fibrin [denoted as 'CF' corresponds to control fibrin] or (iv) only medium ['CM' corresponds to control medium] applied at the SCI by injection were included. A sham group with laminectomy alone but no injury was also included in the study. The distribution of animals in different groups is indicated in table 3.

Group I	Test (T)	Cells transplanted in fibrin matrix
Group II	Control 1 (C)	Cells transplanted in medium
Group III	Control 2 (CF)	Fibrin matrix only
Group IV	Control 3 (CM)	Medium only

Table 3. Allocation of animals for *in vivo* studies

3.5.2. Preparation and labelling of NPCs for transplantation

The rat NPCs on 5^{th} day of *in vitro* culture on fibrin composite matrix were harvested as they are shown to be immature neurons with β III-tubulin expression. About 10^4 cells/animal were used for the experiment. Prior to transplantation, the cells isolated on day6 of culture were labelled with fluorescent membrane-intercalating dye PKH26 (red fluorescence, Sigma) according to manufacturer's instructions. Briefly, the cells were harvested by enzyme-free flushing out in culture medium with a final wash in serum free medium. The cells were then

suspended in the given diluent (0.5ml), mixed with the dye solution in diluent (0.5ml), and incubated for 5min at RT with periodic mixing in dark. After stopping the reaction by adding 1ml of serum to the cell suspension with dye for 1min, which will bind the excess dye, the suspension was centrifuged at 400*g*, for 10min at 22^{o}C. The cells were then washed with 10% serum containing media twice to remove the unbound dye and cells were used for transplantation.

3.5.3. Creation of SCI model and transplantation

The rats were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg, intra peritoneal route). The animal's back was clipped and sterilized with antiseptic betadine. Laminectomies were then performed at L1 position after making an incision and clearing off the paravertebral muscles from T12 - L3 region. All the spinal contusions were induced by an impactor that is standardized to create ~ 150-kdyne force. The animals were evaluated for the development of SCI by assessing the BBB locomotor score and other clinical symptoms of SCI, on the day the injury was given.

Immediately after creating the SCI, transplantation to acute injury was carried out. About 10^4 cells suspended in fibrinogen were injected along with thrombin at the site of injury for test animals. Fibrinogen was used at a concentration of 20mg/ml and thrombin at a concentration of 1IU/ml to form a clot. In C, 10^4 cells suspended in media was applied; in CF and CM either fibrin alone or medium alone, respectively, was applied. About 15µl liquid (either control or test) was injected in all animals using a Hamilton syringe. All procedures were carried out with the help of a dissection microscope. The postoperative care procedures

involved administration of antibiotics as well as painkillers and manual emptying of bladder was done twice a day during the course of experiment.

3.5.4. Tracking of transplanted cells

3.5.4.1. Imaging of gross tissue

The SCI tissue was collected in 4% paraformaldehyde after trans-cardiac perfusion fixation with the fixative, 8 days post transplantation. The tissues are then washed in PBS and used for imaging (IVIS Spectrum Preclinical *in vivo* imaging system, Perkin Elmer, USA). The injury site in all treatment groups were imaged simultaneously under similar conditions. The excitation wavelength used was 535nm and the emission was measured at 580nm. NPC retainment in the lesion site was assessed by fluorescent imaging of the explanted tissue area.

3.5.4.2. Histochemical staining for assessing the injury site

The excised SCI tissues were fixed for 72h in paraformaldehyde and were processed for paraffin embedding using standard histology technique. Briefly, the formaldehyde fixed sections were processed in 70% alcohol overnight, followed by three changes of acetone (20min each), two changes of xylene(10 and 15min respectively) and two changes of paraffin wax (60min each). Then the tissues were embedded in paraffin wax. Transverse tissue sections were obtained at ~5μm thickness using a microtome (Leica RM 2255, Germany). The sections were heated in hot air oven for 60min, de-paraffinised with xylene (3changes, 15min each), rehydrated in descending grades of isopropanol series (100% for 3min, 95% for 3min, 80% for 3min and 70% for 3min), washed in tap water for 5 min and stained. Different sections from each animal tissue were stained using Harris's hematoxylin and eosin (H&E), Toluidine blue (TB), periodic acid-Schiff-Alcian

blue (PAS-AB) and Phospho Tungstic Acid-Hematoxylin (PTAH). All the stains and chemicals used for staining were purchased from Sigma Chemicals, USA.

For H&E staining, the rehydrated sections were submerged in Harris's hematoxylin for 15min, washed in tap water for 5 min, differentiated in 1% acid-alcohol (1-2 fast dip) and blued with 0.2% ammonia water for 1min. The slides are then washed with tap water for 5min, counterstained with 1% eosin Y for 5min; dehydrated in isopropanol (95% for 5min, 100% ethanol for 5min-2 changes); cleared in xylene (3 changes, 15min each); mounted in DPX and viewed under microscope.

For TB staining, the rehydrated sections were treated with TB stain for 45s, washed in tap water, dehydrated in 95% alcohol, cleared in xylene, mounted in DPX and viewed.

For PAS-AB staining, the hydrated sections were treated with 1% periodic acid for 10min, washed in distilled water and kept in Schiff's solution for 30min. After washing thoroughly in running tap water, the sections were counter stained with Alcian blue solution for 30min, washed in running tap water for 2min, dehydrated, cleared and mounted.

For PTAH staining, the hydrated sections were placed in acid dichromate solution for 30min, washed in tap water, treated with acid permanganate solution for 1min and again washed in tap water. The sections were then bleached in 1% oxalic acid, rinsed in tap water and stained with Mallory's PTAH stain overnight. The sections were then dehydrated through graded alcohols, cleared mounted and viewed under microscope (Leica Microsystems, DMIRB, Germany).

3.5.4.3. Immunohistochemical tracking of transplanted cells

After fluorescent imaging, some of the SCI specimens were post-fixed in 4% paraformaldehyde solution overnight. The cords were then cryoprotected in 30% sucrose solution in PBS for making frozen sections. The spinal cord was then embedded in tissue-freezing media (Jung, Leica Microsystems, Germany) and cut into10µm sagittal sections using a cryostat (Leica, Germany). The sections were then washed in PBS and permeated with 0.1% Triton X-100 for 5 min. After washing in PBS, the sections were blocked with 5% bovine serum albumin (BSA, Sigma) and incubated with primary antibodies against neurons (β-III tub, 1:100, Epitomics, an Abcam company, Burlingame, CA, USA) and functional neuronal marker synaptophysin (Syn, 1:100, R&D systems, USA). The corresponding Alexa Fluor 488 conjugated secondary antibodies from Abcam (Cambridge Science Park, Cambridge, UK) were used to develop the antigens. To identify the lineage commitment of transplanted NPCs, dual-labelled cells (PKH26 and neuronal marker) were analyzed. The nuclear stain used was DAPI. Fluorescence intensity of PKH was quantified using Image J software from at least 5 consecutive 10µm sections per animal around the transplanted area imaged at 20x, each section with a length of 28128µm and breadth of 30769µm.

The macrophage marker ED-2 was stained to identify their infiltration and distribution in the injured area. The paraffin-embedded sections were deparaffinised and rehydrated using reducing grades of ethanol followed by distilled water rinse. The tissue sections were then incubated with antigen-retrieval buffer (citrate buffer - pH.6) at 95^{o}C, for 20min. Then the peroxidase activity of the tissue was blocked by addition of 20% hydrogen peroxide for

20min and washed two times in phosphate buffered saline. The sections were then incubated with 5% FBS in PBST for 30min to block non-specific sites. The primary antibody, ED-2 (mouse monoclonal, macrophages marker, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added for 1h at RT and washed with PBST three times. Sections were incubated with appropriate HRP conjugated secondary antibodies (Abcam) for 1h and washed with PBST thrice. To develop the antibodies, DAB solution was added and the reaction was stopped by washing in water. The sections were then dehydrated, cleared in xylene, mounted in DPX and viewed under a microscope. All images were obtained using fluorescence microscope (Leica Microsystems, DMIRB, Germany).

3.6. Analysis of clinical outcome

Hind limb function was assessed from the day of injury till the last day of evaluation period using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995) in an open field which is non-slippery. In each testing session, the rats were observed for 4 minutes by two independent evaluators who were trained to score 0 (no observable movements) to 21 (normal gait). The evaluators were blinded about the group to which each animal belonged.

3.7. Statistical analysis

Statistical significance was calculated by two-way analysis of variance (ANOVA) for all quantitative data using GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA). One-way ANOVA followed by the Tukey-Kramer test was performed to determine the significance in variation between the control and test and to determine variations at different time points within the group. Mean values

and standard deviation (SD) were calculated for all parameters and are represented in graphical form. Significance is labelled in the graphs with '***' ($P < 0.0001$); '**' ($P < 0.01$); and '*' ($P < 0.05$). The number of replicate experiments carried out is indicated in the legends of each figure.

CHAPTER 4

4. RESULTS

This chapter illustrates study result which is divided into 4 main parts. The first part focuses standardisation of fibrin-based niche for NPC isolation and lineage commitment. The second part demonstrates the slow and steady differentiation of NPCs. The third part shows that Wnt signaling emanating from the matrix has a crucial role in the transformation of PBMNC derived NPC to neurons. The fourth and the final part show the application of NPC for transplantation purpose in rat spinal cord injury (SCI) model. Each stage involved systematic results of experiments which were done to optimize the conditions required for the best outcome, keeping in view that the results should have clinical use in the future. The goal of the study is to explore the possible use of autologous PBMNC- derived NLCs for SCI treatment, which can be extended to other patient specific NS injuries/diseases as well.

4.1. *In vitro* niche standardisation using human PBMNC

For successful cell transplantation, two major requirements are to obtain homogenous cells and obtain substantial numbers of cells. The primary experiments were to isolate NPC from a heterogeneous population of PBMNC. Also, because only very low numbers of NPC is available in peripheral circulation these cells need to proliferate *in vitro* to obtain sufficient numbers of cells for transplantation. In this study, the strategy used was to provide biomimetic niche to allow the NPCs to get selected and eliminate all other cell types. Therefore, standardisation of matrix and

media components that can select NPCs and promote proliferation and differentiation towards NLCs was carried out by changing matrix compositions, medium compositions and culture conditions. The experiments were based on the information from an earlier study which was already published from the same laboratory (Jose and Krishnan, 2010). Results of the subsequent modifications and standardization of the earlier findings are presented in the first part of the results.

4.1.1. Neuronal Induction by the optimised niche

The optimised niche favoured induction of neurons from PBMNC derived NPCs. When the partially attached cells collected from uncoated TCPS was seeded on to the FC matrix, groups of MAPCs other than lymphocytes and neutrophils were seen (**Figure 1 A**). A seeding density of $\sim 5\times10^5$ leukocytes cm^{-2} (counted by a hematology analyzer) was found to be optimum to result in colony formation and neural-like cell elongation. The cells attached and colonized on the fibrin-coated TCPS within an hour of seeding. Frequent media changes during the initial period resulted in the removal of nonspecific cells from the culture. By day 4, NLCs were found sprouting out from the colonies (**Figure** 1 **B**). By day 8, the cells further elongated (**Figure 1 C**) and by day16, the NLC density was maximum (**Figure 1 D**).

However, to obtain the optimized niche, the composition and concentration of various growth components had to be adjusted which is discussed in the coming section.

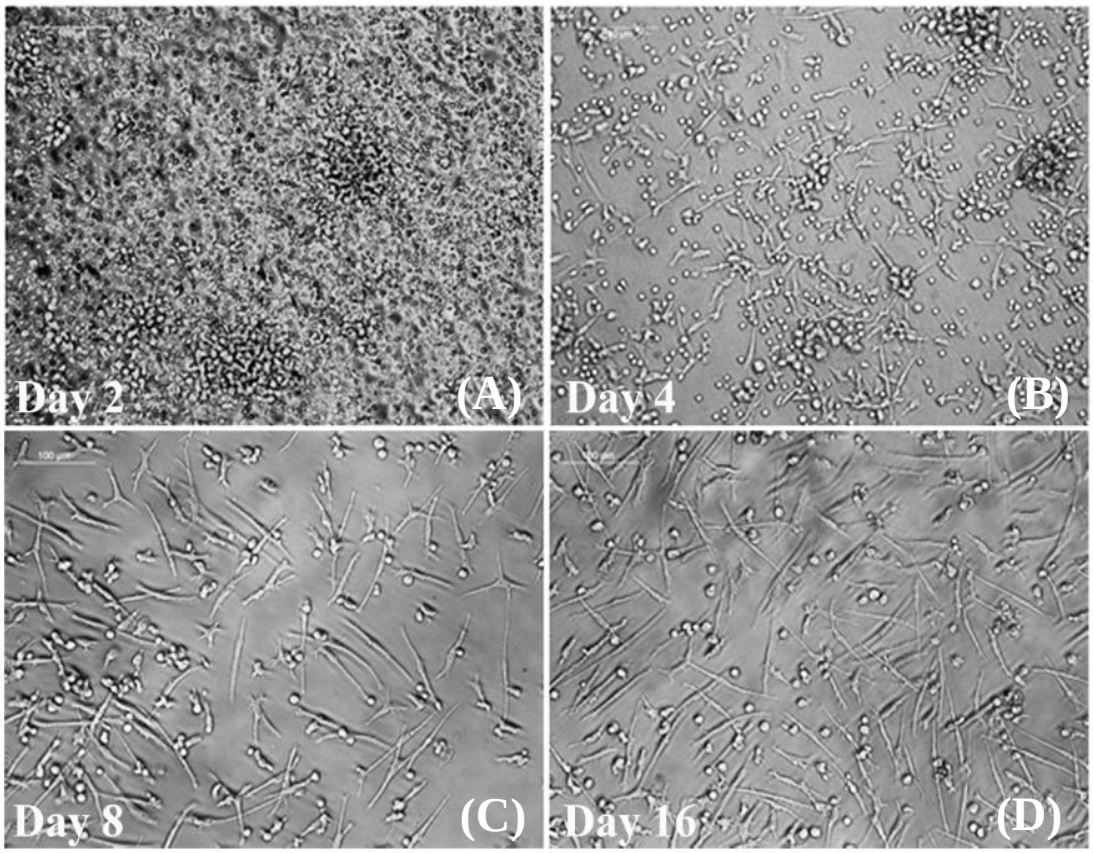


Figure 1. Illustration of neuronal induction from human PBMNC culture. (A) Appearance of cell colonies at 2nd day of culture (B) Micrograph showing sprouting out of neural cells from colonies by 4th day (C) Elimination of non-specific cells and elongation of neural cells by 8th day culture which was maintained till 16th day (D). Scale bar: 100 µm

4.1.2. Optimisation of matrix composition

The components in the matrix showed significant influence on the morphology of NLCs derived through PBMNC culture in the niche. Elongated cells were observed in most of the PBMNC culture wells; however, there was a marked difference between the different compositions and culture conditions. The media used in all cases was the CNIM. Elongated cells were seen in uncoated dishes (**Figure 2 A**) as well as in HA treated dishes (**Figure 2 B**), but with less neurite-like branching. With the progression of the culture period, these cells also exhibited apoptotic blebs and did not survive beyond eight days. Cells grown on Gel-TCPS had a mild tendency to

branch (**Figure 2 C**). The culture on the surface treated with HA+ Gel appeared healthier and showed better branching (**Figure 2 D**).

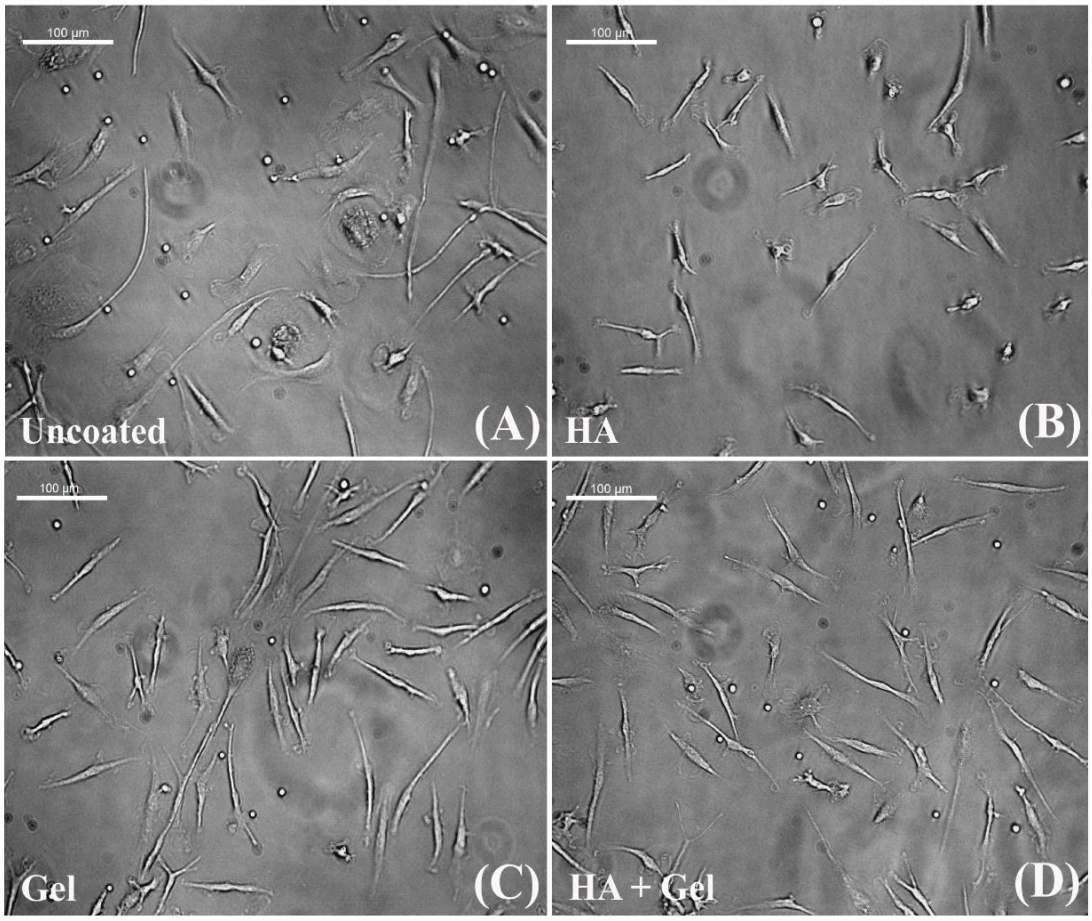


Figure 2. Effect of Gel and HA on PBMNC. Representative phase-contrast micrographs of cells on (A) Uncoated (B) HA-treated (C) Gel-treated (D) HA+Gel-treated TCPS. The images were acquired on sixth day of seeding and the magnification is indicated using scale bar (100 µm).

The cells with neural morphology were observed in the fibrin-coated surface (**Figure 3 A**). However, most of the cells were shorter. Macrophages like cells were present and the cell survival was poor. Incorporation of HA in the fibrin matrix increased the cell length and survival (**Figure 3 B**). With the addition of Gel into the fibrin, cell density was improved (**Figure 3 C**). Irrespective of the similar seeding density, the frequency of cells which exhibited neural-like morphology was more per field when

HA and Gel were added together with Fib (**Figure 3 D**). Cell density and cell-to-cell proximity improved when GFs were also included in the fibrin based matrix (**Figure 3 E**). Cellular extensions and cell-cell proximity improved considerably upon addition of KCl into the culture medium (**Figure 3 F**).

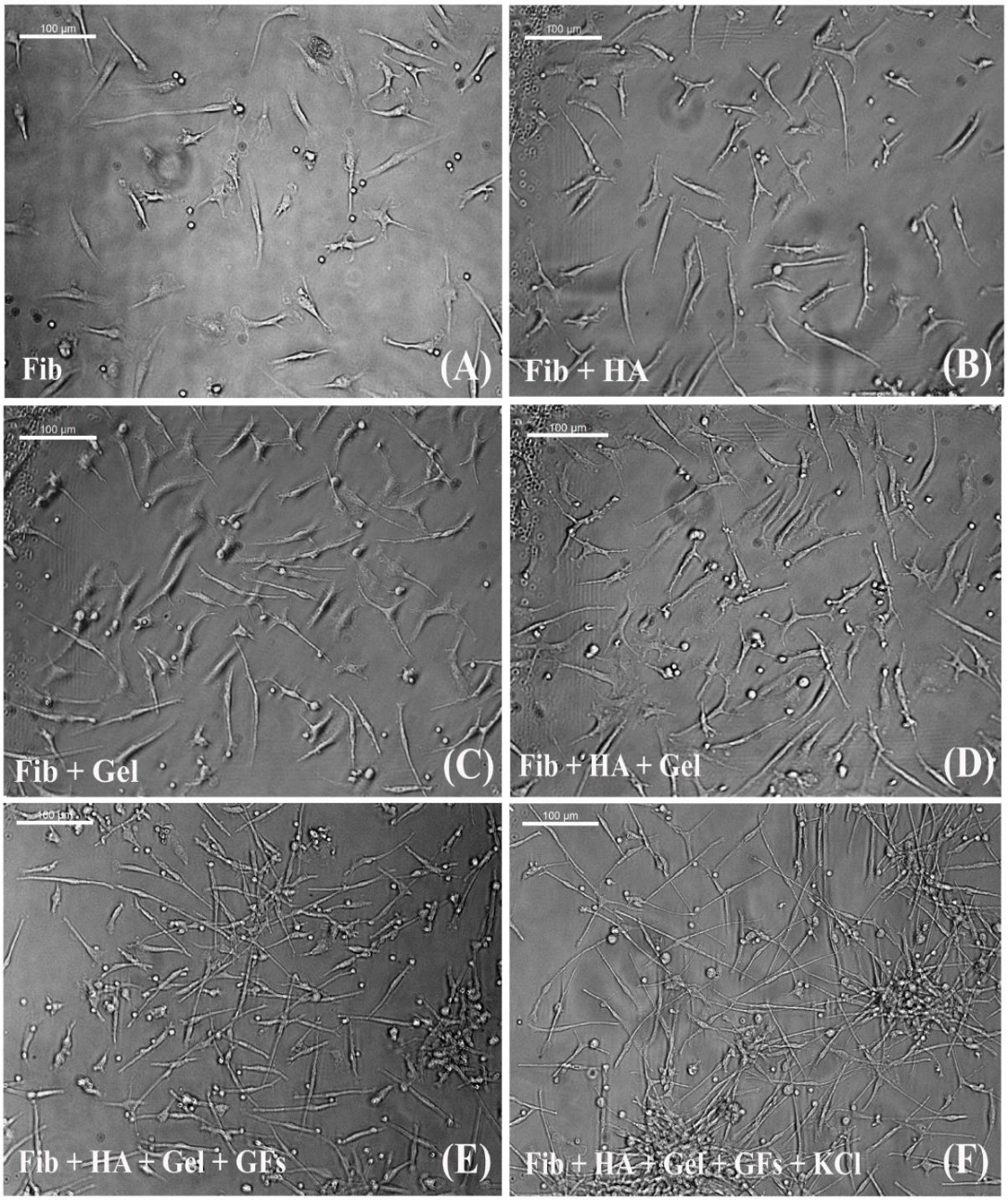


Figure 3. Influence of matrix constituents in fibrin composite. Representative micrographs of cells cultured on dishes coated with (A) Fib (B) Fib+ HA (C) Fib+Gel (D) Fib+HA+Gel (E) Fib+HA+Gel+GFs (F) Fib+HA+Gel+GFs+KCl. The magnification of images is indicated using scale bar (100 µm).

The most striking effects of various niche components on NLC properties are summarized in table 4.

Uncoated	Long and narrow cells, no neurite-like sprouting, short survival, round, macrophage-like cells present
HA	Long and narrow cells, no neurite-like sprouting, improved survival
Gel	Long cells, improvement of cell density, neurite-like sprouting in some cells
HA+Gel	Long cells, improvement of cell density & survival, neurite-like sprouting in more cells
Fib	Poor cell density, less cell survival, broader cell body, more branching, no cell-cell contact
Fib+ HA	Long cells, broader cell body, more branching, no cell-cell contact
Fib+Gel	Long cells, broader cell body, more branching, no cell-cell contact
Fib+HA+Gel	Long cells, broader cell body, more branching, no cell-cell contact
Fib+HA+Gel+ GFs	Longer cells, higher cell density, small outgrowth colonies, better cell-cell contact
Fib+HA+Gel+ GFs+KCl	Longest cells, higher cell density, larger and more frequent outgrowth colonies, very prominent cell-cell contact

Table 4: Summarised effect of various niche components on cell growth

Even though the fibrin coated surface seemed to induce NPCs in PBMNCs to form NLCs, a combined effect of different matrix components was found to increase cell numbers with better and stable neuron-like morphology.

The dose dependent effect of proteoglycans, chemical inducers, growth factors and glycosaminoglycans is tabularised in table 5 and table 6 respectively.

4.1.3. Effect of Proteoglycans and chemical inducers

The addition of certain proteoglycans (PGs) and chemical inducers caused no added effect on neural morphology upon differentiation, instead caused structural deterioration in some cases leading to cell death.

Molecules used	**Concentration**		**Effect**
	Matrix (µg/ml)	**Medium (µg/ml)**	
CSPG	0	-	Addition to matrix did not produce any change. Addition to medium was found toxic to cells. Hence excluded from the niche.
	25	+	
	50	+	
All-trans RA	-	1µM	No marked morphological change. However, glial cells seemed to be more. Excluded from niche. This indicates that the matrix directs differentiation of NPC without chemical stimulation.
	-	5	
	-	15	
cAMP	-	500µM	Retardation in neural cell elongation was observed in all concentrations used when added to matrix/medium. Therefore excluded from the niche.
	-	750µM	
	-	1mM	

Table 5: Effect of proteoglycans and chemical inducers on neuronal differentiation

4.1.4. Optimisation of GFs and GAGs

Inclusion of GFs and GAGs were found to be essential for the neuronal differentiation. The effect of different concentrations was looked at and the best concentration selected.

Molecules used	**Concentration**		**Effect**
	Matrix (µg/ml)	**Medium (10µg/ml)**	
HE	0	+10ug	Inclusion of HE was found to be important for optimum neuronal growth. Best neural growth was observed with 100µg/ml concentration and hence selected that.
	50	+	
	100	+	
	250	+	
PGF	0	+(1ug)	PGF was found to be essential for desirable differentiation towards neurons. However, there was no observable difference and hence selected the least concentration.
	10	+	
	50	+	
	100	+	
HA	5	+	There was no observable difference in neural morphology or marker expression. Since the cell survival was more, 100µg/ml concentration was selected for further studies.
	50	+	
	100	+	

Table 6: Effect of growth factors and glycosaminoglycans on neuronal differentiation

Based on these observations, we came to the final composition of matrix and media with which all further studies were carried out.

4.1.5. Tracking of lineage commitment of NPCs with the optimised niche

The niche favoured isolation of neural progenitor cells during the early days of culture. The cells expressed the progenitor marker nestin (**Figure 4 A**) which confirmed the presence of progenitor cells in circulation. The cells also possessed the proliferation potential as seen with the expression of Ki 67 (**Figure 4 B)**. The isolated NPCs also had the ability to differentiate in to immature and mature neurons which was confirmed by the expression of specific markers of lineage progressions, β-III tub (**Figure 4 C**) and MAP-2 (**Figure 4 D**) respectively.

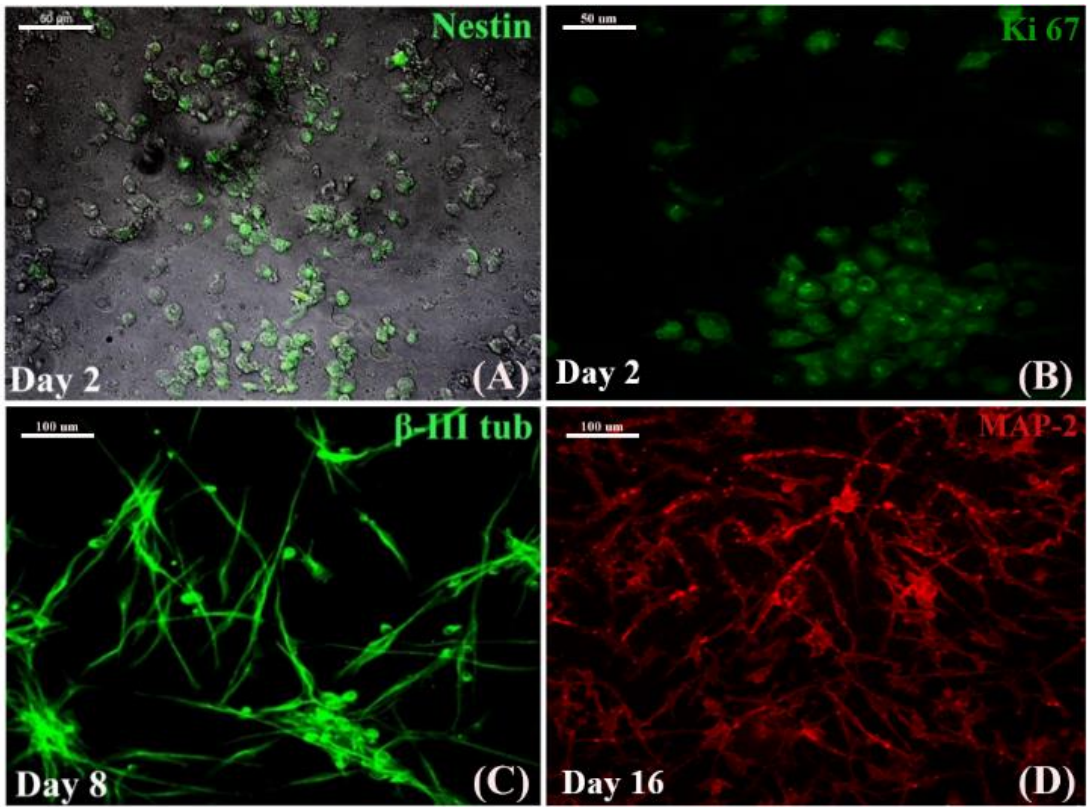


Figure 4. Evidence for induction of PBMNC derived NPCs to neural lineage. (A) Immunofluorescent micrograph of nestin-stained cells on 2d of seeding on FC dishes (B) Immunofluorescent image of Ki 67 displaying proliferation potential of the isolated NPCs (C) Immunofluorescent micrograph of β-III tub^{+} cells on 8d of culture demonstrating commitment towards neural lineage (D) Fluorescent image of MAP-2^{+} cells showing further differentiation and maturation. Magnification of each image is marked using scale bar- 50μm for A & B and 100μm for C & D.

4.1.6. Identification of key molecules in the matrix composite

It was demonstrated that the proteins and GFs, which are known to play a role in neuronal differentiation of stem cells, are present in various cocktails used such as fibrinogen concentrate, HE and PGF. Western blot analysis (**Figure 5 A**) confirmed the presence of FGF, BDNF and VEGF in the HE/PGF cocktail, used for the preparation of FC. All three tested GFs were clearly detected in HE. The specific band corresponding to FGF was prominent in the PGF cocktail as compared to BDNF and VEGF bands. The presence of FN, La and Fib in the fibrinogen concentrate used for matrix construction was confirmed by the precipitin lines formed against anti-FN, anti-Fib and anti-La (**Figure 5 B**).

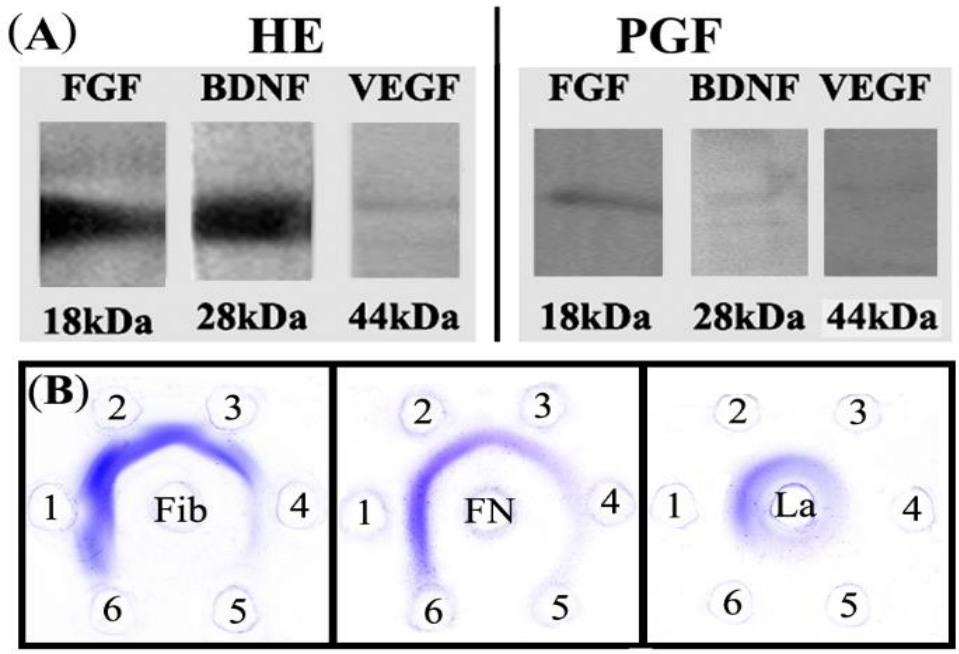


Figure 5. Identification of important constituents in fibrin composite. (A) Identification of important GFs in HE and PGF cocktails using Western blot analysis. Lanes are marked for FGF (18 kDa), BDNF (28 kDa) and VEGF (44 kDa) (B) Identification of Fib, FN and La in fibrinogen concentrate using immunodiffusion. Concentrations of total protein added in wells were (1) 2 mg; (2) 1.6 mg, (3) 1.2 mg; (4) 0.8 mg, (5) 0.4 mg, (6) 2 mg BSA (negative control). Precipitin lines formed with each specific antibody confirm the presence of Fib, FN and La in cryoprecipitate. No precipitin line is formed against/around BSA (negative control).

4.2. Regulated differentiation of NPCs to NLCs

It is crucial to use proliferating and immature neurons for transplantation to achieve a successful outcome. Only a gradual differentiation program can enable the collection of immature neural progenitors from *in vitro* culture. Successful *in vitro* differentiation should be a slow and steady process to enable the harvest of cells at the required level of lineage commitment, which is achieved with our optimised niche.

4.2.1. Indicators of regulated differentiation

The differentiation of NPC to NLC was found to be a slow and steady process. Cells positive for the specific neural marker, nestin was estimated to be ~1 to 9% of the partially attached PBMNC population, i.e., the cells before seeding onto the FC matrix (**Figure 6 A**). As the differentiation of NPC to NLC progressed, the percentage of β-III tub+ cells increased and it was maximal on 8d. The nestin+ cells were not detectable by this time at either transcriptional or translational levels. A significant increase in β-III tub+ cells ($P < 0.0001$) was observed with the progression of the culture period, i.e. from 18% on 4d to ~95% on 8d. The histogram shift of β-III tub+ cells towards the right on 8d indicated strong expression of antigen on the progenitors (**Figure 6 B**). The β-III tub expression was reduced to ~67% on 16d and further to ~27% on 24d (**Figure 6 B**). By 16d, the fluorescence intensity of β-III tub+ cells diminished; by 24d, the signal was further lowered in most of the cells. The histogram shift signifying MAP-2 expression was seen on 16d (**Figure**s **6 C** and **D**). This indicates neuronal maturation. Expression of MAP-2 by a significant number of cells (~34%) and a simultaneous reduction in the β-III tub+ cells on 16d of

culture confirms differentiation of PBMNC derived lineage committed NPCs to mature NLCs. The addition of KCl made no significant difference to the β-III tub/MAP-2 expression profiles. By 24d, expression of both markers reduced. Other specific markers for glial cells, such as GFAP, were negligible and O4 was ~10% positive in flow cytometry.

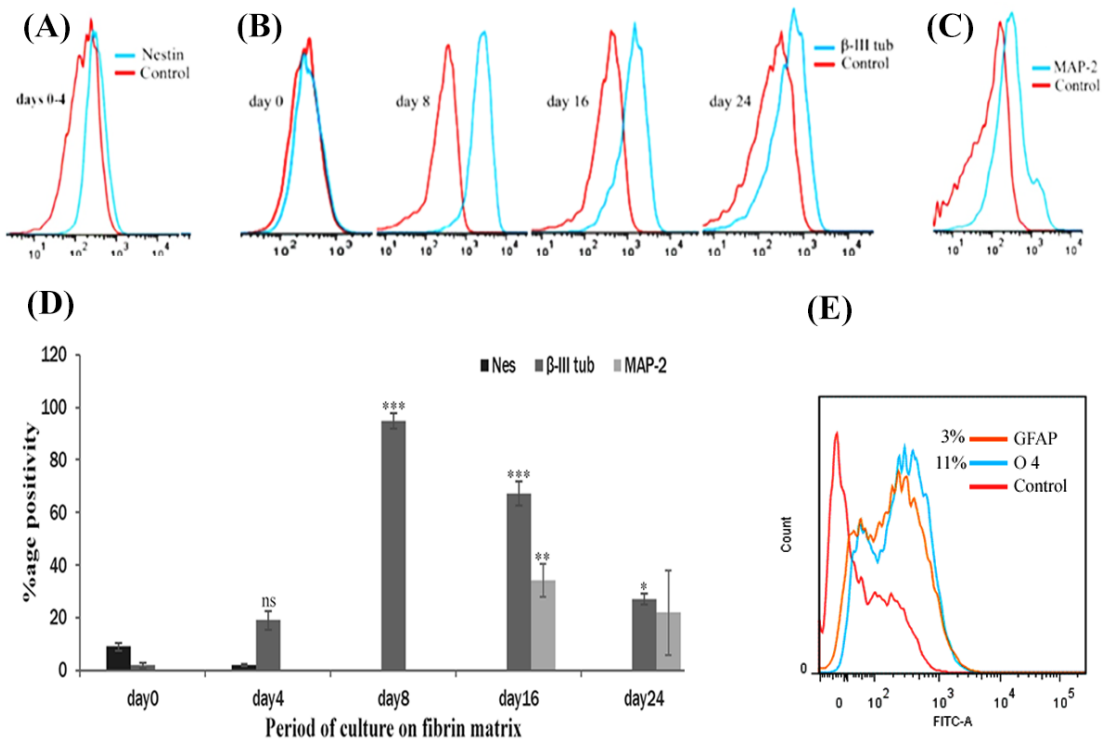


Figure 6. Estimation of neural markers during specific culture periods. (A) Flow cytometry histogram shift of nestin+ cells as compared to unstained (control) on 0d of culture, presenting the few positive cells that are present in the PBMNC fraction (B) Histogram demonstrating progressive shift in β-III tub+ cells as compared to control with progression of culture from 0d to 24d of culture (C) Histogram of MAP-2+ cells on 16d of culture showing shift towards right as compared to control, indicating positive cells on 16d (D) Compiled data of markers at various culture points is represented graphically. The statistical analysis demonstrates a significant difference for both β-III tub and MAP-2 upregulation at different time periods ($P < 0.0001$). Dynamic changes in the expression of differentiation markers was observed with nestin expression during the initial days, maximum β-III tub+ signal by 8d which reduced from 16d. MAP-2 signal was detectable by 16d indicating appearance of mature neurons in culture (E) Histogram shift indicating expression of GFAP and O4. Data is presented as average ± SD. '***' ($P < 0.0001$), '**' ($P < 0.01$) and '*' ($P < 0.05$).

4.2.2. Effect of added KCl in the niche on neuronal morphology

Other parameters analysed in the presence of excess KCl (test) and the normal medium without added KCl (control) clearly demonstrated influence of the electrolyte in signaling (**Figure 7 C** versus **7 A**). Improved colony formation (**Figure 7 C**), neural extensions and cell-cell proximity (**Figure 7 D**) were visible in the KCl^+ cultures until 16d. In the control, neuron-like cell lengthening was low compared to that in test cultures (**Figure 7 B** versus **7 D**). Quantitatively, estimated cell length is presented to compare the influence of KCl in the niche (**Figure 7 E**). A significant increase in cell length ($P < 0.0001$) was observed 3d after the addition of KCl into the medium (8d of culture) as compared to control cultures. The cell length was ~51μm on 4d i.e. before KCl addition. The length increased to ~147μm in the control and ~283μm in the test on 8d, the difference exhibited by the KCl^+ cells being almost double. This increase was steady until 16d. By 24d in both test and control cultures, the cell length decreased to ~100μm.

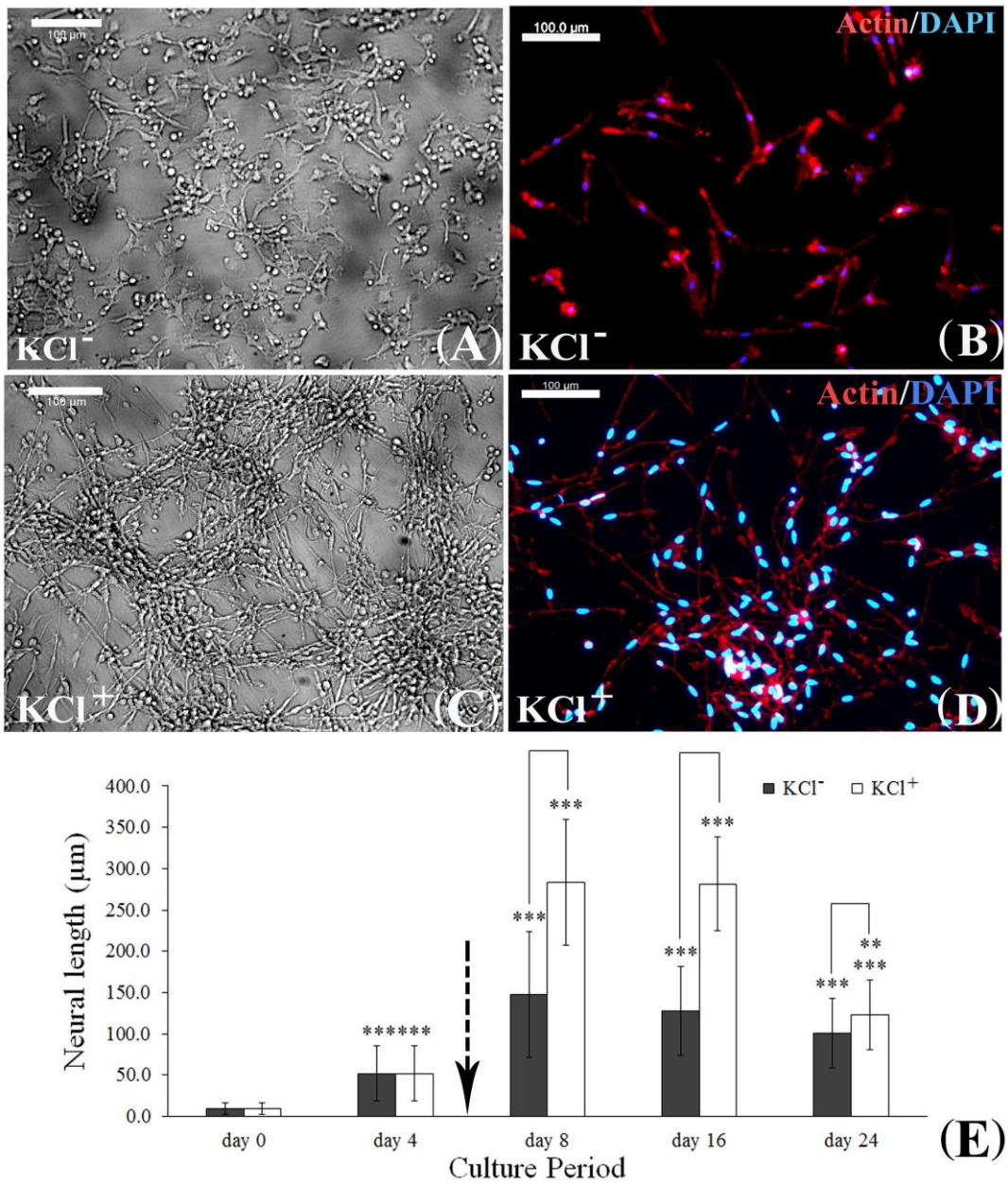


Figure 7. Effect of excess KCl on NLC length. (A) and (C) Phase contrast micrograph showing morphology and distribution of cells on 8d in $KCl^{-}$and KCl^{+} culture. Dotted arrow indicates the period of KCl addition (B) and (D) Fluorescent micrograph of Actin/DAPI stained KCl−and KCl^{+} cultures showing improved colonies and cellular contacts in KCl^{+} cultures (E) Quantitative data on cell length showing a significant difference in cell length between $KCl^{-}$and KCl^{+} cultures at definite periods of culture ($P < 0.0001$). To analyse the variation at each point, the progressive cell length on each day was compared with that on 0d; i.e. cell length of PBMNC on the day of seeding. The effect of added KCl was studied by comparing the length of the cell without and with KCl added cultures on each day of analysis. High statistical significance is noticeable. ***indicates $P < 0.0001$ and **indicates $P < 0.01$. Magnification used for calculation was 20x for all images.

4.2.3. Effect of niche on NLC proliferation and survival

The proliferation potential of cells upon culture on the FC matrix was found to be promising (**Figure 8 A**). Increase in colony size and cell density was evident under the microscope and the proliferation quantified by flow cytometry showed the highest percentage of Ki67+ cells on 8d. An average of 42% of cells was Ki67+ on 0d; the elimination of nonspecific cells by medium change resulted in 73% of proliferating cells by 4d. The proliferating cells increased to ~90% on 8d. During the 0d–8d period, mature neuronal markers were sparingly detected, suggesting the progenitor status of cells with proliferation potential. The proportion of proliferating cells decreased to ~76% by 16d and it further declined to ~36% on 24d. The presence of excess KCl in the medium did not influence proliferation marker expression.

Deterioration in the neural morphology was noticed by 24d, resulting in a decrease in cell length and their flattening (**Figure**s **8 B & C**), which occurred regardless of KCl addition. Cell-cell contact was also diminished significantly. This untoward change in the morphology was also accompanied by apoptosis, which was confirmed using the Annexin V binding assay. More cell death by apoptosis was evident on 24d of culture as compared to 16d (**Figures 8 D and E**). Increased fluorescence intensity resulting in the histogram shift to the right was evident, more conspicuously in KCl^{+} cultures.

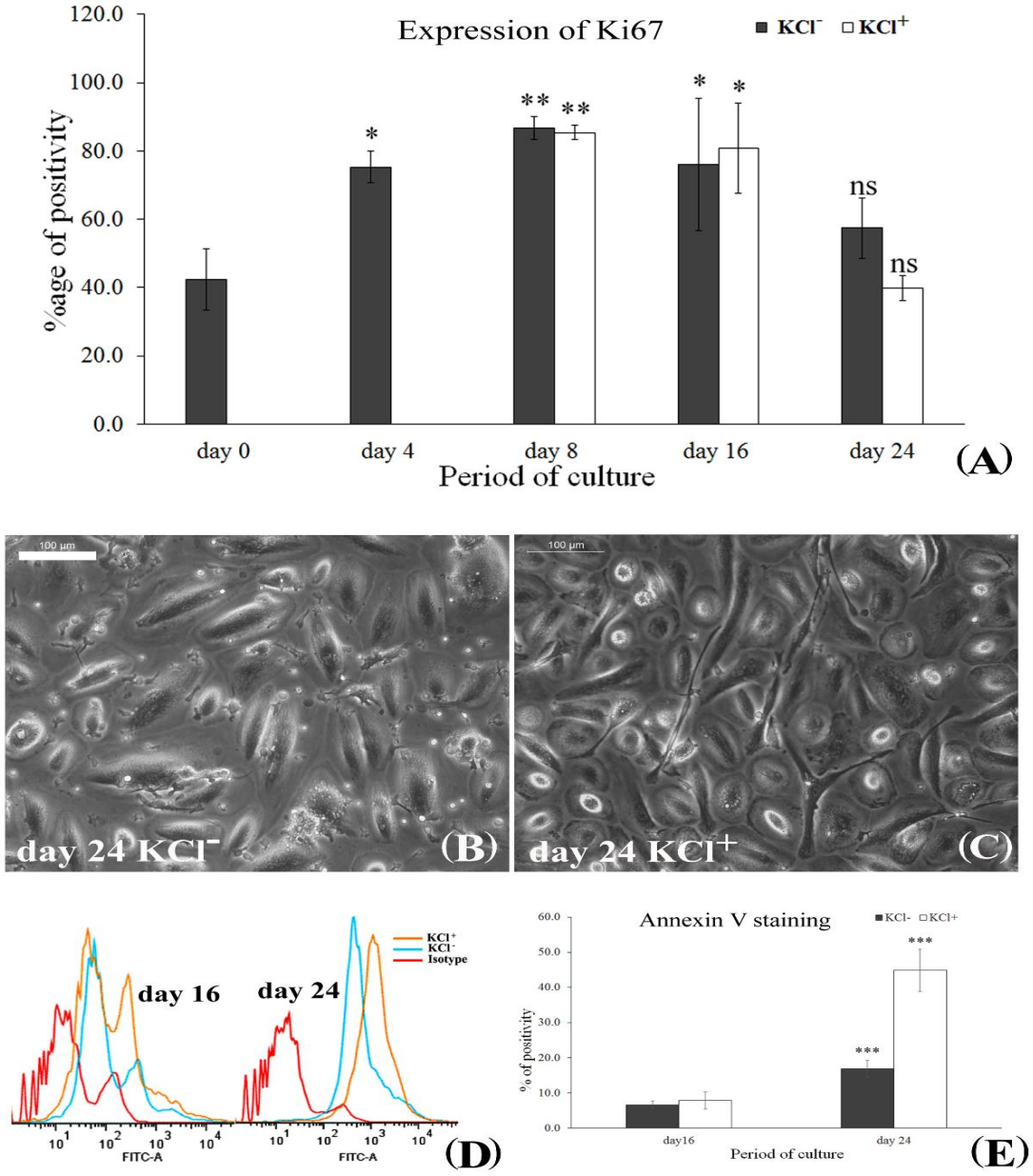


Figure 8. Proliferation and apoptosis of PBMNC in culture. (A) Proliferation profile estimated using Ki67 indicates significant difference in proliferation ($P < 0.0001$) at different culture periods. However, there was no effect on proliferation due to the addition of KCl to the niche (B&C) Phase contrast images of NPC cultures on 24d exhibiting flattened cells without neural morphology, in the absence and presence of excess KCl (D) Histogram of apoptotic cells on 24d as compared to 16d, presenting Annexin bright+ cells in KCl^+ and Annexin dim+ cells in KCl^-cultures (E) Compiled graphical representation of apoptosis (average ± S.D) demonstrates a significant increase in cell death on 24d of culture as compared to 16d, and is significantly high in KCl^+ cultures ($P < 0.0001$). Magnification is indicated using scale bar (100µm). '***'($P < 0.0001$), '**' ($P < 0.01$) and '*' ($P < 0.05$).

4.2.4. Expression of functional proteins

The genomic stability of cells is evident from GAPDH expression throughout the culture and is presented in **Figure 9 A**. Cell-cell contact was more evident in KCl^+ cultures. Concomitantly, weak expression of Syn gene was detected on 8d which increased by 16d; the expression decreased by 24d (**Figure 9 B**). The Syn expression at mRNA and protein levels were found to be significant in KCl^+ cultures, suggesting the possibility of the differentiated NLCs undergoing vesicle formation and synaptic transmission. The qPCR demonstrates maximum Syn gene expression on 16d of culture in the matrix with respect to 0d; there was nearly a 75-fold upregulation of Syn gene expression on 16d in KCl^+ cultures as compared to a ~5-fold increase in KCl^-cultures (**Figure 9 C**). In the flow cytometry histogram, the signal was found to be brighter for cells from KCl^+ cultures as compared to the diminished signals in cells from the KCl^-culture (**Figure 9 D**). Significant numbers of cells (~55%) were positive for Syn on 16d in the KCl^+ cultures as compared to ~32% in the KCl^-group, which is an important observation (**Figure 9 E**). Typical granular and string-like immunofluorescence of Syn was observed along the long axon-like structure of cells in KCl^+ culture (**Figure 9 F**); microscopically, the immunofluorescence of granular structure was not distinct in the control (not shown). By 24d, Syn expression decreased in both test and control cultures.

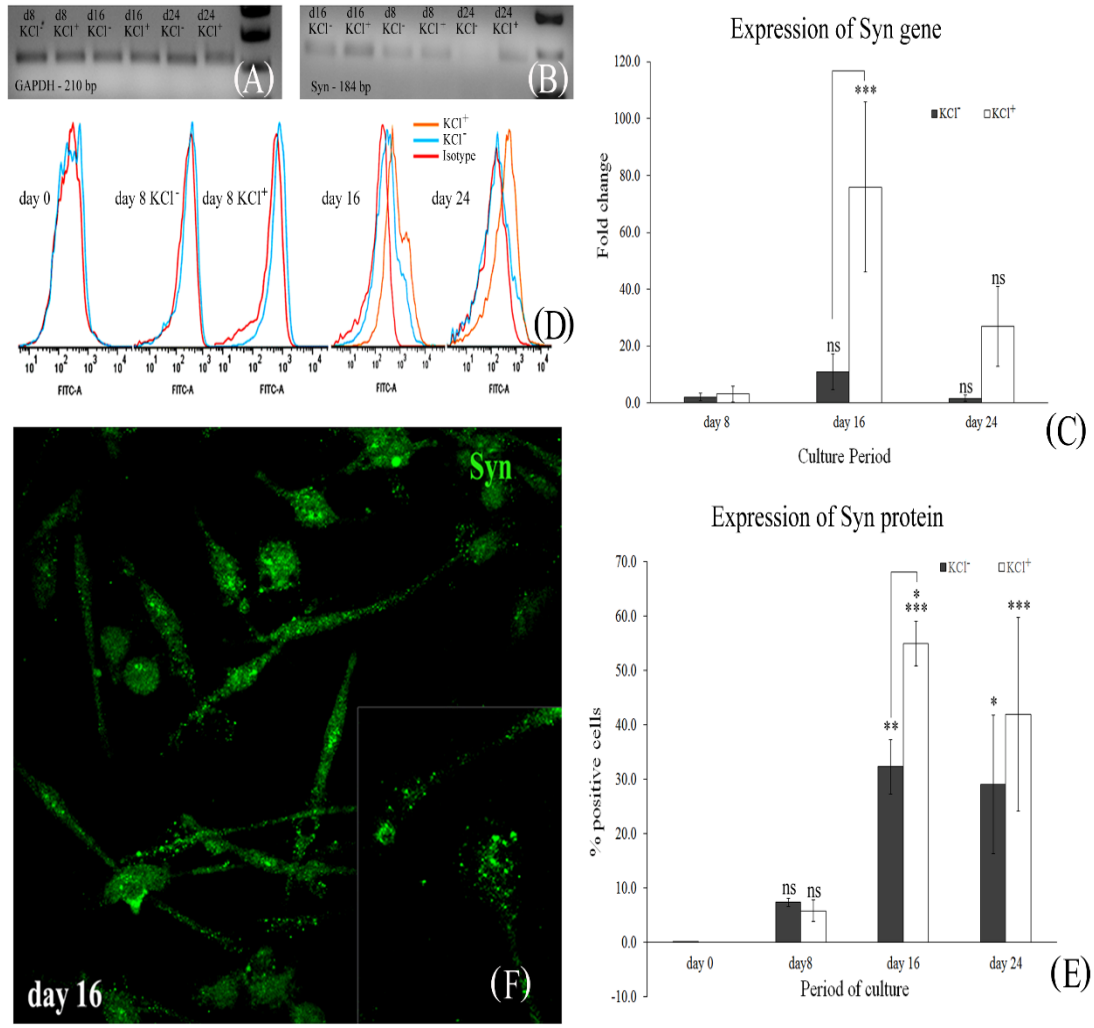


Figure 9. Expression of synaptophysin by PBMNC derived NLCs. (A&B) AGE of qRT-PCR product of GAPDH and Syn, respectively, on different days of PBMNC culture, showing an increased expression of Syn on 16d as compared to 8d (C) Quantitative data of mRNA expression during culture showing difference in the Syn gene expression upon addition of KCl ($P < 0.05$). Addition of KCl upregulated the expression of Syn. The profile of expression was 16d > 8d > 0d (D) Histogram shift of fluorescence intensity as compared to unstained control in flow cytometry, indicating maximum Syn bright+ cells on 16d in KCl^+ culture and Syn dim+ cells in KCl^-culture (E) Compiled data on quantification of Syn by flow cytometry is represented in graphical format which displayed significantly higher positive cells ($P < 0.0001$) when compared between KCl^+ and KCl^-groups ($P < 0.05$) and among culture periods. Maximum expression was on 16d in the former (F) Immunofluorescence of NLCs on 16d of culture, demonstrating granular appearance of Syn in cytoplasm. Magnification of each image is marked using the scale bar (50 µm); insert is a region of interest magnified three times to illustrate granular Syn. '***' ($P < 0.0001$), '**' ($P < 0.01$), and '*' ($P < 0.05$).

Expression of TH, which is a key enzyme involved in the dopamine synthesis pathway, was also expressed at the mRNA level (**Figure 10 A**). The flow cytometry histogram indicated TH strong+ cells in KCl^{+} cultures (**Figure 10 B**). Quantitative data of the FACS analysis revealed ~85% TH+ cells in KCl^{+} cultures, both on 8d and 16d, as compared to 25–30% in KCl^{-}cultures (**Figure 10 C**). Microscopically, the axon-like elongated structure was immunofluorescent against TH in KCl^{+} cultures, but not in the KCl^{-}control (**Figure 10 D**). Thus, the engineered niche directed differentiation of circulating NPC to NLCs, which also expressed functional proteins.

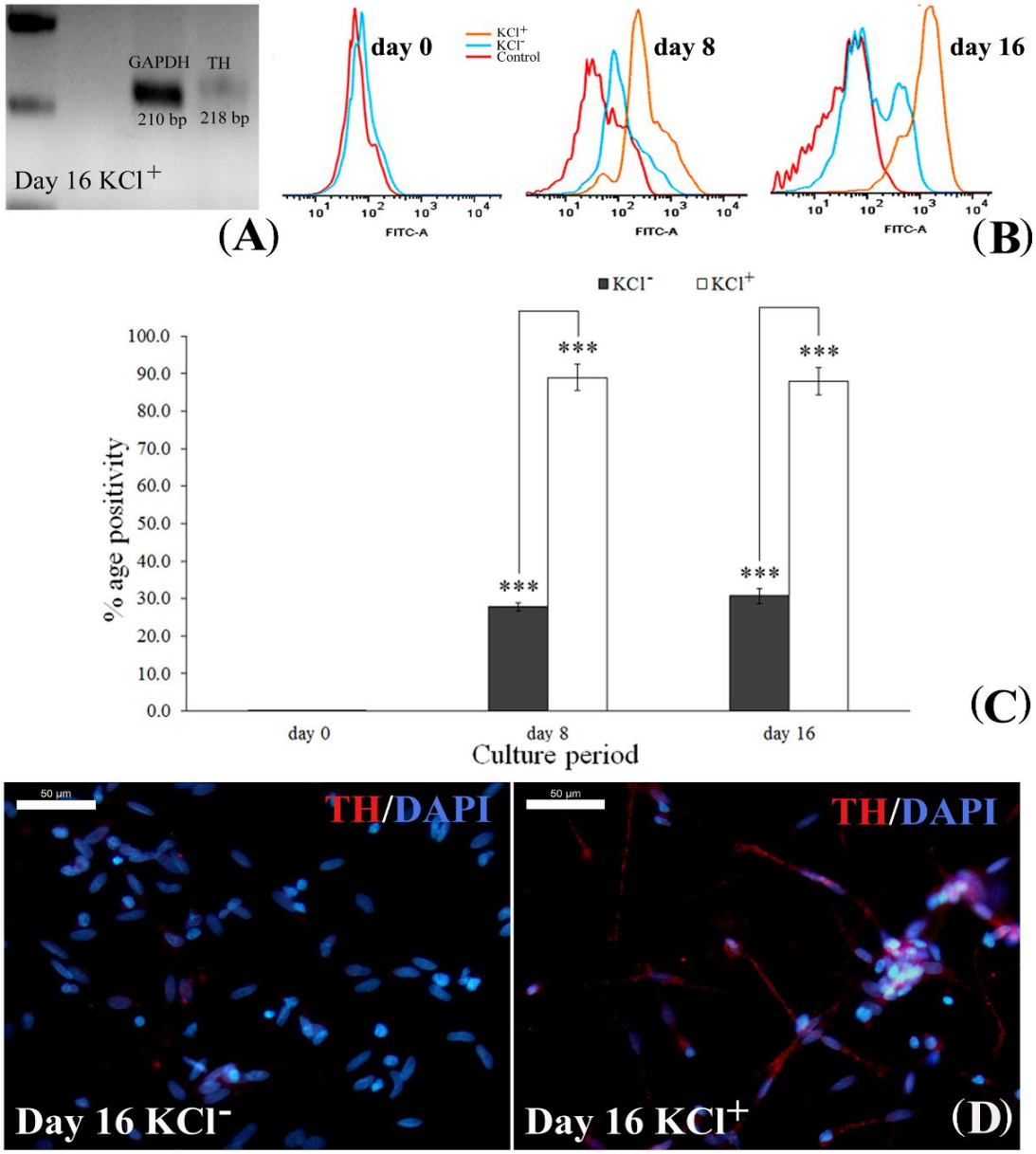


Figure 10. Tyrosine hydroxylase expression in NLCs. (A) AGE of RT-PCR amplified products on 16d of NPC culture on FC (amplicon size given in table 1) (B) Histogram shift of fluorescence intensity in flow cytometry indicating maximum TH bright+ cells on 16d in KCl^+ culture and TH dim+ cells in KCl^-culture (C) Compiled FACS analysis data represented graphically revealed significant difference in TH expression when compared between KCl^+ and KCl^-groups and at different periods of culture ($P < 0.0001$). Greater numbers of TH+ cells were found in KCl^+ cultures as compared to KCl^-cultures (D) Immunofluorescence of cells grown in the FC matrix for 16d in $KCl^-$and KCl^+ media, respectively. Neural extensions were stained conspicuously for TH in KCl^+ cultures as compared to feebly stained cells in KCl^-ones. Magnification is indicated by the scale bar (50µm). '***' ($P < 0.0001$), '**' ($P < 0.01$) and '*' ($P < 0.05$).

Altogether, the transition of neural progenitors from circulation into neuron-like cells was a controlled process. The molecular and cellular changes during the differentiation process are summarized in **Figure 11**.

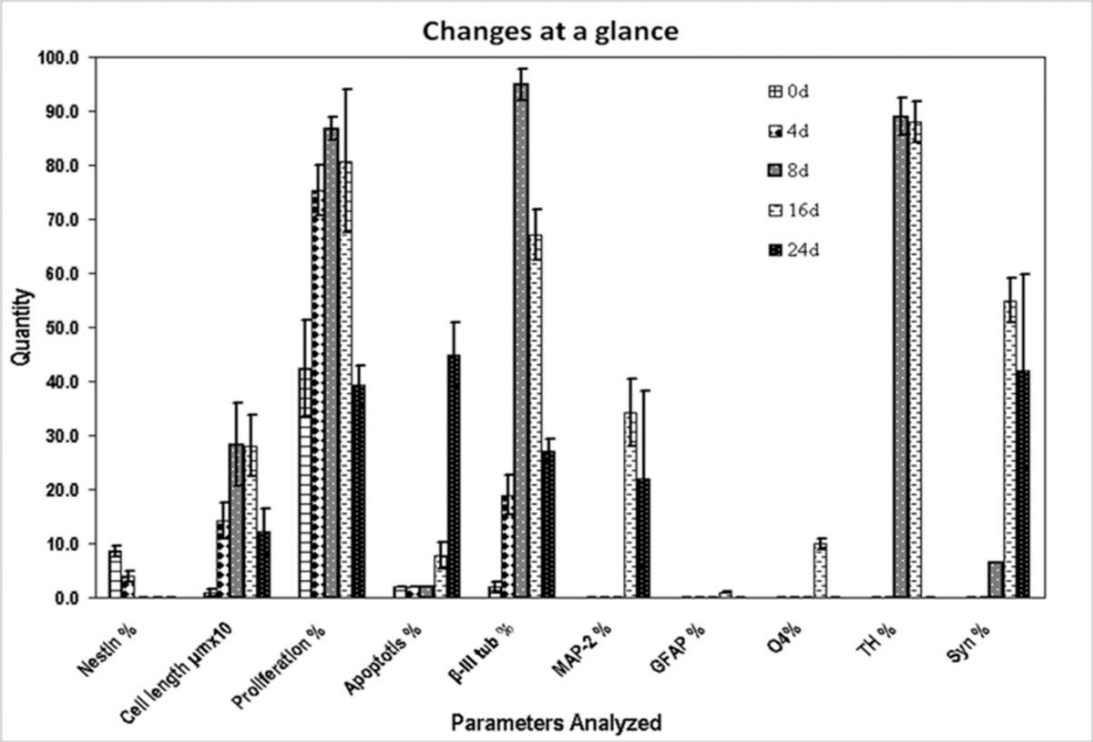


Figure 11. Summary of changes observed upon PBMNC culture. Nestin, the early progenitor marker was detected on 0d through 4thd of culture indicating presence of the NPC in circulation. The lengthening of cells was evident from 4d with maximum on 8d to 16d. The percentage of proliferating cells was high on days 4, 8 and 16. By 24d, apoptosis was detected. The immature marker β-III tub expression was remarkable by 4d with exponential increase by 8d. The mature neuronal marker, MAP-2 expression was measurable only by 16d. The expression of astrocyte (GFAP) and oligodendrocyte (O4) markers were low or not detectable. The functional protein tyrosine hydroxylase (TH) was expressed by 8d; the synaptophysin (Syn) was detected on 8d but was remarkable by 16d. Once the neurons showed mature markers, proliferation potential started declining. By the time apoptosis was significant, maturation and functional markers weakened.

4.3. Confirmation of signal induction by niche components

In literature, it has been well described that different signaling mechanisms are involved in the neuronal development out of which Wnt pathway was selected for demonstrating the role of different matrix components in the constituted *in vitro* niche. The strategy used was to first add a Wnt-3a inhibitor to show Wnt pathway is involved. Once it was proven that added inhibitor is effective to block differentiation, the adhesive proteins were blocked one by one and altogether to show the importance of adhesive proteins in the neuronal differentiation of NPC. Molecular markers of Wnt pathway and neuron-specific markers were used to demonstrate the role of the niche in the Wnt-3a mediated neuronal induction. The culture protocol was modified to prevent growth factors present in the bovine serum from induction of the neuronal differentiation.

4.3.1. Derivation of neurons confirmed in the standard niche

The modified protocol for culture of cells with gradual depletion of serum and growth factor induced neuronal differentiation of PBMNC derived NPCs within 8 days of culture (**Figure 12**). Cell colonies started appearing within a day after the partially attached PBMNCs were seeded onto the fibrin matrix (**Figure 12 A**). By 2d, slightly elongated cells could be seen protruding from the cell colonies throughout the culture area (**Figure 12 B**).With media changes, most of the non-specific cells were eliminated by 4d and the slightly elongated cells were seen further elongating attaining typical neuronal morphology (**Figure 12 C**). By 8d, the cells attained maximum length with much improved neurite-like branching and cell-cell proximity (**Figure 12 D**). The cells attained homogeneity as well.

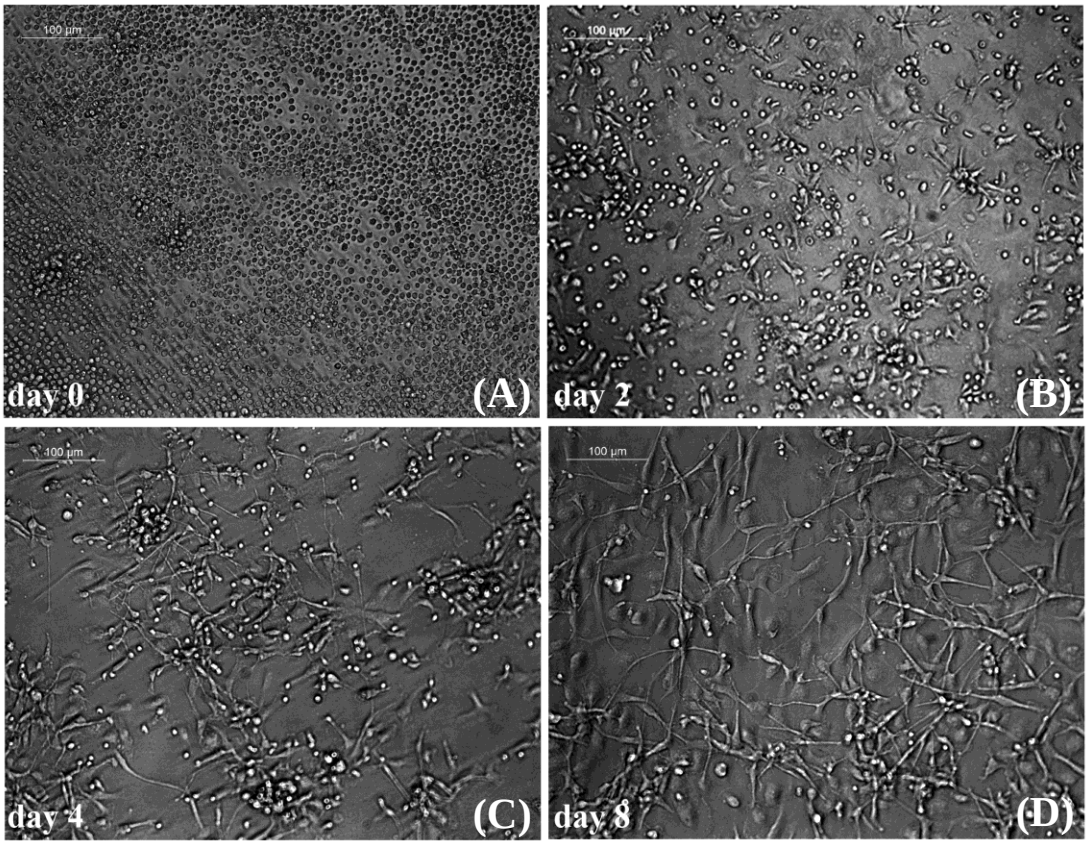


Figure 12. Derivation of neurons in the standard niche. (A) Day0 PBMNCs seeded onto the Std niche forming cell colonies (B) The colonies of cells giving rise to NLCs by 2d of culture on the Std niche (C)Non-specific cells are removed and the NLCs getting elongated (D) The cells further elongate and branch filling the entire culture dish by day8.

4.3.2. Effect of Wnt-3a signals on neural morphology and cell length

The concentration of added Wnt and its Inh was optimised to 150ng/ml and 25ng/ml after culturing the cells in various concentrations of these factors and looking into the effect. The period of addition of these components, that too in to the medium, was optimised after trying out various time points in medium alone, matrix alone and in both. For studying the effect of Wnt and Inh, both components were added from the first day of seeding on to the fibrin-coated dishes. Wnt-3a was withdrawn after 96h. Inh was added for the entire period of culture.

The colony formation was observed in all culture systems i.e. the Std niche, Wnt+ as well as Inh+ cultures (**Figure 13 A-C**). There was no significant difference in neural morphology between the Std niche as well as Wnt 3a added cultures indicating no additional influence upon Wnt protein addition (**Figure 13 A and 13 B**). Commercial Wnt inhibitor adversely affected cell morphology. There was a drastic decrease in the ability of cells to elongate. Most of the cells remained rounded and they detached from the fibrin niche within 9 days of culture (**Figure 13 C**). The neural length measurement showed significant decrease in the Inh added cultures indicating that Wnt-3a might be present in the matrix which is getting inhibited upon Inh addition (**Figure 13 D**). This suggests that Wnt-3a has a role in cell attachment and elongation. The cell length was ~75µm upon culture on the Std niche. Wnt addition increased the cell length to ~80µm, but it is not significant. Inh addition decreased the cell length significantly to ~25µm.

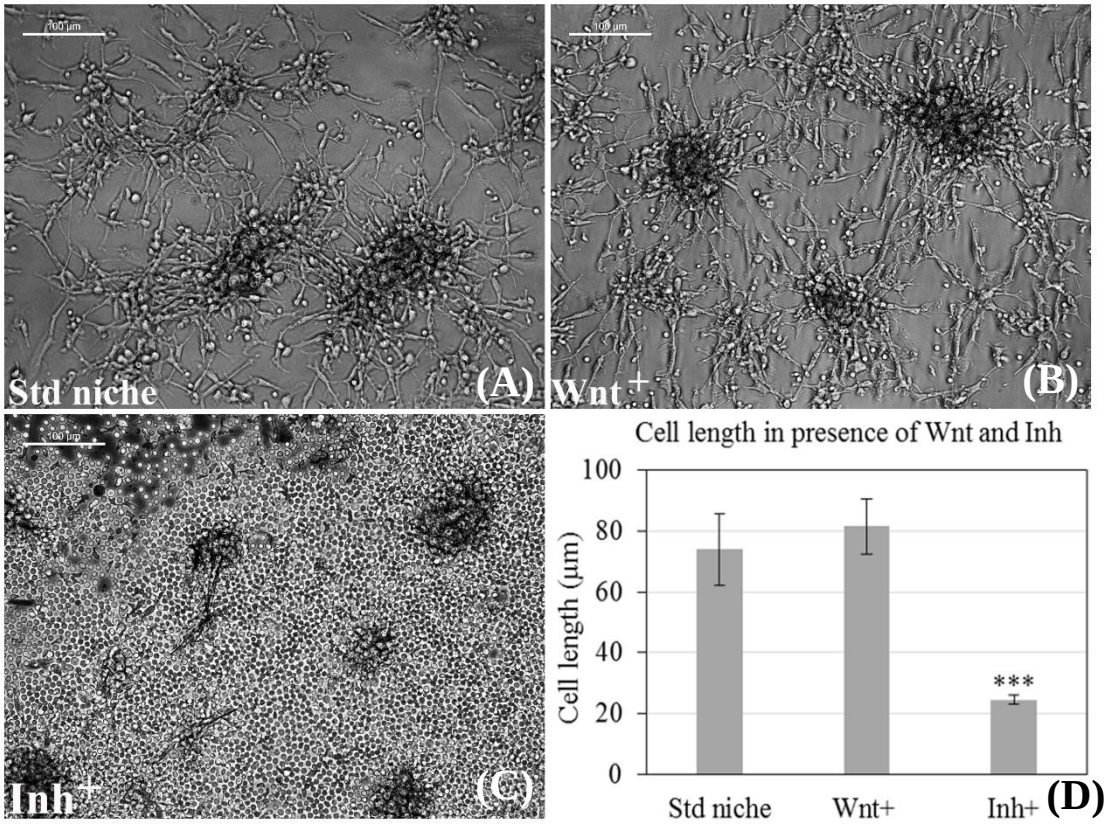


Figure 13. Effect of Wnt-3a signals on neural morphology. (A & B) No morphological difference in the derived NLCs between the Std niche and the Wnt+ cultures (C) Drastic difference in cell morphology with Inh addition (D) Quantitative data of neural cell length showing the difference between the Std niche, Wnt+ and Inh+ cultures.

4.3.3. Involvement of niche components for Wnt-3a signaling

Blocking individual constituents separately or collectively inhibited Wnt-3a signaling from matrix as evident from cell morphology (**Figure 14 A-F**). The cell growth was normal in the Std niche (**Figure 14 E**). In comparison to the Std niche, cell attachment and elongation was seen drastically affected upon blocking of FN component (**Figure 14 B**) and was maximum affected when Fib, FN and La were blocked together. This effect on cell morphology is comparable to the effect of the

Inh (**Figure 14 F**). When Fib and La were blocked individually, the effect on cell morphology was mild (**Figure 14 B, C & D**).

Analyses of day 8 cells (**Figure 14 G**) clearly indicate that the average cell length was maximum i.e. ~85µm in the Std niche cultured cells. La and Fib blocking did not have significant impact on cell length. The cell length was ~78µm and ~85µm, respectively, which is not a significant change in comparison to Std niche. FN blocking decreased the cell length to ~60µm and was more significantly reduced to ~39µm when all 3 components were blocked together.

This observation suggests the important role of insoluble matrix components present in the niche for inducing Wnt-like signaling to facilitate cell attachment and differentiation.

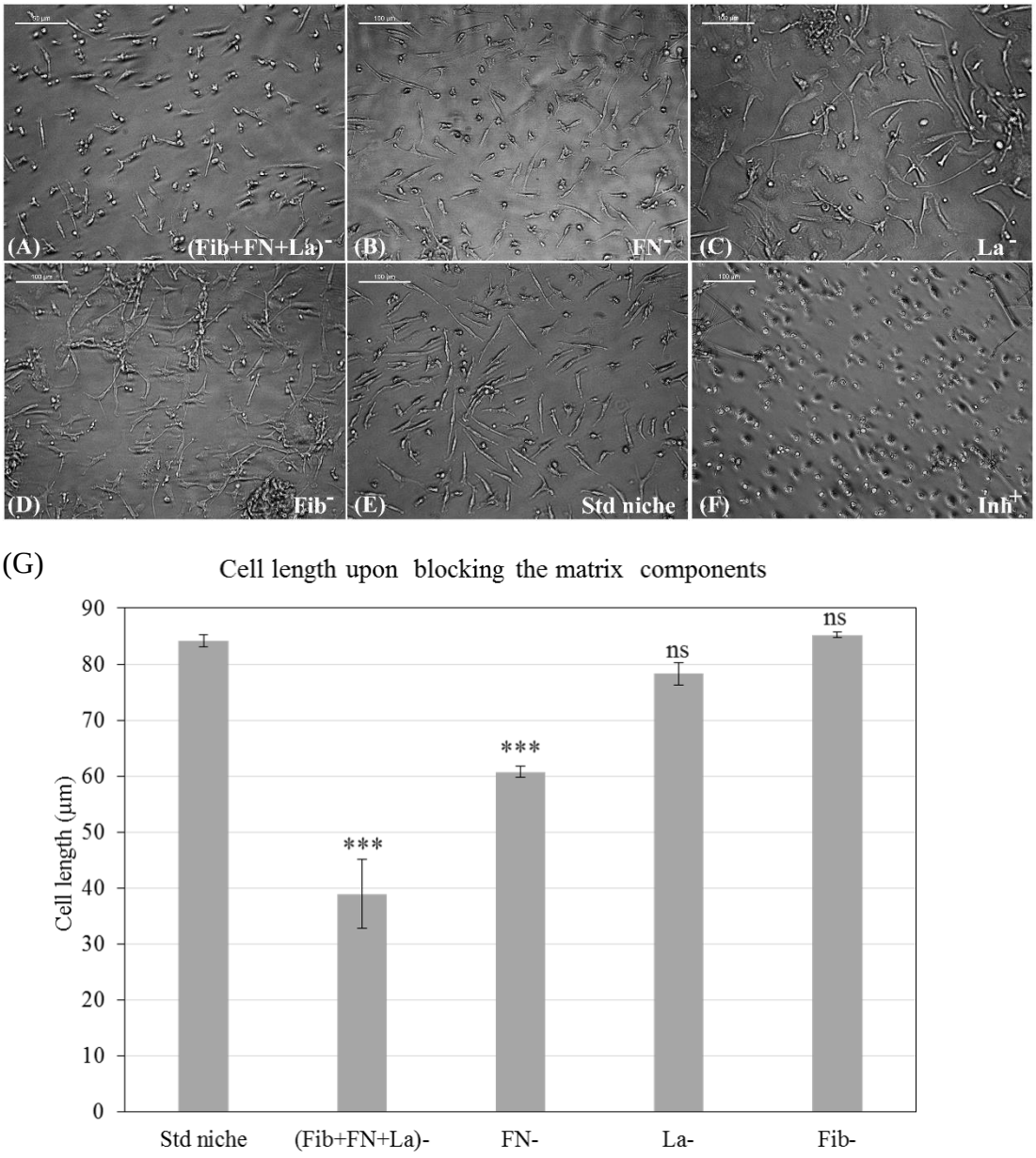


Figure 14. Involvement of niche components in differentiation. Blocking (A)Fib, FN &La (B) FN alone (C) La alone (D) Fib alone (E) without blocking (F) Inh added which is the positive control (G) Quantitative data of neural cell length of matrix blocked cultures in comparison to Std niche.

4.3.4. Activation of Wnt signal transducers

In order to confirm that the cells in culture are responding to Wnt signaling, the expression of Wnt target genes in NPCs, such as LEF1, Cyclin D1 and Axin2 were investigated. Wnt-3a addition upregulated all the three Wnt target genes as compared

to standard niche even though, morphologically there was not a significant difference (**Figure 15**). Addition of Inh to the Std niche downregulated the Wnt target genes. The increase in LEF, CycD1 and Axin2 expression upon Wnt protein addition were about 3.5 fold, 7.5 fold and 7 fold respectively as compared to Std niche, while the decrease in LEF, CycD1 and Axin2 expression upon inhibiting the Wnt signals were 0.5, 0.005 and 0.1 fold respectively. Thus, the Wnt addition appeared to act synergistically with the matrix components to upregulate expression of Wnt target genes. Since the Wnt target genes were downregulated upon addition of Inh, it is confirmed that Wnt signaling from the matrix components is responsible for the niche mediated NPC differentiation into neuron like cells.

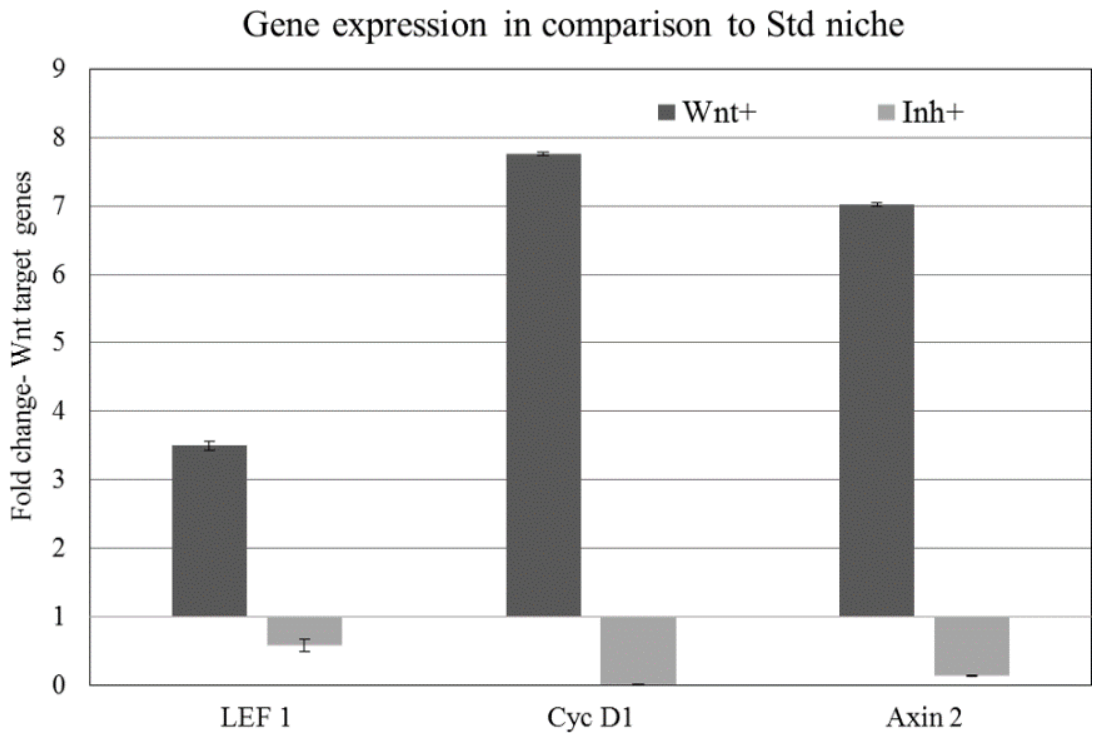


Figure 15. Activation of Wnt target genes. LEF1, Cyc D1 and Axin 2 were the target genes studied. The up/down regulation of the target genes upon Wnt/Inh addition as compared to the Std niche was represented graphically.

4.3.5. Effect of matrix blocking on Wnt target genes

The observation in 4.3.4 is reinstated by looking at the Wnt target genes upon blocking signals from matrix components with antibodies. It can be seen that matrix blocking individually or in combination downregulated all the target genes studied confirming Wnt signaling from each and every component of the matrix (**Figure 16**). The downregulation of LEF, Cyc D1 and Axin2 were ~0.3, 0.05 and 0.1 fold respectively for (Fib+FN+La)- matrix; 0.5, 0.1 and 0.5 fold for FN- matrix; 0.5, 0.2 and 0.25 for La- matrix and 0.7, 0.4 and 1.5 for Fib- matrix. The downregulation of Wnt genes is comparable to that of Inh added ones and the fold changes were 0.5, 0.005 and 0.1 respectively for LEF, Cyc D1 and Axin2. This confirms origin of Wnt signals from matrix that is actively participating in the pathway.

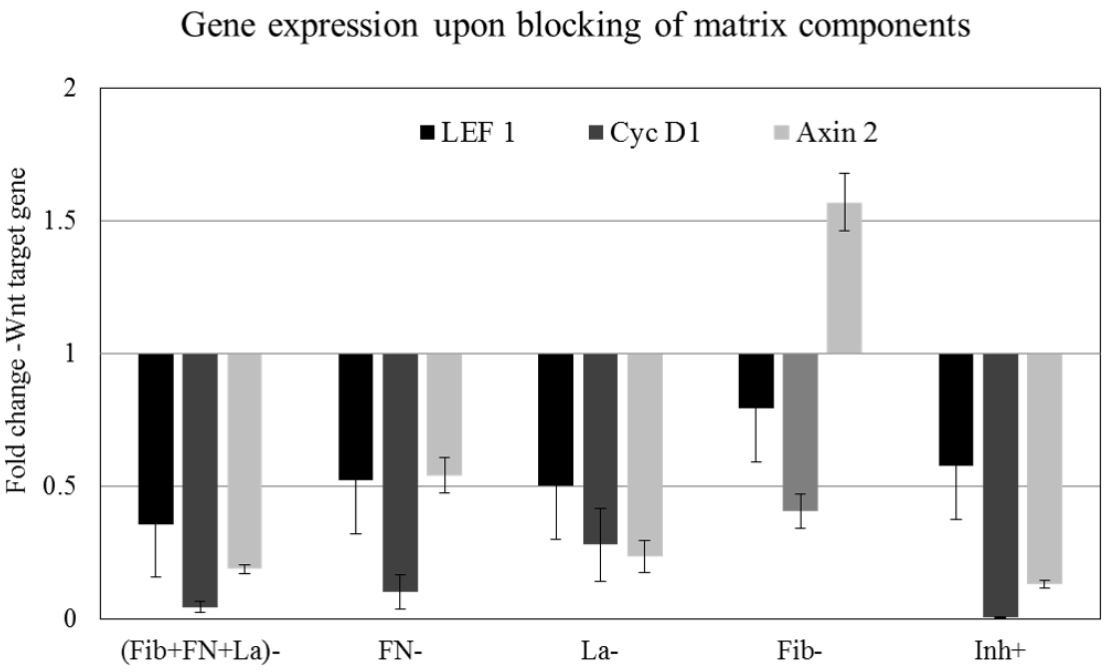


Figure 16. Effect of matrix blocking on Wnt target genes. The graphical representation of the effect of combined as well as individual matrix components on the regulation of target genes LEF, Cyc D1 and Axin2.

4.3.6. Effect of Wnt-3a from niche on proliferation

The cells cultured in the Std niche were found to proliferate. As compared to Std. niche, Wnt-3a addition marginally upregulated proliferation whereas addition of Inh downregulated proliferation marker PCNA. Blocking of FN and (Fib+FN+La) together downregulated proliferation markers similar to what was observed with Inh addition. The PCNA was quiescent upon blocking Fib and La (**Figure 17**).

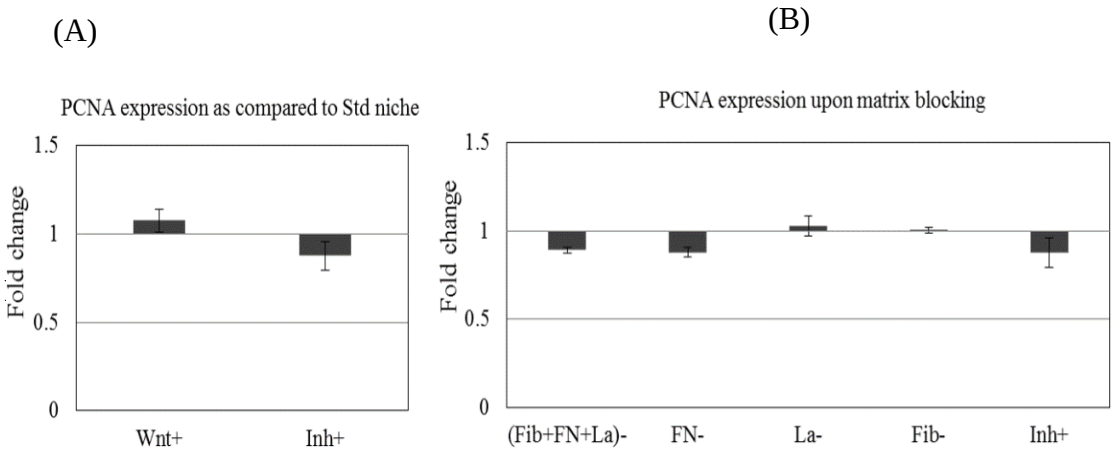


Figure 17. Effect of Wnt signals on proliferation. Up/down regulation of PCNA gene upon (A) Wnt/Inh addition in comparison to Std niche (B) matrix blocking individualy/combined.

4.3.7. Effect of Wnt signals on neural gene expression

Wnt-3a protein addition to the Std niche upregulated Nestin expression which is a progenitor marker and downregulated NSE expression which is a mature neuronal marker. These results indicate that the additional Wnt-3a have an effect on maintaining the progenitor status of cells (**Figure 18**). The expression of Syn, a functional neuronal marker was also downregulated. β- III tubulin, an intermediate neuronal marker and the oligodendroglial marker, Oligodendrocyte specific protein (OSP) were expressed marginally as compared to Std niche. The astrocyte marker,

GFAP was not expressed in any of the cultures. Addition of inhibitor downregulated all tested neural gene expression including immature and mature neuronal markers as well as glial, indicating the importance of Wnt signals from Std niche for neural differentiation.

This result confirmed the importance of Wnt signals elicited from the niche for cellular and molecular level regulation of proliferation and differentiation of circulating progenitors.

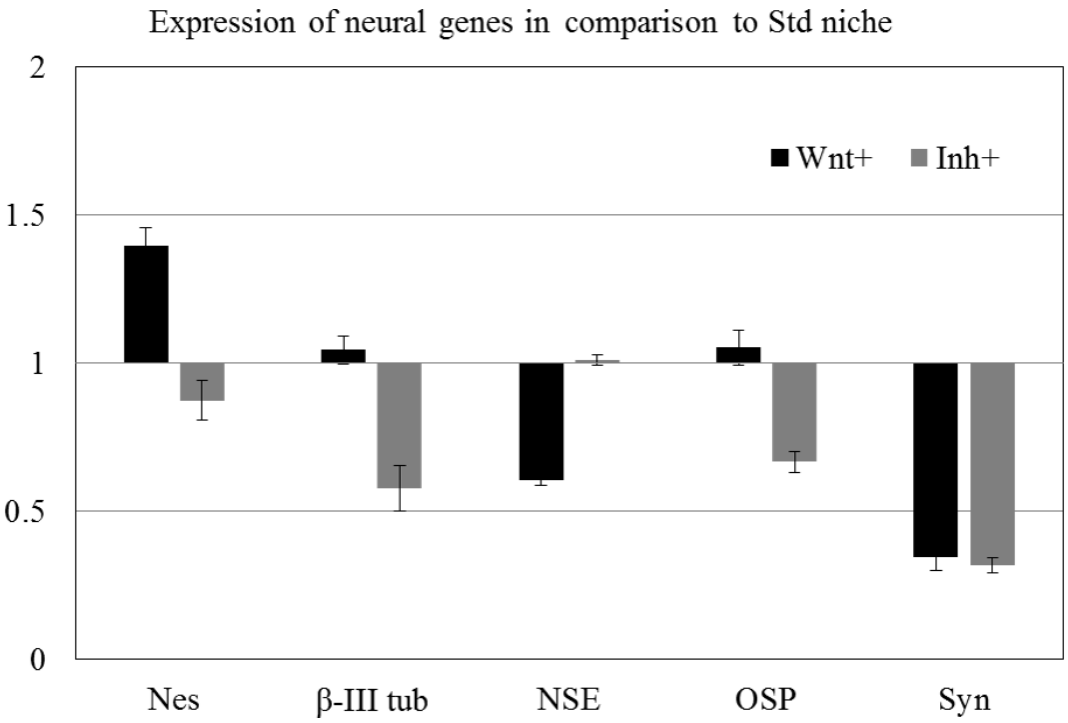


Figure 18. Effect of Wnt signals on neural gene expression. Graph signifying the effect of Wnt/Inh on the up or down regulation of neural genes Nes, β-III tubulin, NSE, OSP and Syn.

4.3.8. Effect of matrix components on neural gene expression

Upon blocking of important niche constituents, a mixed effect was observed (**Figure 19**). Blocking of 3 adhesive proteins together resulted in upregulation of progenitor marker Nestin. All other neuronal markers, which are involved in lineage

commitment as well as maturation which include β-III tubulin and NSE were found to be downregulated. The results confirm the role of all three matrix components in combination for regulating proliferation and promoting differentiation. OSP was also downregulated indicating the role of matrix in promoting oligodendrocyte differentiation as well. The expression of Syn was marginal. The mixed effect on individual component blocking could be because there is involvement of signaling cascades other than Wnt in the differentiation process.

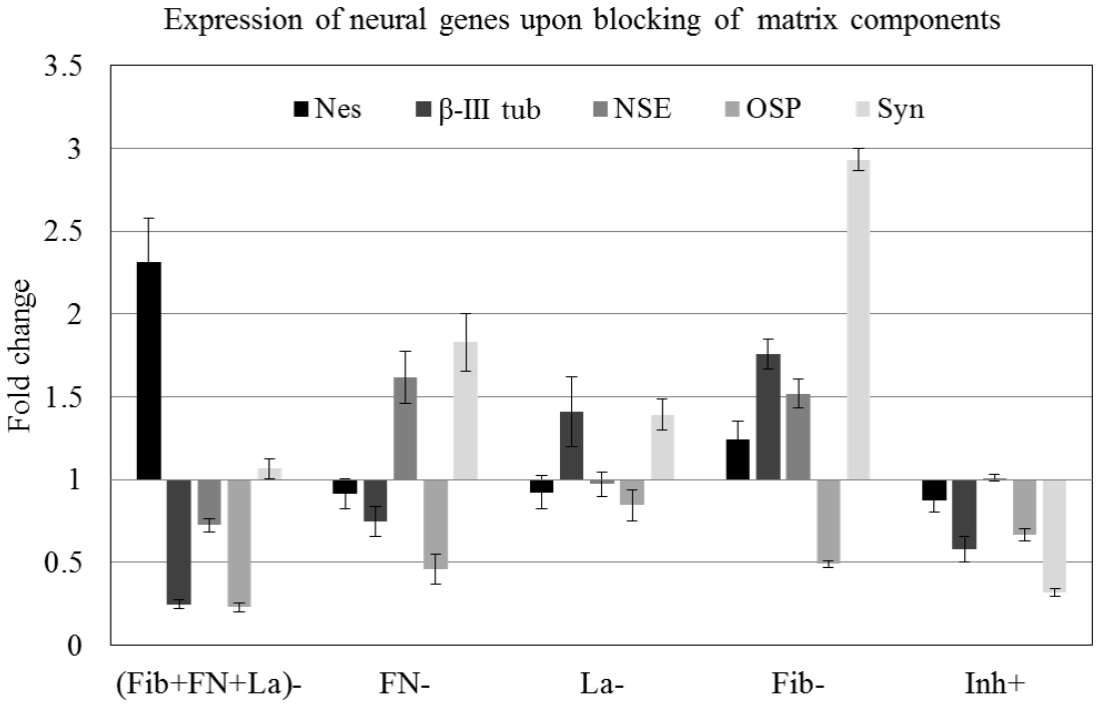


Figure 19. Effect of matrix blocking on neural gene expression. Effect of matrix components individually or in combination on Nes, β-III tubulin, NSE, OSP and Syn expressions is evident in the graph.

4.3.9. Effect of matrix blocking on neuronal protein markers

As compared to Std niche, in the Inh+ culture, downregulation of marker proteins was evident (**Figure 20A**). Similarly, the expression of all markers studied was also downregulated in (FN+Fib+La)- cultures (**Figure 20A**). Quantitative data of protein

band intensities obtained from three replicate experiments is presented in graph 20B. Downregulation of all markers was more intense in (FN+Fib+La)- as compared to 'T' and the negative control.

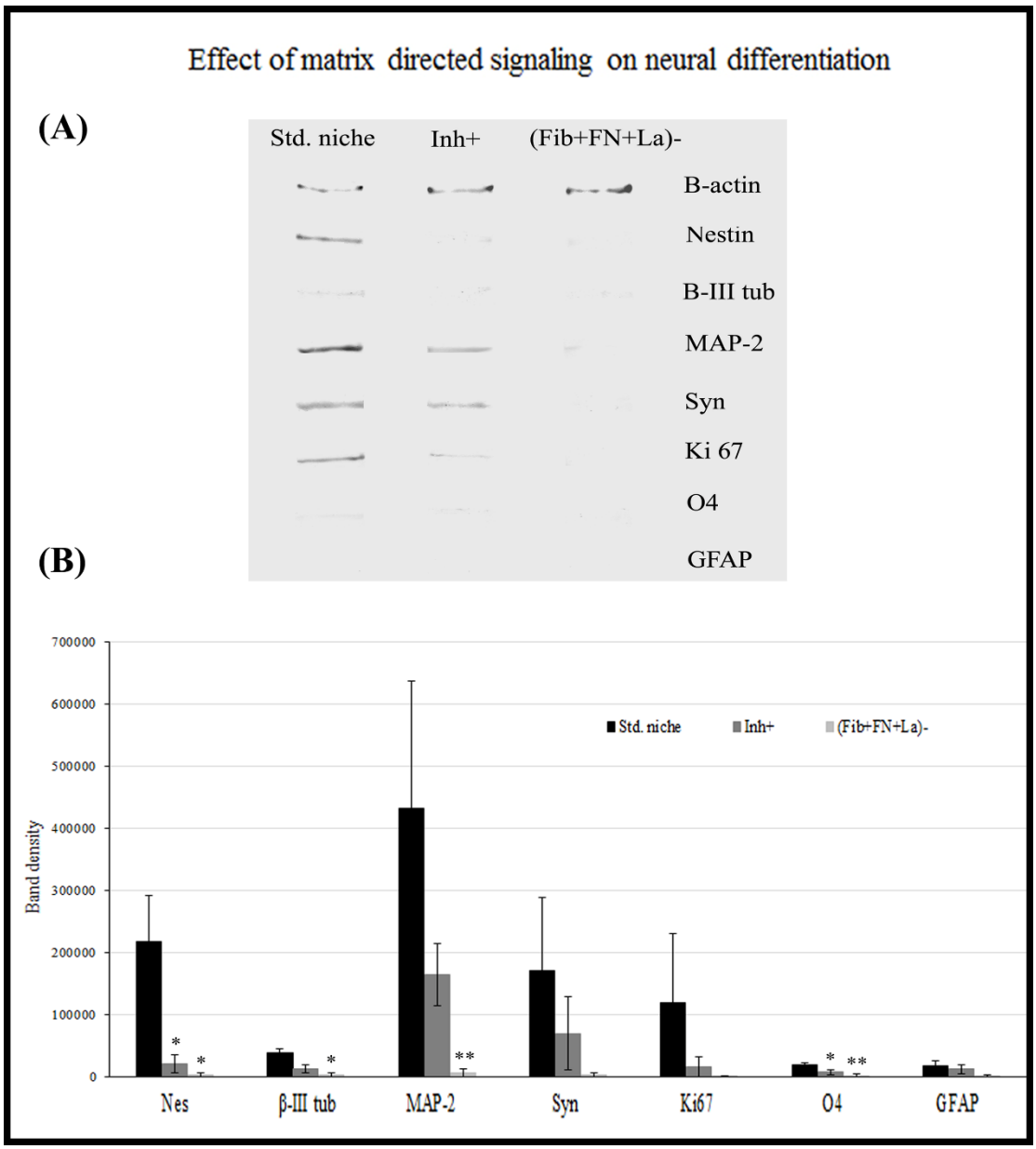


Figure 20. Effect of matrix blocking on neural protein expression. (A) Effect of matrix components on the expression of Nes, β-III tubulin, MAP-2, Syn, Ki67, O4 and GFAP proteins (B) Quantitative analysis of band intensities of the proteins studied is represented in the graph.**indicates P<0.01 and *indicates P<0.05.

The summary of #4.3 is represented in Figure 21.

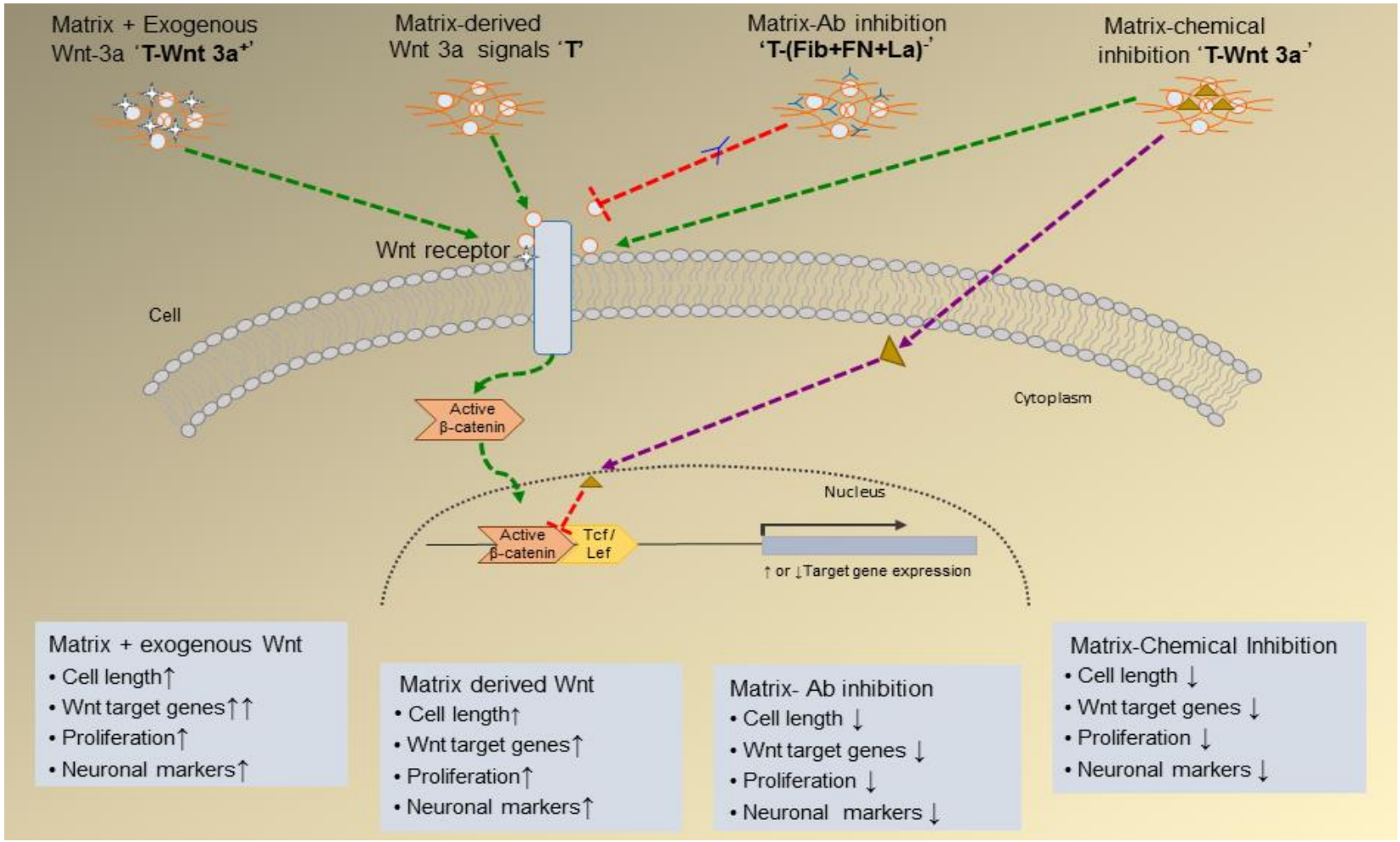


Figure 21. Summary of Wnt signaling elicited by the niche

4.4. Cell transplantation and evaluation

This part of the study involves rat NPC isolation, effect of 3-D fibrin niche on NPC, identification of suitable matrix composition for cell transplantation and evaluation of transplanted site.

4.4.1. Rat NPC isolation

Rat NPC was isolated and demonstrated potential to differentiate into neurons to use it as an autologous cell source to carry out cell transplantation experiments in rat SCI model.

4.4.1.1 Morphological characterisation

The fibrin composite matrix selected NPCs efficiently from the heterogeneous population of rat PBMNC and neuron like cells (NLCs) were derived upon culture on the same niche standardized for human cells. The transition of progenitors to neuron-like outgrowth colonies appeared on culture area from 2^{nd} day of culture (**Figure 22 A**). Upon subsequent media changes, non-specific cells were eliminated and a more homogenous population with neural morphology was seen by 4^{th} day (**Figure 22 B**). More NLCs sprouted out from the colonies, migrated and the culture surface was populated with more cells. By 8^{th} day, many cells showed elongated NLC appearance (**Figure 22 C**)**.** By 16^{th} day, the cells lengthened further and cell-to-cell closeness was visible upon examination of cultures under light microscope (**Figure 22 D**).

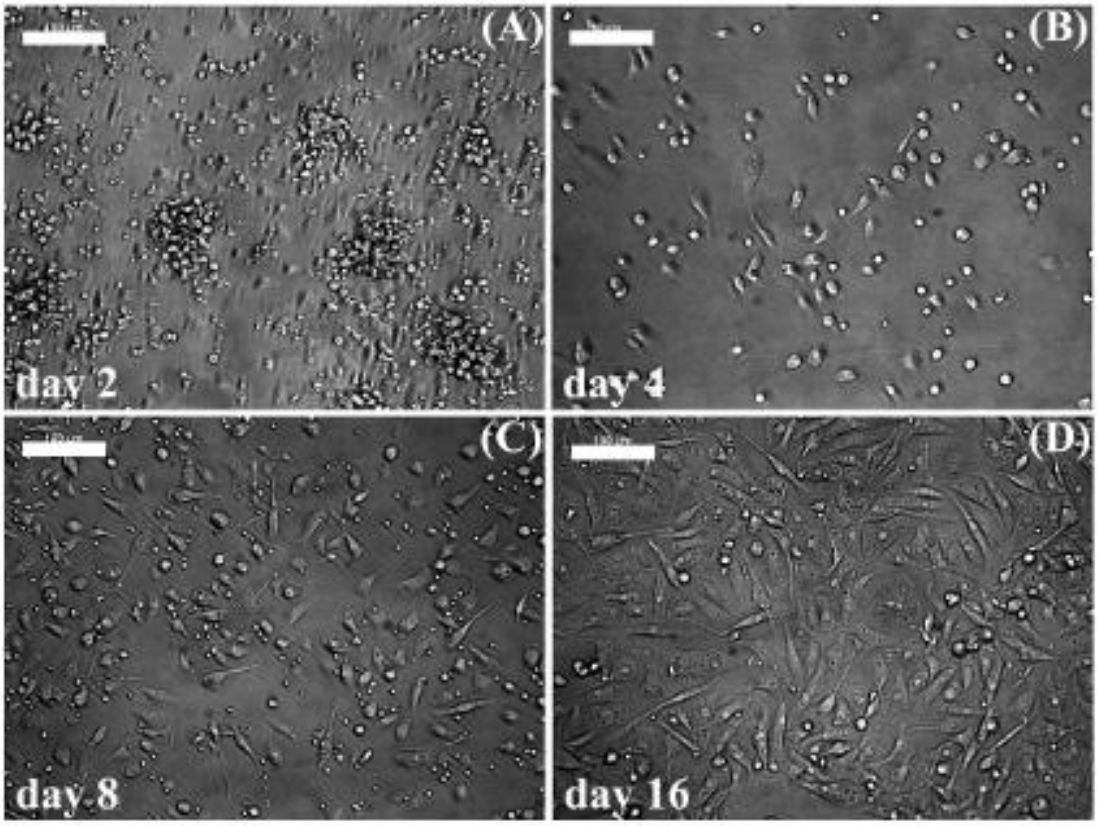


Figure 22. Culture of rat PBMNCs on a fibrin based neuronal niche. (A) Appearance of colonies after 2 days (B) Micrograph showing morphological change by 4th day (C) Demonstration of elongated cells and associated elimination of non-specific cells by 8th day culture (D) Indication of proliferation of NLCs filling the culture area by 16th day. Scale bar: 100 µm

4.4.1.2. Immunochemical characterization

Cells grown for specific periods were identified using fluorescent/immuno-markers. The individual and merged images are represented in **Figure 23**. By the 8th day of culture, majority of the PBMNC-derived NPCs expressed the immature neuronal marker β-III tub (**Figure 23 A1-A3**) indicating further commitment of progenitors towards neuronal lineage. Expression of Ki67 during the period of lineage commitment confirmed the ability of NPCs to proliferate (**Figure 23 D1-D3**). Upon culture for up to 16 days, the cells expressed the mature neuronal marker MAP-2

(**Figure 23 B1-B3**)**.** By this period, the cells also expressed Syn, suggesting vesicle formation and synapse formation (**Figure 23 C1-C3**).

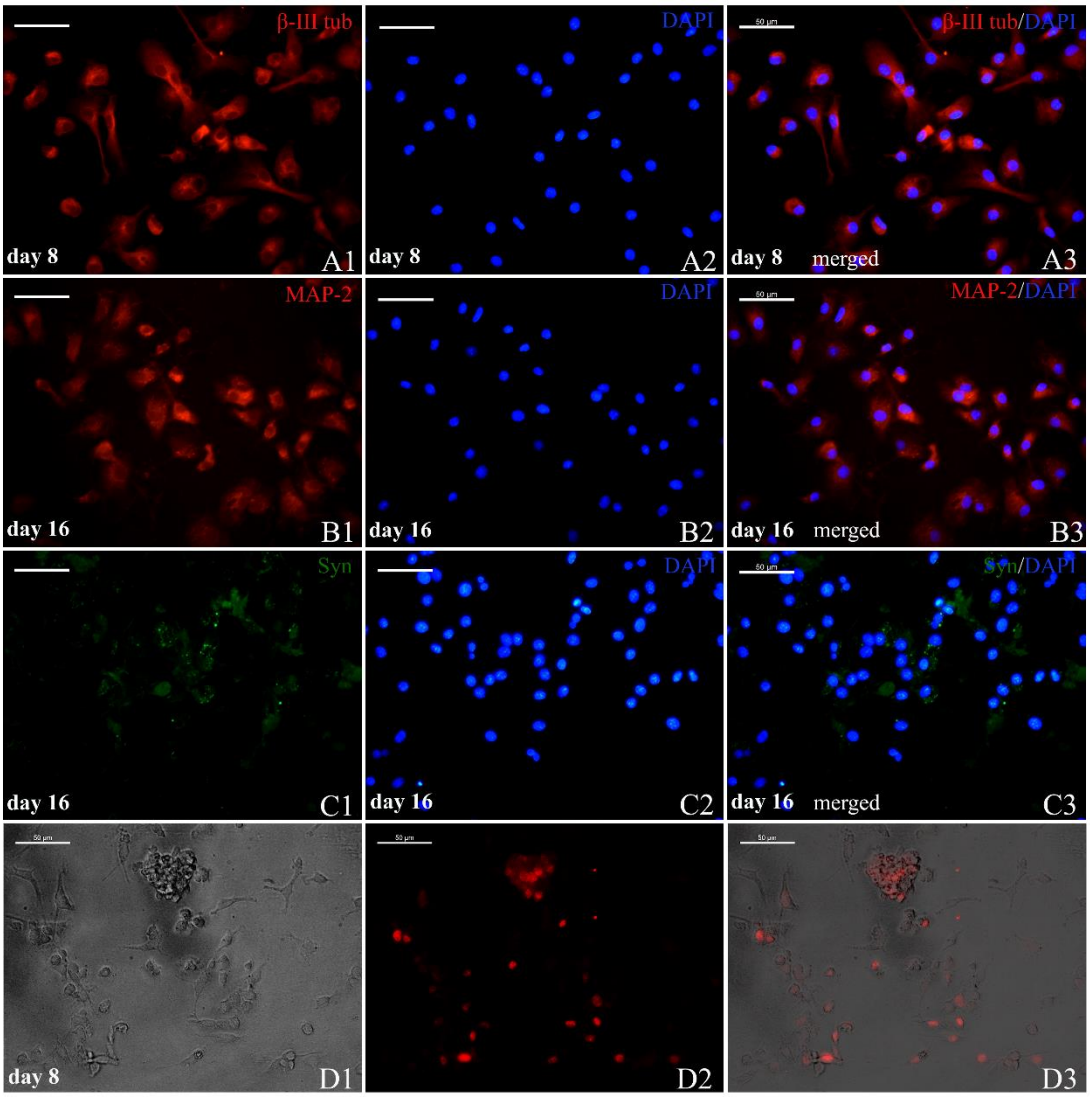


Figure 23. Identification of NLC by immuno-staining. (A1- A3) On the 8th day of culture, expression of β-III tub counterstained with DAPI identifying immature neurons was seen (B1-B3) On the 16th day of culture, expression of MAP-2 counterstained with DAPI identifying matured neurons (C1-C3) On the 16th day of culture, expression of Synaptophysin counterstained with DAPI indicating possible synapse formation (D1-D3) On the 8th day of culture, phase contrast micrograph show colonies and fluorescence indicating expression of proliferation marker Ki67. Magnification of each image is marked using scale bar (50μm).

Quantitative estimation of cells using flow cytometry showed that in isolated PBMNC, ~1.3% of the cells were positive for the progenitor marker nestin on day 0, even before culture on NPC niche (**Figure 24 A**). The nestin expression persisted during the initial culture days which declined during the later period. The expression of β-III tub and MAP-2 was minimal on day 0 as seen in **Figure 24 B and 24 C**. By day 8, nearly 80% of the cells were positive for β-III tub (**Figure 24 D**). As the culture progressed to day 16, approximately 60% of the cells expressed MAP-2 (**Figure 24 E**). During the same period, almost 30% of the NLCs were positive for Synaptophysin (**Figure 24 F**). The data from replicate experiments were compiled is presented graphically (**Figure 24 G**). The expression of neuronal markers on rat PBMNC-derived NPCs were cross-verified from data on mRNA amplification. Nestin was expressed on day 0 and at later period, β-III tub and Syn. Thus, the markers of neuronal lineage commitment were expressed both at transcriptional and translational levels (**Figure 24 H**). The cells from 5 to 8 days seemed suitable for transplantation as they are immature with potential for proliferation and neuronal differentiation.

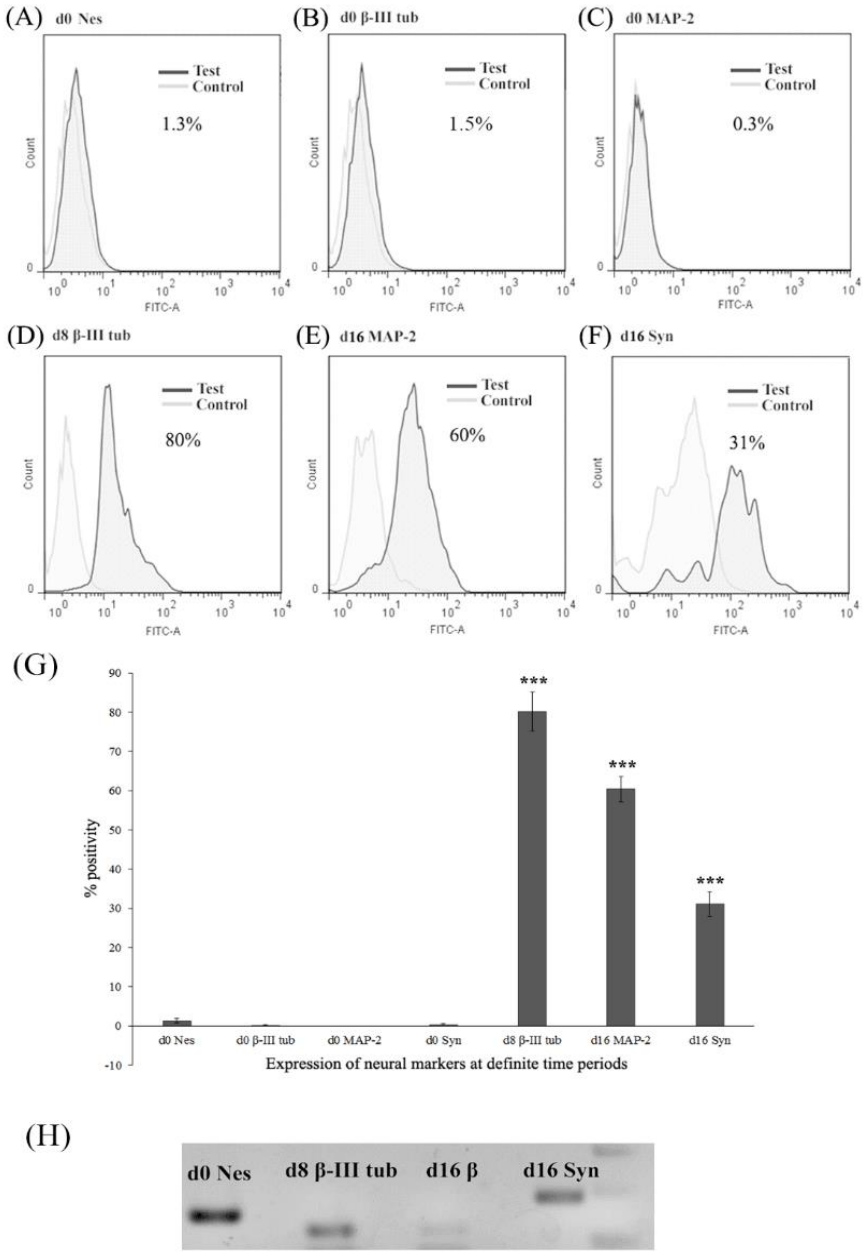


Figure 24. Flow cytometry and RT-PCR data. (A) ~1.3% cell in culture expressed Nestin was positive on day 0 (B-C) show low numbers of cells expressing β-III tub and MAP-2 (D) ~80% of the cells expressed β-III tub on day 8 (E) By day 16, ~60% of the cells expressed MAP-2 (F) ~30% of the cells expressed Syn indicated by day 16 (G) Graphical representation of quantitative data (Average ± SD; n=3) acquired by flow cytometric analysis for nestin, β-III tub, MAP-2 and Syn at different time periods. Statistical analysis showed significant change w.r.t day0 indicated '***' ($P < 0.0001$), '**' ($P < 0.01$) and '*' ($P < 0.05$) (H) AGE showing amplified gene products of nestin, β-III tub and Syn; correlating transcription and translation during the period of analysis for each marker.

4.4.2. Niche optimization for in vivo cell delivery

4.4.2.1. Quality of 3-D scaffold

Upon E-SEM analysis, the fiber thickness and porosity of the polymerized fibrin was found to be influenced by the concentration of thrombin used for clotting fibrinogen (**Figure 25**). The fibrin strands appeared thicker when 1.0 IU thrombin was used (**Figure 25 A**) as compared to the strands formed with 5IU. The pore size also decreased with higher thrombin activity (**Figure 25 B -D**). When cells were cultured on the 3-D fibrin matrix, their attachment was observed independent of thrombin concentrations used for clotting fibrinogen. Cell colonies from which NLCs sprout, migrate and proliferate were observed at all concentrations (**Figure 25 E**). However, semi-quantitative measurement using ImageJ software revealed that cell length varied between 1µm to 125µm within 72h of seeding on to the matrix (**Figure 25 F**). It was observed that ~23% of the cells exhibited >50µm cell length when thrombin concentration was 1IU, whereas only ~4% of the cells showed >50µm length in the presence of 5IU thrombin. Only ~2% of the cells exhibited >50µm length when the fibrin matrix was produced using 10IU and 15IU thrombin. Thus, the extent of cell lengthening and the proportion of cells with NLC-like morphology were more when thrombin activity was low in the matrix.

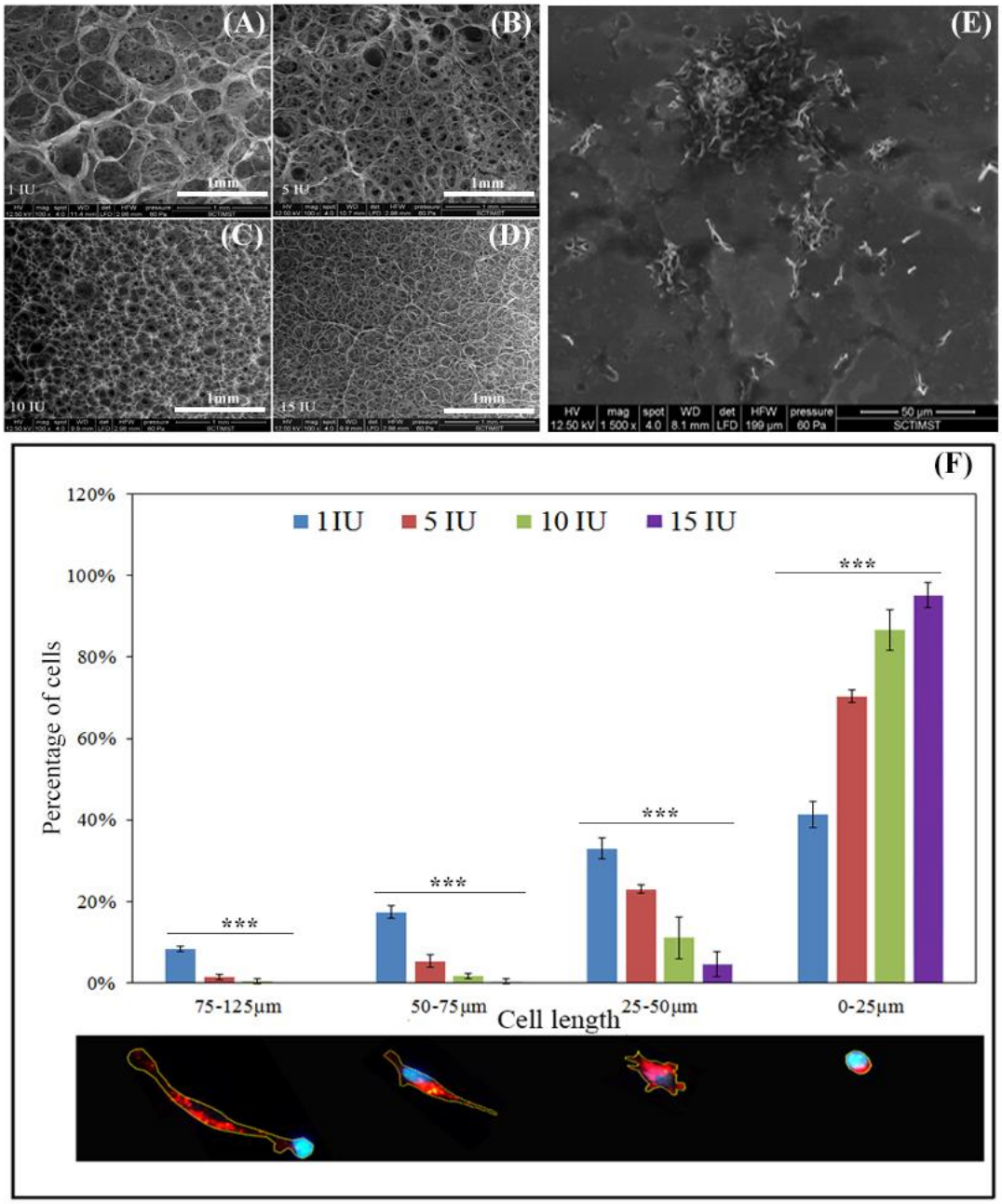


Figure 25. *In vitro* characteristics of 3-D fibrin niche formulated. (A-D) Environmental scanning electron micrographs showing the effect of thrombin on scaffold porosity and fibre morphology- magnification scale bar 1mm (E) Colony formation and sprouting of PBMNC seeded on 3-D fibrin niche-magnification scale bar 50µm (F) Data on quantitative analysis of the effect of thrombin on cell lengthening on 3-D fibrin niche measured by Image-J software- higher thrombin reduces numbers of longer cells.

When thrombin was added to already committed and lengthened NLCs, they were unaffected by the presence of 1IU (**Fig 26 A-D**) as compared to unadded cultures (not shown), but 5IU, 10IU or 15IU thrombin made drastic change to the cell morphology and reduced cell frequency, probably due to their death. Quantitative data showing the effect of different thrombin concentrations on cell length and cell number/mm^2 of lineage-committed cells is represented as a combo graph (**Fig 26 E**). Maximum cell length of ~160µm was observed with 1IU thrombin concentration as compared to ~110µm for 5IU thrombin. The cell length further decreased to ~80µm for 10IU and ~59µm for 15IU thrombin concentration. A similar trend with varying thrombin concentrations on cell number was also observed. Maximum cell number of 54/mm^2 was observed with 1IU thrombin which decreased to 30/mm^2, 14/mm^2 and 10/mm^2 with increase in thrombin concentrations.

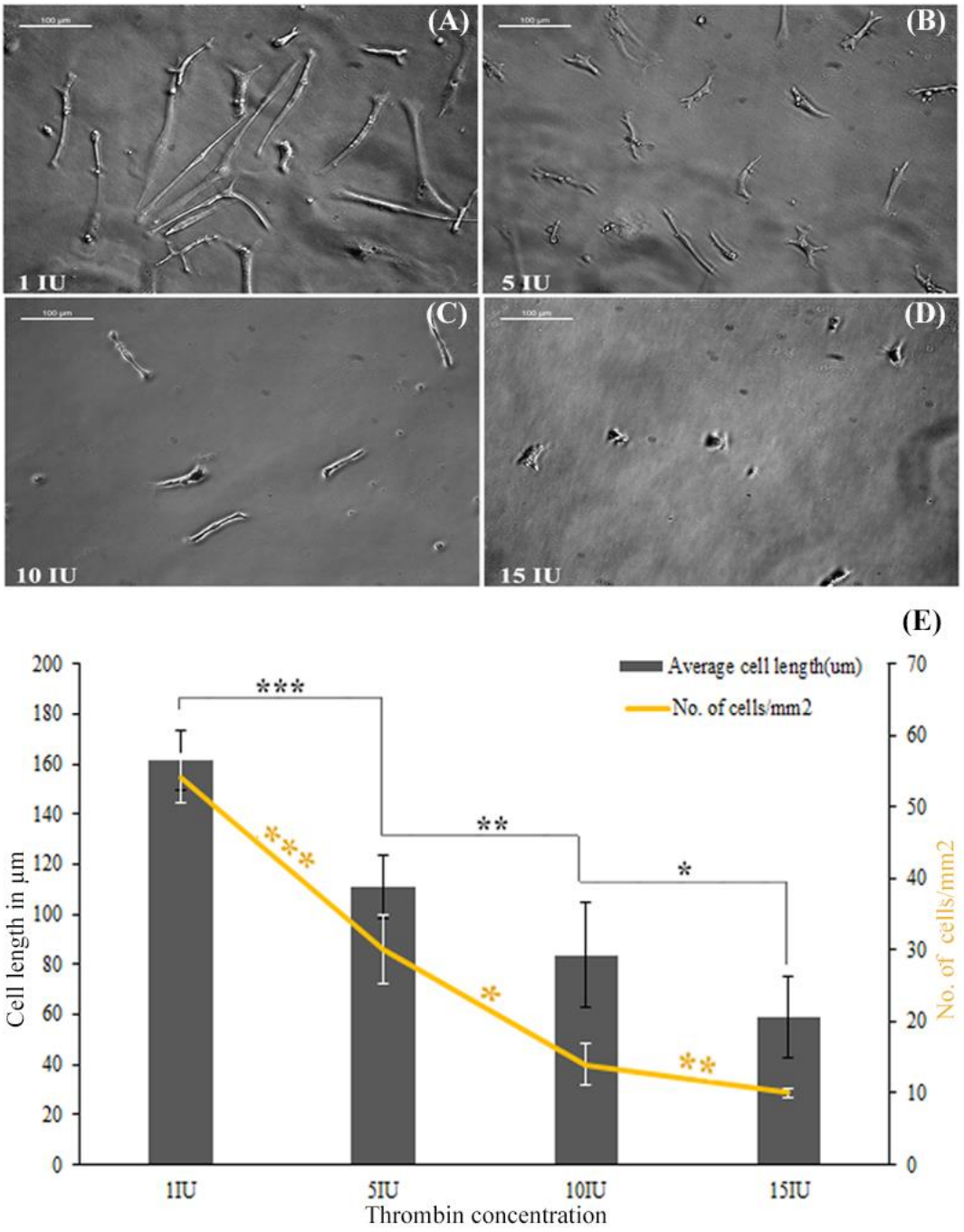


Figure 26. Effect of additional thrombin on cells cultured on 2D fibrin niche. (A-D) Photomicrographs showing effect of graded thrombin concentrations added to lineage committed NPCs-scale bar 100µm, indicating better maintenance of NLC morphology in the presence of low thrombin activity (K) Quantitative data of the effect of graded thrombin concentrations to lineage committed cells showing decrease of cell length and cell no/mm^2 with increased thrombin. Significance is indicated as '***' ($P < 0.001$), '**' ($P < 0.01$) and '*' ($P < 0.05$).

4.4.3. Cell transplantation and evaluation

4.4.3.1. Creation of SCI model and cell transplantation

Upon laminectomy and induction of contusion injury at L1 vertebra using the impactor (**Figure 27 A-C**), the animals developed symptoms of SCI including development of paraplegia and urine retention (**Figure 27 F**). Cells/ Fibrin+cells/ fibrin/ medium were transplanted at the SCI site (**Figure 27 D-E**).

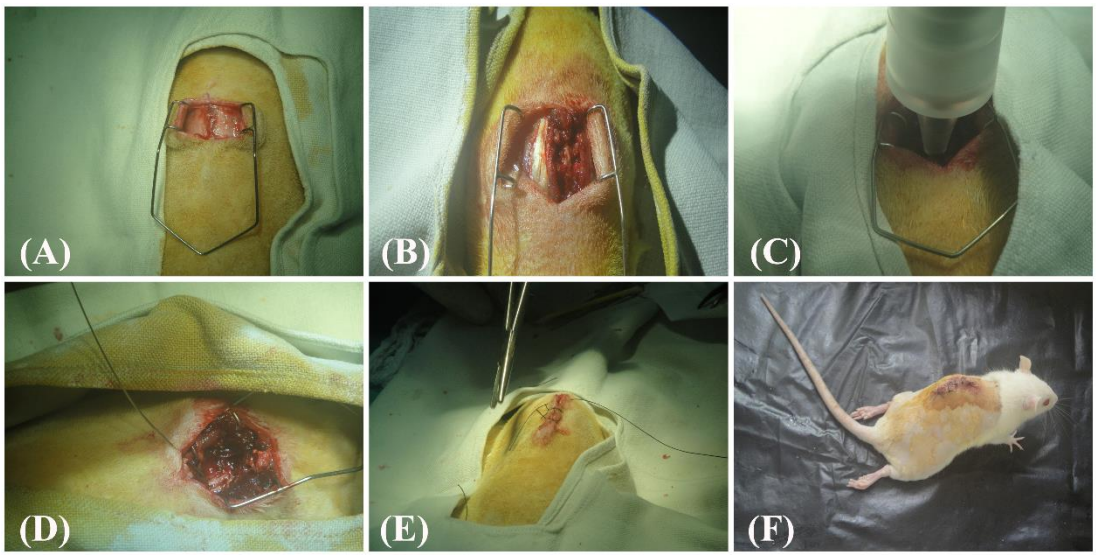


Figure 27. SCI model development and transplantation. (A-B) The skin was incised and the paravertebral muscles removed. (C) Laminectomy was then performed to expose the spinal cord at L1 vertebra. Then a contusion injury was given using an impactor (D) Transplantation was carried out at the injury site (E-F) The incisions are sutured back and SCI animals were allowed to survive for 8 days.

4.2.3.2. Survival of transplanted cells

The protocol used for labelling was successful and all cells in the culture appeared fluorescent red at the time of transplantation. After 8 days of transplantation, SCI tissue showed more red fluorescence corresponding to PKH26 labelled cells closer to the site of injury in the test group (**Figure 28 A**). Even though, the same number of cells was injected, the intensity of fluorescence was weaker in the control SCI to

which cells were delivered using the culture medium. There was no fluorescence at the injury site in the CF and CM animals which eliminated possible auto-fluorescence due to the cell carrier fluid. The extent of spinal cord injury produced after creating contusion injury is represented in **Figure 28 B**.

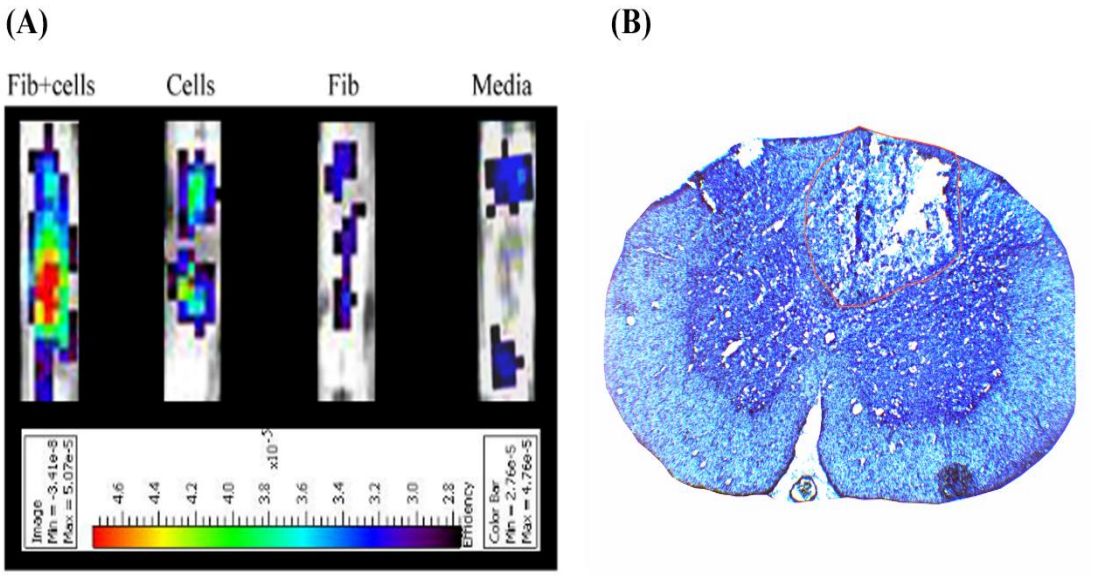


Figure 28. F**luorescence of transplanted cells** (A) Prominent red fluorescence corresponding to PKH26 label in T as compared to C and absence of auto fluorescence in CF and CM (B) Cross section of injured spinal cord stained with toluidine blue acquired at 10x magnification demonstrating the injury.

4.4.3.3. Characteristics of stained tissue sections

The injury sites of both the cell-less controls - CF and CM - exhibited pronounced deterioration in cell morphology. However, in the cell transplanted control and test, the injured areas were seen to be occupied by the transplanted cells, which in turn, appeared to have influenced the tissue architecture. Even though transplanted cells were present in both ‘T’ and ‘C’ animals, the cells were found to be more frequent and the tissue architecture better and more consistently preserved in ‘T’ animals **(Fig**

29 A-D and F-I). The transplanted cells were found to be healthy with typical nuclear and cytoplasmic staining. Neither significant proteoglycan deposition nor granular tissue formation was observed in any of the animals by day 8 **(Fig 29 F-I)**. Also, less glycolipid deposition was observed in the fibrin-injected animals ('T' or 'CF') as compared to 'C' or 'CM', indicating better tissue clearance at the injury site (**Fig 29 K-N**). PTAH staining was positive in the 'T' and the 'CF' animals. Patches of dark blue stains in the sections demonstrate the presence of fibrin, signifying the stability of injected fibrin even after 8 days of transplantation **(Fig 29 Q-T)**. Sections of laminectomy group stained for respective stains were included as an additional control **(Fig 29 E, J, O, U)**. There were more cells positive for macrophages in the 'C' and 'CM' animals as compared to 'T' and 'CF' animals (**Fig 29 V-Y)**. The quantitative data of ED2 positive cells showing significant difference between the 'C' animals and the 'T' animals is represented graphically **(Fig 29 Z),** suggesting an immunomodulatory role for fibrin.

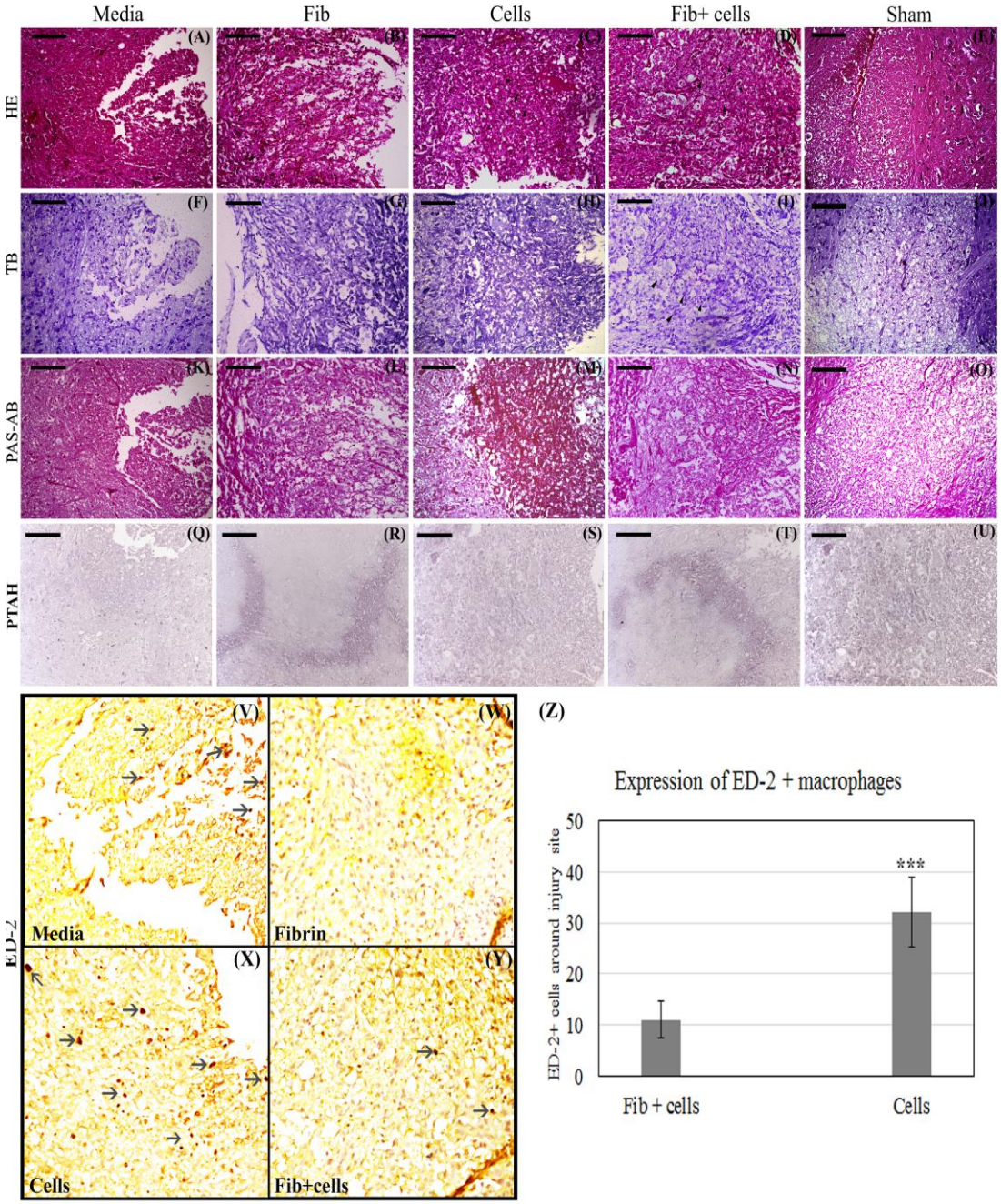


Figure 29. Representative images of histochemical-stained SCI sections. (A-D) Sections of SCI stained with H&E corresponding to CM,CF,T & C (F-I) TB corresponding to CM, CF,T & C (K-N) PAS-AB corresponding to CM, CF,T & C (Q-T) PTAH corresponding to CM,CF,T & C (E,J,O,U) Sham/uninjured controls for H&E, TB, PAS-AB and PTAH stains respectively (V-Y) Sections of SCI immunostained for ED-2 corresponding to CM,CF,T & C (Z) Compiled data on quantitative analysis of ED-2+ cells. Scale bar in all cases 100μm except from Q-T where it is 50μm.

4.4.3.4. Identification of the transplanted cells in vivo

Fluorescent imaging by microscopy revealed that transplanted cells were present at the injury site in both the 'T' and 'C' animals consistently; however, fluorescent spots were more frequently dispersed in the former (**Figure 30 A**) as compared to the latter (**Figure 30 B**). The DAPI staining of tissue sections demonstrated nuclear colocalisation with PKH^{+} transplanted cells. In the 'CF' and the 'CM' animals, there was no fluorescence suggesting the specificity for PKH label in 'T' and 'C' (**Figure 30 C & D**). The magnified images of the transplanted site of respective study groups are represented from **Figure 30 E-H**. These results are consistent with the bio-imaging results, which further confirm the role of fibrin for not only retaining cells but also protecting them. Again, quantitation of fluorescence intensity (red PKH fluorescence) using ImageJ software indicates statistically significant fluorescence in the test animals as compared to experimental and media controls (**Figure 30 I)**.

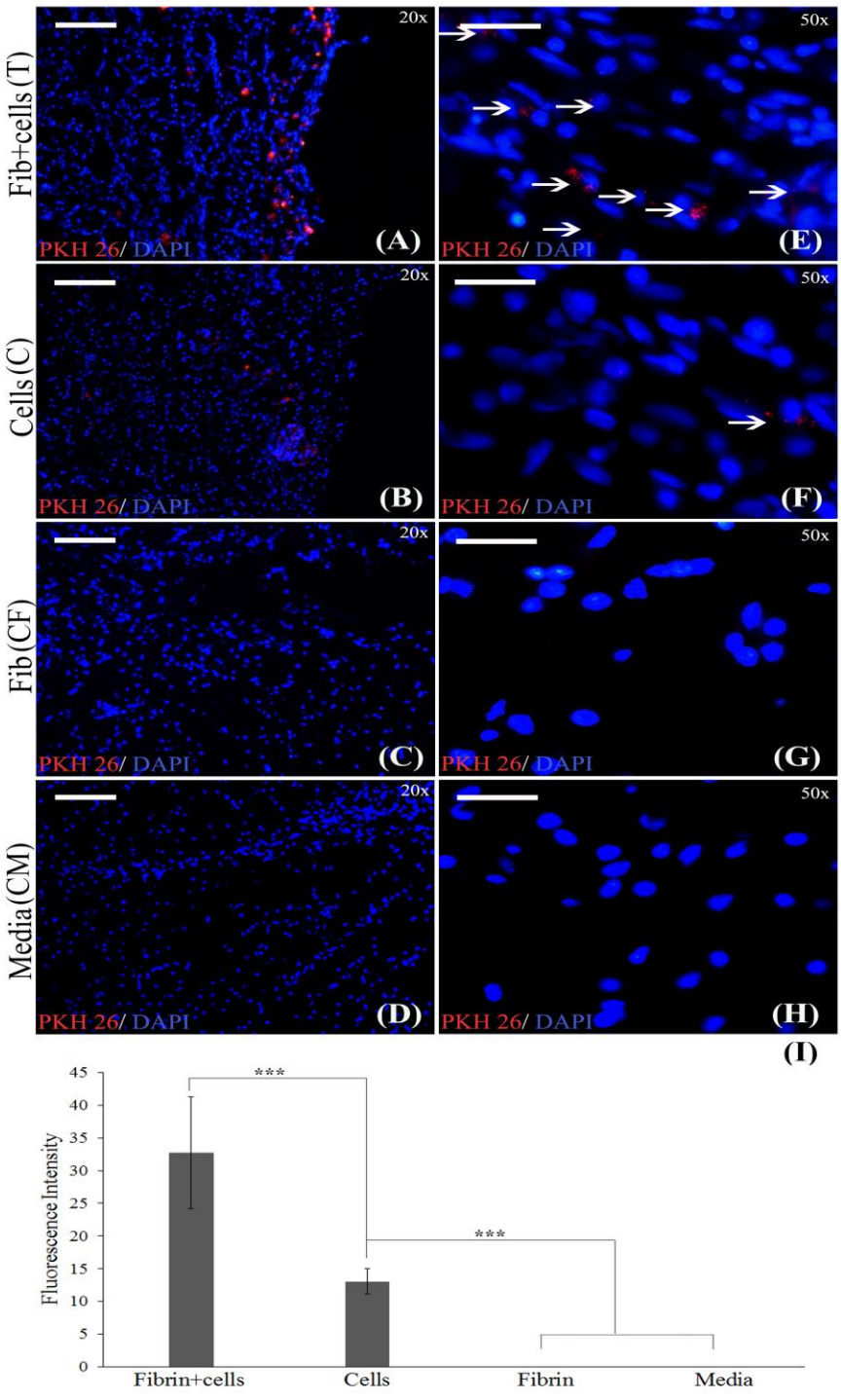


Figure 30. Fluorescent images of microsections of transplanted cells at SCI. (A&E) The red fluorescence of PKH26 in T sites (B&F) The red fluorescence of PKH26 in C sites (C&G ; D&H) Absence of red fluorescence in CF sites and CM sites. Magnification 25µm in A,B,C&D and 50µm in E,F,G&H. All the sections were counterstained with DAPI (I) Quantitative data of average fluorescence intensity of labelled cells for the test and control groups measuring T >C>CF/CM. Significance is indicated as '***' ($P < 0.001$), '**' ($P < 0.01$) and '*' ($P < 0.05$).

4.4.3.5. Fate of transplanted NPCs

The transplanted cells co-expressed green fluorescence of immunostaining for β-III tub and Syn respectively, with the red fluorescence of PKH-label at varying degrees. The co-localisation of fluorescence represented in the **Figure 31** panel reveal that many of the transplanted NPCs retained in the 'T' animals may have progressed into β-III tub^+ intermediate neurons and a few cells differentiated into Syn^+ mature/functional cells (**Figure 31 A and E**). In the 'C' animals, very few cells expressed β-III tub and Syn (**Figure 31 B & F**). In the 'CF' and 'CM' tissues, red fluorescence of PKH26 was not detected (**Figure 31 C, D, G & H**).

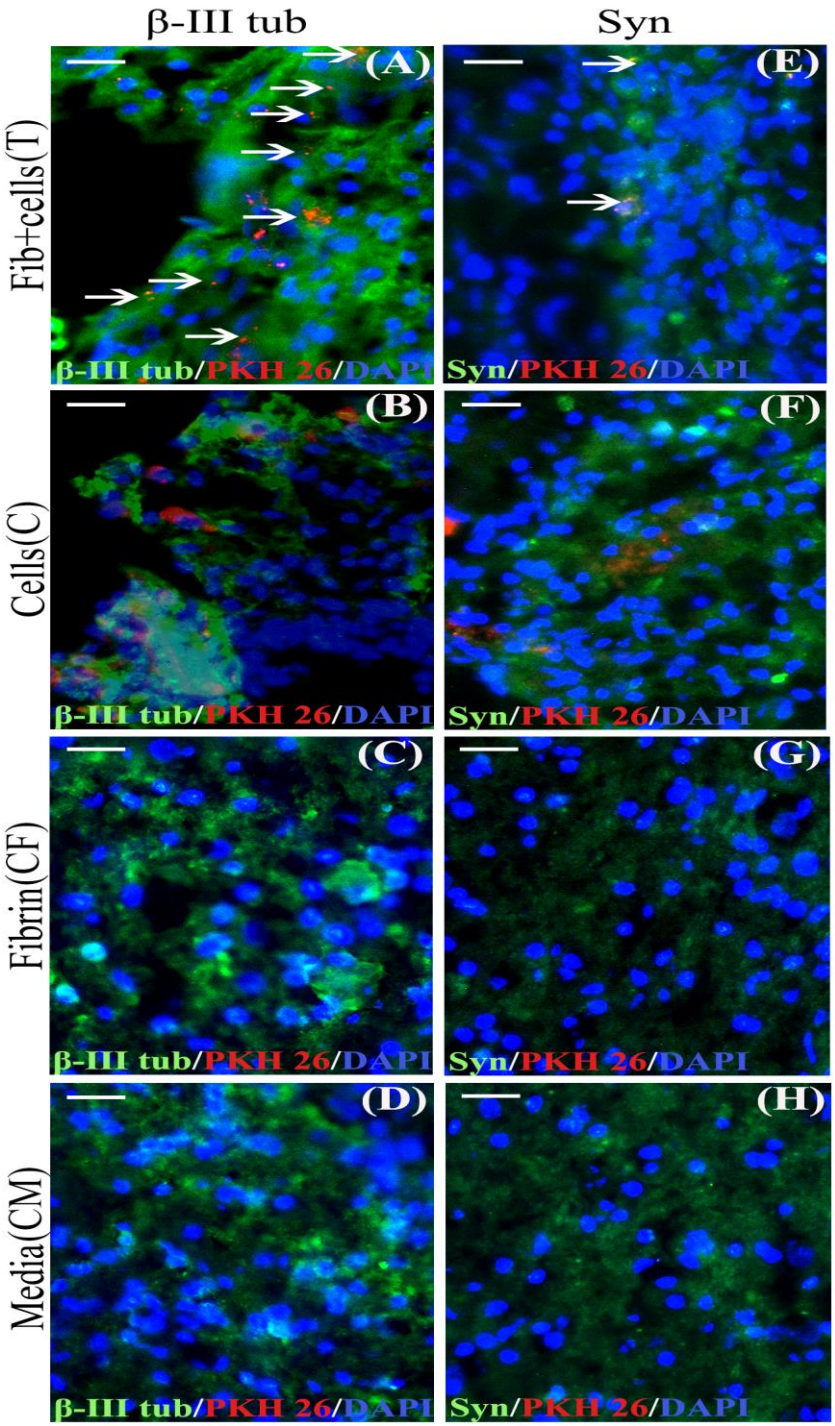


Figure 31. Co-localization of PKH26 and neural marker in transplanted cells. (A, B, C & D) Merged images of β-III tubulin and PKH26 (E, F, G & H) Merged images of Syn and PKH26 (A& E) correspond to T; (B&F) correspond to C; (C&G) correspond to CF; (D&H) correspond to CM. Merged images showing green fluorescence of Alexafluor-488 conjugated to secondary antibody used for developing neural markers, red fluorescence of PKH26, co-localized area of both red and green showing orange colour in the midst of blue fluorescence of DAPI. Magnification scale bar is 25μm.

4.4.3.6. Clinical outcome

All animals showed a BBB score of 1 after the injury which is represented as day 0 in the graph (**Figure 32**). The score improved to ~3 by day 4 in all animal groups. By day 8, there was an improvement in the score to ~8 in the test animals as compared to ~6 in cells group and ~5 in vehicle controls. Even though there was a slight improvement in the test group scoring in this proof of concept study, further evaluation is needed to see if the difference will be also reflected in the long-term study.

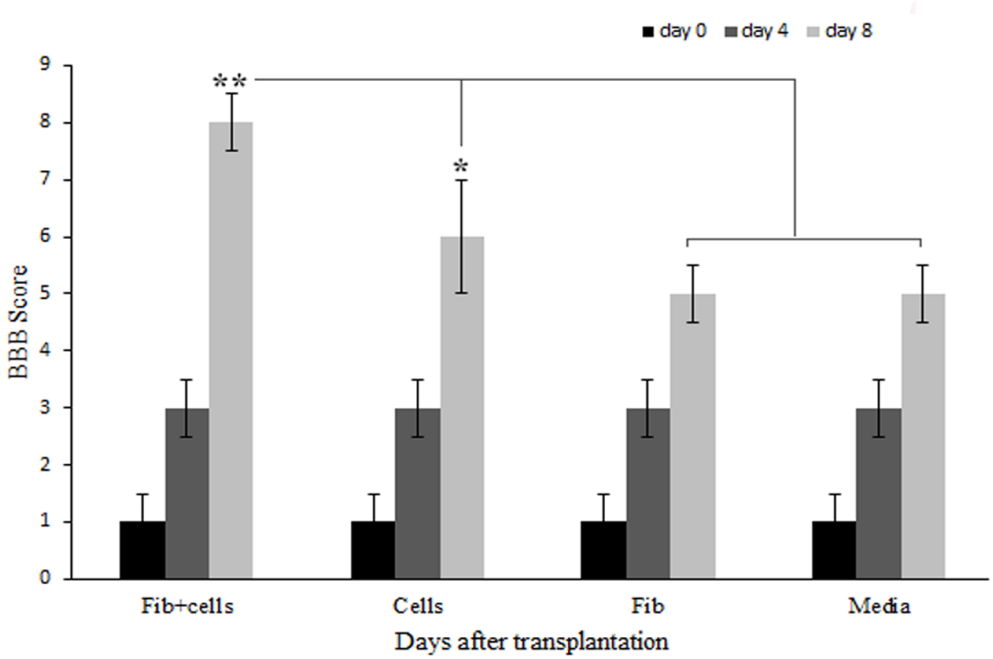


Figure 32. Functional evaluation. BBB score of all groups studied for 3 time periods showing an improvement in locomotor function for the test group on day8 as compared to the controls. Significance is indicated as '***' ($P < 0.001$), '**' ($P < 0.01$) and '*' ($P < 0.05$).

The summary of # 4.4 is presented in **Figure 33.**

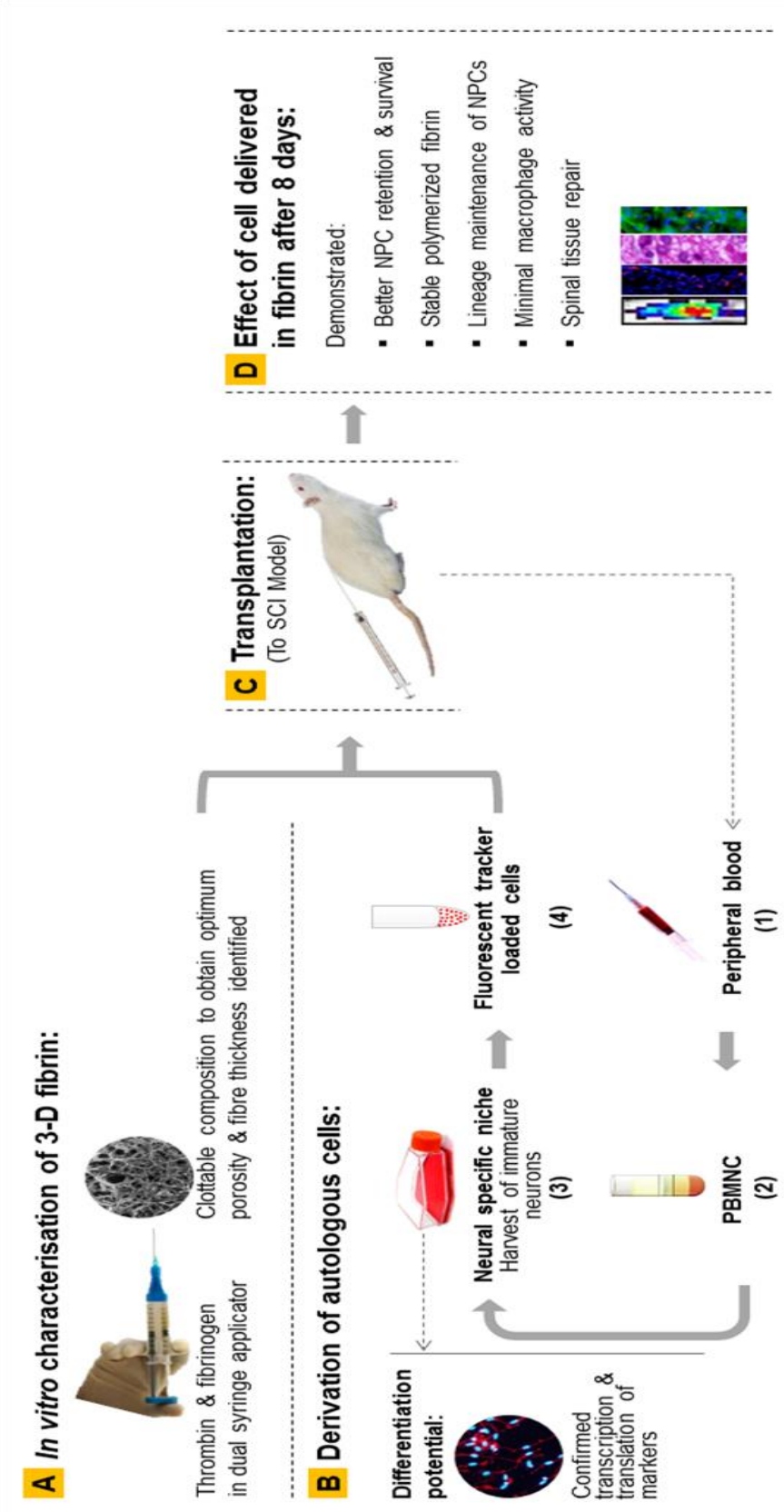


Figure 33. The summary of cell transplantation and evaluation section

CHAPTER 5

5. DISCUSSION

The important results of the study that are illustrated in chapter 4 are discussed in this chapter. Major findings of the study are correlated to recently published literature in the relevant field of study and interpretations are made wherever possible. The limitations of the study are also discussed.

5.1. Standardisation of fibrin composite niche

Autologous transplants may be beneficial since they can avoid common complications such as rejection and immunosuppression associated with allogeneic cell sources. The neural stem cells (NSCs) available in CNS are limited and their use is not practical for therapeutic purposes. Therefore, an easily accessible autologous stem cell source needs to be identified for clinical use. The concept that NPCs in PBMNC might differentiate into nearly functional NLCs using a niche-directed strategy was successfully demonstrated in this study. The results suggest that autologous immature neural cells could be obtained from peripheral blood for patient-specific regenerative therapy without much risk. The development of an optimised niche for the differentiation of circulating NPCs to NLCs was also achieved. Due to the enormous proliferation potential and multipotency of BMDSCs, they are one of the adult cell types in regenerative medicine that is most widely experimented (Goel et al., 2009). The mononuclear cells from BM have been reported to migrate into circulation under many circumstances (Krause et al., 2001). It was found that when the marrow cells were systemically infused into the

peripheral blood of irradiated mice, they migrated into the brain and differentiated into cells of neural lineage, suggesting their ability to cross the blood-brain barrier and differentiate into a specific cell type, by the influence of an appropriate niche (Brazelton et al., 2000). Even though stem cells in peripheral blood were identified decades ago, it has not been long since their ability to differentiate to cells other than blood cells was identified (Zhao et al., 2003). This study results suggest that if bone marrow can be stimulated to release nestin+ progenitor cells into blood, they could be directed to neural lineage by providing appropriate niche conditions *in vitro*. In addition, it is also clear that a specific composition of the niche is essential to differentiate NPC to committed NLCs, *in vitro*. The composition of specific components in the niche has been varied to obtain the final formula that favoured isolation of NPCs with proliferation potential. The potential of these NPCs to differentiate in to neurons was also proven in the niche. It was found during optimisation that the inclusion of certain substances known to promote neuronal differentiation in other stem cell systems not only had any additional benefit when added to the matrix, but also resulted in cellular changes that may result in cell death upon addition to medium. Major components which improved cell growth are fibrin, gelatin, HA and growth factors. Addition of KCl improved cell length and cell-to-cell contact.

Fibrin used for the constitution of matrix has been employed in various clinical and bioengineering applications. It is used clinically as hemostatic glue and in wound repair. Fibrin provides a permissive environment for cell attachment, growth and migration. Fibrin has been found to be effective as a scaffold for vascular, skin and nerve tissue engineering (Sreerekha et al., 2006)(Willerth et al., 2006)(Nair and

Krishnan, 2013). In the current study, fibrin-based niche was formulated using fibrinogen and thrombin isolated from human blood plasma. Fibrin interacts with integrins and cadherins to promote cell attachment. The bioactivity of fibrin makes it an attractive matrix for stem cell differentiation, tissue engineering and cell delivery. The ability of fibrin to harness GFs has been exploited for the differentiation of embryonic stem cells (ESCs) to neurons (Willerth et al., 2007). An analogous approach was used in this study to differentiate circulating NPC to neurons. Fibrin sealant has been known to be useful for promoting the repair of severe peripheral nerve injuries for a long time (Jubran and Widenfalk, 2003). Use of fibrin for differentiating mouse/human ESCs to neural/astroglial lineages by modulating its properties has also been reported (Janmey et al., 2009). The effective fibrin scaffold composition varies with cell type. Therefore, there is a need to optimize cell specific concentration to limit the extent of cell death and to facilitate cell adhesion and migration (Willerth et al., 2006).

In this study, the combination of fibrin and other components that produced lineage-committed neural cells and that appeared most healthy was selected for tracking the extent of differentiation. The presence of BDNF, FGF, and VEGF were demonstrated in the cocktails of GF used. It is considered that BDNF is crucial for neural differentiation, proliferation, survival, synaptic function and neurite regeneration in both *in vitro* and *in vivo* conditions. The presence of PDGF in the PGF cocktail is well established and has been reported to play a role in neural differentiation as well (Johnson et al., 2010b). Thus, other than the presence of essential adhesive proteins, the GF components that are known to support NPC growth and differentiation were demonstrated in the matrix composed. Although

FGF, VEGF and PDGF are not classical neurotrophic factors, they are mitogens and inducers for *in vitro* growth of glial precursors. Stem cell survival and differentiation into specific neural lineage is known to be influenced by PDGF (Willerth et al., 2007). In the context of ASCs, bFGF was used to differentiate mouse bone marrow stromal cells towards neurons (Yang et al., 2008). The GF when supplied at the injury sites, FGF promoted neural progenitor proliferation, survival, and enhanced recovery. Thus many experiments demonstrated direct and important roles for VEGF in regulating neural progenitor proliferation, their survival, migration, axon/dendrite patterning, differentiation, synaptic function, neuroprotection and glial growth (Mackenzie and Ruhrberg, 2012)(Kim et al., 2010)(Fournier et al., 2012). Receptors for VEGF are present in neurons and the neurogenesis occurs in close proximity to growing blood vessels (Fournier et al., 2012). Treatment of neuronal cultures with VEGF has enhanced survival and neurite growth independently (Rosenstein et al., 2010). All these reports on the effect of GFs on neural cell growth and differentiation formed the basis for the use of the GF cocktail in this study for differentiating the circulating NPCs to NLCs. It was also proven that these GFs were present in the cocktails used. Hyaluronic acid plays a leading structural role in the formation of ECM of central nervous system. It has been shown that when HA-poly-D-lysine hydrogels were implanted into traumatic mouse brain, the matrix promoted CNS regeneration (Tian et al., 2005). The sprouted neural outgrowth colonies obtained upon the culture of PBMNCs on the FC matrix in our study resembled neuronal outgrowth cells attained from embryoid bodies by other investigators (Dottori and Pera, 2008). Adhesive proteins FN and La are found to be crucial for *in vitro* neural cell growth (Jose and Krishnan, 2010). Examination of in-house

prepared fibrinogen concentrate (Krishnan et al., 2003) used for matrix construction demonstrated the presence of both of these proteins using a simple immunodiffusion experiment; therefore, exogenous addition of these components into the matrix is not an essential requirement for obtaining neural differentiation. If there is an additional benefit, if exogenous La and FN are supplemented was not tested in this study.

5.2. Regulated proliferation and differentiation

The lineage commitment of peripheral blood derived NPC progressed stage by stage; indicating stability of the process. Most of the time spontaneous biological changes may turn out to be reversible or detrimental to cells causing cell death and necrosis. The cellular changes that occurred in this protocol was found to be slow and steady; Because of this advantage, NPCs at a suitable differentiation/proliferation status could be selected for translational purpose. The results imply that the intermediate NPCs obtained from culture may differentiate into functional neurons *in vivo*, upon transplantation. The critical nature of *in vitro* differentiation niche suggests the importance of the availability of a suitable signal for the success of *in vivo* transplantation as well.

The progressive transition of circulating progenitors was found to be promising. Expression of nestin was followed by β-III tub and later by MAP-2. The progression of differentiation appeared to be a slow and steady process. The number of NPCs obtained from circulation was low, but NPCs selected by the niche proliferated during the initial phase of lineage commitment. It is well known that proliferation is inversely related to differentiation and the results of this study also showed a similar trend. Maximum proliferation was observed when only the immature neural marker

β-III tub expression was predominant. The cells were >90% positive demonstrating the purity of neurons obtained, which is remarkable. The observed proliferation profile suggests that the cells have not turned into mature NLCs until 8d because at this stage NPCs still retained the ability to divide. By the time the mature neuronal marker MAP-2 was detected, proliferation had also declined. The mature NLCs derived from the MAPC population expressed key functional proteins such as Syn and TH. The interesting observation in this study was the rare glial cell types obtained. Myelin forming oligodendrocytes were 10% of the differentiated population. In the context of regeneration of CNS injuries, more oligodendrocytes involvement is required; it may be beneficial to have them at a higher proportion. However, in order to get those, a separate cell-specific protocol may be necessary. Since this protocol produced less glial scar forming astrocytes (3%), the cells produced using this protocol may show less adverse effect upon transplantation. Potassium chloride is known to regulate the neural activity, which in turn is responsible for controlling a variety of functions including memory, synaptic transmission, neurite outgrowth, differentiation, synaptogenesis, cell death and survival (Kim et al., 2010). Treatment of the NPC culture with excess KCl as compared to what is available in the standard culture medium had profound influence on Syn and TH expression. Exposure to excess KCl has been shown to result in membrane depolarisation and Ca^{2+} influx through voltage-sensitive calcium channels, and subsequently triggers several calcium-dependent signaling pathways that eventually lead to changes in gene expression. It has been shown that the exposure of developing sensory neurons to KCl depolarizing stimuli increased the proportion of TH^{+} neurons (Brosenitsch et al., 1998). The marked increase in neural length just

after 3d of KCl addition signifies its importance in cell stimulation. The ion channels through which the signaling molecules act are known to be involved in the regulation of cell differentiation. The results of this study also signify that KCl had a profound influence to improve neural morphology and neural protein marker expression, making it a suitable method for achieving NPC differentiation for translational purpose. The reduction in both proliferation and cell length was found to parallel the reduced cell survival, reflecting on the apoptosis assay. It was interesting to note that differentiation and associated cell death was higher under the influence of additional KCl. Expression of mature markers was down-regulated when the culture period was extended to >16d; more of it when culture niche contained excess KCl. Generally, mature primary neurons do not survive in culture *in vitro*. Therefore, it is more likely that down-regulation of neural genes/proteins is a consequence of cell death. It may also be interpreted that because NLCs derived from circulating progenitors reached the fully mature stage on the niche, they did not survive long term in the culture. The behaviour of transplanted NPCs may not follow the same profile because other *in vivo* signals may act and prevent cell death. Since *in vitro* differentiation of NPCs into NLCs on fibrin-based niche is proven, an equivalent niche may serve as a cell delivery vehicle for successful transplantation of lineage- committed neural-like cells *in vivo*.

5.3. Identification of Wnt signaling

This study identified Wnt signaling as a crucial factor involved in the lineage commitment of NPCs present in the MAPCs towards neurons. Wnt-3a signals were found to be emanating from the niche. The signals were active and induced the

downstream Wnt target genes. Wnt signals were also implicated in the morphological, cellular and molecular level changes occurring during the lineage commitment process of NPCs.

Wnt family of secreted signaling proteins play very important role during the development of nervous system as well as in adult brain. Its primary mode of action is through β-catenin and cadherin pathways for cell-cell adhesion where it plays role in organisation and maintenance of stem cells (Dravid et al., 2005). Wnt signaling activation leads to the accumulation of β-catenin in nuclei, where it binds to high mobility group (HMG) box transcription factors of the T-cell factor (TCF) and lymphoid enhancing factor (LEF) families and promotes situation- dependent changes in transcription (Moon, 2005). It also has role in regulating cell fate decisions (Teo et al., 2006). They regulate the proliferation of NPC and their differentiation to neurons in the neurogenic regions of the brain (Alvarez-Palazuelos et al., 2011). Wnt signaling is also an obligate component involved in NPC differentiation into neurons (Cajánek et al., 2009). This multiple roles of Wnt indeed led this study to know the role of Wnt in our MAPC system. In order to carry out this study, we adopted a gradual serum-free protocol to culture cells first in bFGF. Then bFGF was withdrawn to allow the process of differentiation. Serum supplementation initially helped in the attachment and initial growth of cells. The serum was then depleted to eliminate the effect of other serum components on Wnt. Wnt-3a is an activator of canonical Wnt signaling in a variety of cell systems (Lee et al., 2000).The inhibitor, PNU74654 inhibits the interaction between β-catenin and T cell factor 4 (Tcf4) and disrupts the Wnt signaling pathway.

The Wnt and the inhibitor concentration used in this study were selected after many experiments. A range of concentrations were tried for both Wnt as well as Inh (Kondo et al., 2011)(Demilly et al., 2013) and the optimum concentration that produced an effect at which it is also non-toxic to cells was selected for the study. Similarly, Wnt and Inh addition and withdrawal time period was also optimised using cell culture in the presence of both molecules at various time points. Since addition of Wnt or Inh to the matrix did not cause any significant difference, the components were added only to the medium for all studies. Since there was no morphological difference between the Std niche and the Wnt+ cultures, and the addition of Inh affected the cell morphology, it was assumed that Wnt would be present in the niche. After 8 days of culture, there was a drastic decrease in cell number in the Inh added culture. The rounded-off, detached cells were seen in culture, probably be due to the lack of signals for attachment and migration. This suggests a role for Wnt-3a in promoting cell attachment and elongation of PBMNC-derived NPCs. There are reports stating that Wnt-3a signal has role in reorganising the cytoskeleton. Wnt causes rearrangement of F-actin, other actin associated proteins and villin (Shibamoto et al., 1998). Blocking of matrix components inhibited cell lengthening with maximum effect upon combined blocking of Fib-FN-La. Fibronectin is already known to direct cell adhesion, spreading, outgrowth and migration (Chen et al., 2012). Fibrin and La are also identified to have effect on cell attachment. Fibronectin binding to Fib is required for maximal cell attachment (Corbett et al., 1996). When blocking of matrix components occurs, the signals for attachment and binding might be lost.

The activation of Wnt target genes such as Axin2, LEF and cyclinD1 were studied in PBMNC-derived NPC system (Hirsch et al., 2007). It was found that the genes known to be induced by Wnt/β-catenin signaling in murine systems was inducible in human NPCs as well (Hübner et al., 2010). Irrespective of the morphological difference observed upon blocking (Fib+FN+La) or FN alone, almost all genes were downregulated when the matrix components were blocked indicating the role of each and every component at molecular level. There was a marginal increase in cell proliferation in Wnt-3a added cultures and a marginal decrease in proliferation in Inh added cultures as compared to Std niche. Wnt pathway activation by a specific pharmacological inhibitor of glycogen synthase kinase-3 maintains the undifferentiated phenotype in both types of ESCs and sustains expression of the pluripotency-specific transcription factors. Wnt signaling is endogenously activated in undifferentiated mouse embryonic stem cells and is downregulated upon differentiation (Sato et al., 2004). In another study, Wnt-3a conditioned media, but not Wnt-11 was found to maintain ES cells in the proliferation/self-renewal state without differentiation. However, purified Wnt-3a did not maintain self-renewal of ES cells for prolonged intervals. This means that, other factors in the medium conditioned by Wnt-3a expressing cells may have contributed to maintenance of ES cells (Singla et al., 2006). In the retina of adult mammals, Wnt signaling promoted proliferation as well as regeneration (Osakada et al., 2007). The effect of blocking Fib+FN+La or FN was comparable to the action of the Inh, indicating that the matrix components are important for activation of Wnt pathway in the matrix-directed differentiation of PBMNC-derived NPC which is demonstrated in this study.

Wnt-3a addition enhanced the neural gene/protein expression specific for progenitors; those for mature neurons was suppressed. Addition of Wnt inhibitor downregulated expression of immature as well as mature markers studied. This could be because of the effect of Wnt-3a on pushing the progenitors towards differentiation. The Inh action would be β-catenin mediated since the action is through inhibition of β-catenin binding to Tcf. In human NPCs, when the effects of Wnt-3a and β-catenin on the differentiation was examined, it was found that neurogenesis induced by Wnt-3a was independent of the transcriptional activity of β-catenin pathway (Hübner et al., 2010).Wnt-3a signaling mediated by β-catenin promoted differentiation in P19 cell line through axin-down regulation (Lyu et al., 2003). Wnt/β-Catenin signaling blockade using Dickkopf1/ by using cells lacking Wnt 1 or LDL receptor-related protein 6 promoted neuronal induction and dopaminergic differentiation in embryonic stem cells (Cajánek et al., 2009). Several signaling systems including Notch, FGF2 and EGF are known to play roles in the maintenance and differentiation of neural stem cells. Blocking all components together up regulated progenitor and downregulated mature neuronal markers, suggesting the role of matrix in promoting differentiation and regulating proliferation. Individually, the matrix component showed a mixed effect upon blocking probably due to the crosstalk of multiple signaling pathways involved in the differentiation process. Only when different pathways are studied simultaneously, such mechanisms can be elucidated clearly.

5.4. Standardisation of cell delivery matrix

In the absence of pharmacological antidote for SCI, cell therapy may have promising prospects. Identification of suitable cell source and their development *in vitro* into neural progenitors are important steps in designing cell based therapy. In addition,

there is a need to develop a cell delivery strategy which may circumvent problems associated with poor niche conditions at the injured site. The primary focus of this study was to establish that injectable fibrin sealant could be a suitable cell delivery matrix which might support retention of transplanted NPCs. The short-term analysis of transplant site inquired cell retention and immune response status consequent to transplantation of autologous NPCs embedded in natural protein-based delivery matrix. The experimental design involved standardization of cytocompatible matrix composition for cell delivery, sourcing of NPC from circulating rat blood and use of inbred animals as SCI model to test efficacy of the treatment strategy. Better cell retention and immunomodulation owing to the presence of fibrin matrix either with or without NPCs were the striking observations. Even though evaluation of the transplanted SCI site was carried out after a short term, the results of this study is encouraging and promise prospects for advanced study using the same model.

Additional highlights of this proof-of-concept study were obtaining nearly homogenous animal NPC with ability to differentiate into neurons, which could be harvested at an early immature stage from culture for transplantation into SCI. More importantly for the first time this study showed that to have a 3-D fibrin matrix compatible for neural cell growth, thrombin concentration needs to be carefully controlled. It was interesting to observe that both fibrinogen and thrombin should be used at specific proportion to obtain sufficient porosity and fiber thickness which in turn might influence cell attachment, migration and differentiation. Further, it is demonstrated that the injection of *in vitro* standardized composition to the SCI resulted in the formation of *in situ* fibrin which is stable for a week or more to retain the cells embedded in the fibrinogen. It is also noticed that upon delivery of fibrin,

with or without NPCs, presence of macrophage markers were minimal until 8th day of transplantation. Usually, cell mediated immune response is an immediate effect to injury and foreign substance. The transplantation sites were free of any indicator of acute immune response. This is an important observation which should be evaluated in more detail to understand if the immune reaction due to injury can be arrested by the fibrin injected to SCI site immediately upon presenting with injury. In addition to the emphasis on the use of fibrin matrix *in vitro* and *in vivo* for developing a strategy for cell-based therapy, this is a novel attempt to transplant NPC from circulating blood of inbred rats to the SCI site in the species.

Autologous stem/progenitor has an important advantage that immune-suppressive drug administration may be avoided and consequent side effects can be reduced (ANGHEL et al., 2013). To avoid such adverse effects of immune suppressants, many studies employ knockout mice (Sullivan and Stiehm, 2014). Autologous transplantation of stem cells derived from patient's bone marrow and adipose tissue have been reported to show positive outcome (Syková et al., 2006). Autologous Schwann cells derived from peripheral nerves or OECs from the olfactory bulb mucosa have also been shown to mediate regeneration of axons/ recovery of function (Féron et al., 2005). In the current study, circulating NPCs from inbred animals was used and upon analysis of the transplanted site, immune reaction was minimal and macrophage activation was not seen in the presence of fibrin. Eight days were given from the time of injury and transplantation to analyze the tissue for possible immune reaction, which is considered to be a reasonable period for showing up possible immuno-response. However, there is a need for more specific markers to establish immune reaction is minimal. Even with this limitation, the study suggests

that the cells from inbred animals could be suitable for use as autologous cells for studying long-term effect of cell transplantation as well.

Important aspects that need to be considered for increasing the success of cell-based therapy include development of minimally invasive but highly effective delivery strategies, optimal cell dose, proper timing of cell transplantation post injury, survival of transplanted cells in the host and efficacy of transplants to restore function of specific types of damaged or lost cells (Pêgo et al., 2012)(Nandoe Tewarie et al., 2009). Out of these, protection of transplanted cells from the hostile niche was the major advantage of using injected fibrin as the cell delivery vehicle. This observation is of great significance and a more detailed study is indicated from the results of the preliminary study. Since different adhesive proteins are present in the delivery matrix, it is expected that the adhesive forces would promote initial cell retention and long-term cell homing. It appears that when liquid fibrinogen forms into a clot with embedded cells *in situ* upon delivery to the SCI, the resultant fibrin functions as a scaffold for neural engineering. Upon analysis of the gross and sectioned tissue, it is observed that the injected fibrin is retained at the injection site until the termination of experiment in the 'T' animals. Interestingly in both 'T' and 'CF' animals, fibrin was retained and macrophage activity was minimal. Therefore, it is postulated that even fibrin without cells may be beneficial to prevent the injury-associated immune response and more detailed long-term in this respect is proposed.

It has been reported that upon use of fibrin to deliver human MSCs at peripheral nerve injury site, a strikingly diminished invasion by host cells was the outcome suggesting that this natural scaffold *per se* acts as a barrier against cellular invasion from the surrounding host tissue (Kappos et al., 2015). For clinical applications, a

biomaterial must have specific tailored physico-chemical and mechanical properties that allow spinal cord stabilization, cell attachment as well as growth. It must also possess adequate porosity and permeability for the diffusion of ions, nutrients, and waste products. More importantly, it should be biocompatible and biodegradable. The biomaterial should also degrade by the time the new tissue grows in, thus mimicking the natural mechanisms of breakdown and synthesis of ECM in the natural tissues (Silva et al., 2014)(Little et al., 2008). Fibrin is a biomaterial that satisfies most of these criteria. Possibility of maintaining fibrin components at a liquid state during injection and ability to solidify into tissue engineering scaffold *in vivo* was considered the most important benefit of this cell delivery system (Sharp et al., 2012). This study targeted standardization of component composition *in vitro* appropriate for effective gelling into fibrin and for subsequent stability for a sufficient period after cell transplantation. More evaluation is essential to understand how long the fibrin remains and if upon degradation, the degradation products might cause any adverse reaction in the spinal cord tissue. Fibrin based hydrogels seemed to have supported and enhanced mammalian neurite outgrowth of both dendrite as well as axon (Ju et al., 2007). Thrombin, at low concentrations is a potent mitogen. However, recent studies report that exposure to higher doses of thrombin could lead to apoptosis of neurons (Donovan et al., 1997). Prolonged exposure of NPCs to thrombin *in vitro* was found to exert pro-apoptotic effect on cultured vascular smooth muscle cells (VSMCs). There was parallel occurrence of cell retraction and cleavage of fibronectin suggesting that thrombin-induced apoptosis is consecutive to pericellular proteolysis (Rossignol et al., 2004). This thrombin-induced cell death was found to be mediated through the activation of Rho A (Donovan et al., 1997). In

this study, it is noticed that low concentration of thrombin could be used for clotting fibrinogen *in situ* to retain cells, which is unlikely adverse effect on NPC growth and survival. There is enough evidence in this study to suggest that higher concentrations of thrombin could be toxic, affecting cell attachment, growth and survival.

Biomaterial scaffolds and in particular fibrin scaffolds is capable of enhancing survival of transplanted cells to provide a favourable environment *in vivo* as well as for stem cell culture *in vitro* have been shown earlier (Willerth et al., 2006). The preliminary observation that ED-2, a marker for the infiltrating macrophages, was minimal when fibrin was injected with or without cells could be an important point of the study which needs to be pursued in more detail. Immuno-/histochemical analysis of the injury site revealed retention of neural properties by transplanted cells even after 8 days. The results of this preliminary evaluation opened scope to further evaluate if transplanted NPCs progress into neural cells using the same experimental design to confirm role of transplanted matrix and cells in regeneration of neural injuries.

5.5. Limitations of the study

The study has certain limitations. Isolation and differentiation of NPCs from healthy human subjects/rat was established and not diseased patients. Even though there are reports stating the presence of stem cells/progenitor cells in diseased patients, the study may be extended with blood from SCI patients as well. Neuronal sub typing may be performed which would help identify the specificity of the derived cells. Even though, molecular markers suggest possibility of synapse formation patch-clamp technology needs to be applied to establish electrical conductivity when ion channels are stimulated in culture. The preliminary observation with transplantation

studies show promise; however, lack of long-term analysis results reduce the scope for practical application of the scientific observations made.

CHAPTER 6

6. SUMMARY AND CONCLUSION

The expected achievements of this study were i) standardisation of protocol for NPC isolation from adult peripheral blood which could be applicable for nervous system regeneration ii) proving the potential of NPCs differentiation in to NLCs in a controlled manner using a biomimetic approach and be able to harvest transplantable cells iii) confirming the role of regular neuronal signaling mechanism in differentiation of NPCs and iv) transplantation of NPCs in rat SCI model to study the feasibility of proving the concept that a suitable composition of fibrin can be used for successful cell delivery to the injured site. At the end of the study more promising outcomes were achieved such as: (i) NLC differentiation to the extent of expression of synaptophysin and TH were observed when excess KCl was added in the culture medium; (ii) Critical concentration of fibrinogen and thrombin for obtaining *in vitro* and *in situ* fibrin clot with suitable physical and biological properties for optimum neural cell growth identified; (iii) The neural progenitor cells isolated from inbred animals behaved similar to autologous cells making in-bred colony of rat a suitable model for studying the regenerative outcome of cell transplantation; (iv) The fibrin composite injected to the SCI prevented immune response to the injury postulating an inhibitory effect of its injection to acute SCI against immune reaction.

6.1. Summary

The advantage of biomimetic niche produced by combining adhesive proteins, GFs and GAGs over the addition of individual constituents to the fibrin matrix, on neural cell differentiation and proliferation has been proven. The biomimetic niche approach was found to regulate neural induction and growth. The requirement of chemical induction for promoting neuronal differentiation was eliminated. Optimum concentration of GFs and GAGs for neuronal lineage commitment was determined. Potential of the optimised niche for the isolation of nestin+ progenitors from peripheral circulation, and further the action of niche to support proliferation and neural lineage progression was demonstrated at transcriptional and translational levels using neural specific markers. The composed niche was proven to comprise essential adhesive proteins and growth factors for inducing neuronal differentiation by employing simple immunochemical techniques.

Transition of neural progenitors from circulation into NLCs was found to progress in a programmed manner. Progenitor marker nestin disappeared in concurrence with progress in expression of immature neuronal marker β-III tub. Subsequent expression of mature neuronal marker MAP-2 confirmed neuronal differentiation. Appearance of mature marker showed inverse relation to proliferation potential. Synchronised onset of apoptosis and decline of proliferation was also evident. KCl had profound influence on cell morphology and expression of functional proteins such as Syn and TH, with no significant effect on proliferation. Cells between 6 to 8 days of *in vitro* culture showed best neural progenitor properties. Cells between 8-12 days revealed homogeneity, best neural like cell proliferation and survival.

The slow and steady differentiation program suggested possible involvement of regular signaling mechanisms in the transition of cell phenotype. Using inhibitor of Wnt-3a, a crucial role of this molecule in the NPC lineage commitment to neurons was confirmed. It was also proven that Wnt-3a signals was elicited by constituent molecules present in the fibrin-based niche which were responsible for isolation and differentiation of NPCs. The downstream pathways operating in the Wnt-3a signaling was also demonstrated to get inhibited by Wnt-3a inhibition by chemical inhibitor or biological blocker such as antibodies.

Optimum non-toxic thrombin concentration for preparation of fibrin gel that best supports NLC attachment, elongation and survival was identified for potential use as *in vivo* cell delivery matrix. In addition, an optimum concentration of fibrinogen was found to produce 3-D fibrin *in vitro* with suitable porosity and fiber strand dimensions for cell migration and spreading, respectively. The effective clotting of fibrin in situ when the components were injected into the rat SCI was found with the clot stability until 8^{th} day of injection. The niche conditions optimized for human NPC isolation and differentiation favoured selection of rat NPCs from rat circulating PBMNCs. The isolated NPCs were demonstrated to proliferate and commit into neuronal cell lineage for use of the cells from in-bred rats as autologous cells for transplantation to SCI sites.

Contusion SCI animal model was successfully created with resultant clinical symptoms of SCI. The harvest of rat NPCs from PBMNCs before maturation and delivery to SCI employing injection of the 3-D fibrin delivery system allowed retention of more cell for 8 days and retained neural markers as per histology and IF assays. No granulation tissue formation/ PG deposition was observed indicating that

transplanted cells may be well preserved at the injury site. Glycolipid deposition was less in the fibrin- injected animal, with or without NPC suggesting reparative and immunomodulatory effects of fibrin. ED-2 macrophage immunostaining confirmed the immunomodulatory role of fibrin upon cell transplantation.

6.2. Conclusion

1. A bio-mimetic composition was successfully formulated for isolation of human NPCs from circulating PBMNCs
2. The presence of critical adhesive and mitogen molecules in the composed niche was established
3. The composed niche was proven to induce proliferation and lineage commitment of the isolated NPCs to NLCs
4. The formulated niche produced 90–95% homogenous NLCs with good proliferation potential in less than 8 days
5. The differentiation of NPCs to mature NLCs was demonstrated to necessitate additional concentration of KCl in the medium
6. The culture protocol developed in this study promises feasibility of autologous sourcing of NPC from circulating blood
7. The niche-directed progressive differentiation program of NPCs permitted harvest of immature and proliferating cells for translational purpose
8. The steadiness of the differentiation indicated that the niche components elicited regular signal transduction

9. The chemical inhibitor of Wnt-3a acted on the niche and prevented NPC attachment and subsequent differentiation steps drastically.
10. The antagonistic activity of antibodies against adhesive proteins, similar to chemical inhibitor activity, confirmed presence of Wnt-3a signals in the niche
11. The concept of autologous NPC isolation and transplantation was proven using in-bred rats
12. The rat SCI model and NPC isolated from rat PBMNCs were compatible for transplantation
13. Need of a biomaterial such as fibrin niche for successful NPC delivery and retention at the site of injury was established
14. The modifications of clinically used two-component fibrin composition identified critical fibrinogen and thrombin concentrations for cell delivery
15. The injected concentrations of fibrinogen-thrombin combination resulted *in situ* clotting at the SCI site.
16. A contusion type SCI model was successfully created and studied the NPC transplantation effects of fibrin with and without cells
17. The injection of fibrin-based niche prevented immune response to the SCI, independent of NPC embedded in it.
18. The survival and maintenance of the neural like properties for 8 days after transplantation was demonstrated.

6.3. Future prospects

1. Establish electrical communication in the derived NLCs using the patch clamp technique
2. Investigate the long-term outcome of autologous cell transplantation using the same cell transplantation strategy and SCI model
3. Investigate the effect of injected fibrin niche on immunomodulation

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LIST OF PUBLICATIONS

Original papers

1. **Tara S** and Lissy K. Krishnan. Bioengineered fibrin-based niche to direct outgrowth of circulating progenitors into neuron-like cells for potential use in cellular therapy. J Neural Eng 2015; 12:036011. doi: 10.1088/1741-2560/12/3/036011 (Impact Factor 3.415)
2. **Tara S**, Harikrishnan VS, Easwer HV, Sandhyamani S, Lissy K. Krishnan. Role of fibrin-based niche for effective delivery and survival of autologous neural progenitor cells at spinal cord injury site in a rat model (under communication).
3. **Tara S** and Lissy K. Krishnan. Wnt signals from fibrin-based niche facilitate β-catenin mediated differentiation of circulating neural progenitor cells to neurons (under communication).
4. Indu S, **Tara S** and Lissy K. Krishnan. Fibrin-based niche directs bone marrow derived mesenchymal stem cells into neuron like cells (under communication).

Presentations and Conference Proceedings

1. **Tara S**, Harikrishnan VS, Easwer HV, Sandhyamani S, Lissy K. Krishnan. Combining Biomaterials and Circulating Neural Progenitor Cells for Spinal Cord Injury Regeneration, 2015. 4th TERMS World Congress, Boston, USA **(Oral)**
2. **Tara S**, Harikrishnan VS, Easwer HV, Sandhyamani S, Lissy K. Krishnan. Potential application of circulating neural progenitors and fibrin-based niche for spinal cord injury regeneration: Proof of concept. Indo-French seminar on Women in Science, 2015 through CEFIPRA, Bangalore, India **(Poster)**
3. **Indu S**, Tara S & Lissy K. Krishnan. *In vitro* differentiation of bone marrow derived rat mesenchymal stem cells to neuron-like cells using specifically designed niche, 2014.Keystone Symposia meeting on Engineering Cell Fate and Function, CA, USA **(Poster)**

4. **Tara S** & Lissy K. Krishnan. Bioengineered fibrin matrix influence differentiation of circulating multipotent adult progenitor cells to neurons: proof of concept. Science Fete 2014. Sree Chitra Tirunal Institute for Medical Sciences and Technology, India **(Oral - Best paper award)**

5. **Tara S** & Lissy K. Krishnan. Bioengineered fibrin matrix for the *in vitro* differentiation of circulating progenitor cells to neurons and *in vivo* survival in rat spinal cord injury. TERMIS Americas conference 2013, USA **(Poster-Award winning category)**

6. **Tara S** & Lissy K. Krishnan. Effect of matrix composition on the differentiation of circulating neural progenitor cells to neural like cells. "5th Meet and Indo-Us Workshop on the application of flow cytometry" 2013, Kolkata, India **(Poster)**

7. **Tara S** & Lissy K. Krishnan. Fibrin-based niche supports *in vitro* differentiation of neural progenitors from human peripheral blood and *in vivo* survival of transplanted cells in rats. ISSCR 8th annual meeting Proceedings, 2012, USA

8. Lissy K. Krishnan, Renjith P. Nair and **Tara S**. Design of biomimetic niche for adult progenitor cell selection and differentiation. The 4th Annual meeting of TCS 2011, Chandigarh, India **(Oral)**

9. **Tara S** & Lissy K. Krishnan. Effect of matrix composition on the differentiation and survival of circulating neural progenitor cells to neural-like cells. "SCSS Symposium 2011", Singapore **(Poster)**

CURRICULUM VITAE

Academic profile

2009-till date: Research Scholar, Thrombosis Research Unit, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum

2006-2009: Junior Research Fellow, Molecular Reproduction Unit, Rajiv Gandhi Centre for Biotechnology, Trivandrum.

2005-2006: Project Assistant, Toxicology Division, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum

2004-2005: Quality Analyst, M//s Endocrine Technologies (I) Pvt. Ltd, Hyderabad

2002-2004: M.Sc. Biotechnology, Periyar University

1999-2002: B.Sc. Clinical Nutrition and Dietetics, Zoology, Chemistry, Kerala University

Achievements

- Received Burroughs Wellcome Trust fund for participating in 4th TERMIS World Congress 2015
- Best paper award in the Science Fete, 2014 conducted by SCTIMST, Trivandrum
- Poster abstract of TERMIS Americas, 2013 was considered in the award winning category
- Received Stem Cell Society Singapore travel award to attend conference in Singapore, 2011
- Received travel support from DST and ICMR in 2013 & 2015 for attending conferences in USA
- Qualified national eligibility tests CSIR/NET JRF in June 2008, Qualified NET/LS in June 2007
- 1/80 students selected at National level for Biotech Industrial Training Programme, 2003-04
- Top scorer in college during post-graduation, 2004

APPENDIX

PBS (1000ml) pH 7.4

NaCl	- 8g
KCl	- 0.2g
Na2HPO4	- 1.44g
KH2PO4	- 0.24g

Added distilled water to 1000ml, filtered and stored at RT.
PBST was prepared by adding 0.1% Tween-20 to PBS.

HBSS (1000ml) pH 7.4

KCl	- 0.4g
KH2PO4	- 0.06g
NaCl	- 8g
Na2PO4	- 0.0482g

Added distilled water to 1000ml, filtered, autoclaved and stored at 4^{o}C.

SFM

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

Filtered and stored at 4^{o}C

DNA gel loading dye (6X)

Glycerol	- 6ml
0.5M EDTA	- 1.2ml
Sterile water	- 2.8ml
Bromophenol blue	- 2mg
Xylene cyanol	- 2mg

Mixed. Stored at RT

10x TAE buffer (200ml) pH -8

Tris	- 9.86g
0.5M EDTA	- 4ml
Glacial acetic acid	- 2.8ml

Stored at RT. Diluted to 1x upon run.

Resolving gel buffer (1.5M)

Tris base	- 18.15g
Distilled water	- 50ml

Adjusted pH to 8.8. Brought total volume to100ml with distilled water. Stored at RT.

Stacking gel buffer (0.5M)

Tris base	- 6g
Distilled water	- 60ml

Adjust pH to 6.8. Brought total volume to100ml with distilled water. Stored at RT.

Acrylamide-bisacrylamide

Acrylamide	- 29.9g
Bisacrylamide	- 0.8g
Distilled water	- 73ml

Total volume-100ml. Filtered with 0.2µ filter. Keep protected from light and at 4^{o}C.

10% SDS

SDS	- 1g
Distilled water	- 9ml

Gently stirred and stored at RT.

APS

APS	- 0.1g
Distilled water	- 1ml

Vortexed. Prepared freshly.

Sample buffer

Distilled water	- 3.55ml
0.5M Tris HCl(pH 6.8)	- 1.25ml
Glycerol	- 2.5ml
10% SDS	- 2ml
0.5%bromophenol blue	- 0.2ml

Added mercaptoethanol and sample buffer in 1:4 proportion before use.

Coomassie Brilliant Blue

0.1% Coomassie	- 0.1g
10% acetic acid	
40% methanol	
Distilled water	- 50ml

Destain

10% acetic acid	
40% methanol	
Distilled water	- 50ml

5x electrode running buffer pH 8.3

Tris base	- 1.5g
Glycine	- 7.2g
SDS	- 0.5g
Distilled water	- 95ml

Transfer buffer

Tris base	- 0.58g
Glycine	- 0.29g
SDS	- 0.037
Methanol	- 20ml
Distilled water	- 80ml