DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS TO OLIGODENDROCYTE PROGENITOR CELLS

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INTRODUCTION

According to the 2011 annual statistical report obtained by the National Spinal Cord Injury Statistical Center (NSCISC) at the University of Alabama at Birmingham, approximately 12,000 new cases of Spinal Cord Injury (SCI) are reported each year with the average life expectancy decreasing by at least 10 years depending on the severity of the injury[1]. The 2011 annual statistical report by the NSCISC also reports that the yearly expenses of a paraplegic patient post injury is approximated to $66,106 and increases with the severity of injury[1]. It has been identified that 67% of spinal cord injuries occur due to vehicular accidents and falls.

SCI is a physically debilitating and emotionally taxing problem for patients. They can incur a permanent loss of sensory and motor function below the level of injury. Several functions of the autonomous nervous system such as bladder control, urination and sexual arousal can also be affected after a spinal cord injury. Depending on the level of injury and symptoms displayed, spinal cord injuries have different degrees of severity. The most common grading system is the ASIA scoring system described by the American Spinal Injury Association[2]. This grades injuries from A to E, where "A" is a clinically complete injury with no motor or sensory function below the level of injury. "E" is a normal individual with no motor or sensory dysfunction, and the grades in between describe varying degrees of clinically incomplete injury. An injury that lies in the cervical region of the spinal cord ie. C1-C7 can cause weakness of all four limbs known as quadriparesis, or paralysis known as quadriplegia. Depending on the location of injury in cervical spine, the severity of symptoms increase. For

example, a high cervical injury may cause death or ventilator dependency for survival with an increasing gain of function with respect to respiratory control and digestion as the level of injury progresses caudally through C2 – C7. An injury to the thoracic or upper lumbar levels of the spinal cord can cause loss of function to the lower extremities, referred to as paraparesis or paraplegia.

The majority of cases of SCI in humans occur in the form of a contusion. A contusion injury is an impact to the spinal cord that damages the capillary blood vessels causing leakage of blood into the spinal cord. This leakage of blood and blood products into the spinal cord cause a series of prolonged events referred to as "secondary injury". A spinal cord injury is comprised of a primary and a secondary injury. The secondary injury is caused by several cascades of reactions triggered by the primary injury (Fig. 1).

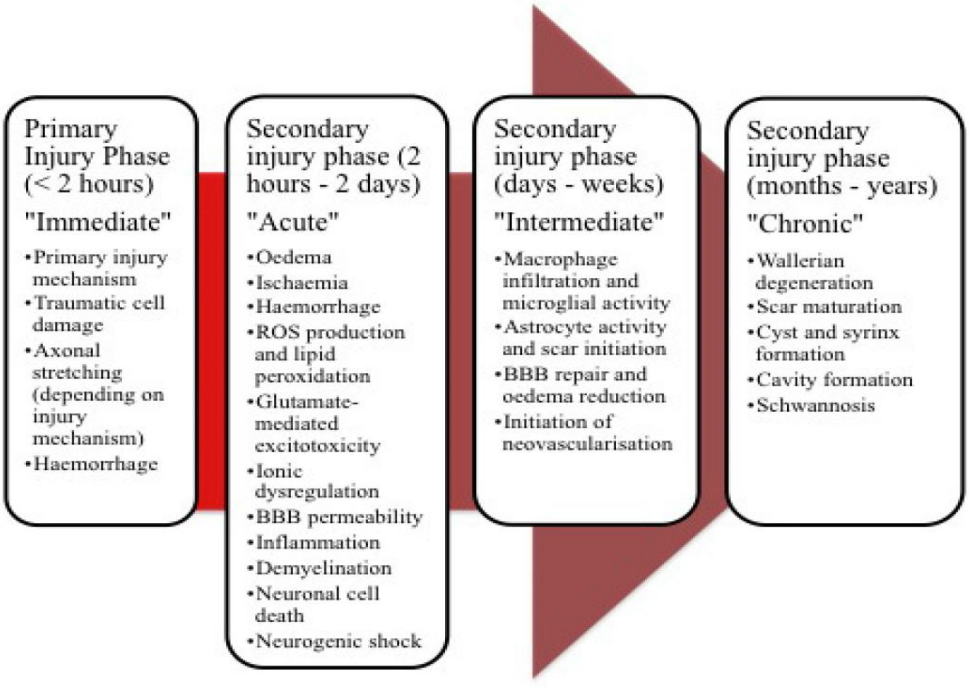

Figure 1. Progressive stages of spinal cord injury and its associated pathological events[3]

Apart from the structural damage to the column, cellular, biochemical and immunological reaction within the injury site can lead to wide-spread loss of neuronal function around the primary injury. Several of these reactions are triggered by the damage to the blood vessels and capillaries within the spinal cord[13].

Damage to the blood vessels triggers several cascades such as the clotting cascade and the complement cascade. Activation of complements and other such cytokines in the primary injury area, invites immune cells such as macrophages and other antigen presenting cells. Hemorrhaging in the gray matter upon contusion injury activates the microglia and astrocytes in the surrounding region to form the glial scar in that location. Invasion of immune cells generates a cyst in the region of primary injury[11,12,13]. This leads to further necrosis of neurons surrounding the glial scar. Permeability of the Blood Brain Barrier (BBB) is also affected thereby, losing its filtration specificity[11,12,13]. Change in permeability of the BBB leads to increased diffusion of neuro-degenerative molecules into the spinal cord. Free radicals are also produced as a reaction to the primary injury.

The free radicals and other ions produced in the injury site induce excitotoxicity. Excitotoxicity forms a major reason for neuronal apoptosis as well as oligodendrocyte apoptosis in the surrounding regions[11,12,13]. Oligodendrocyte apoptosis causes increased demyelination of axons in the white matter. Death of myelinated axons and demyelination events lead to the production of myelin debris in and around the injury location. Myelin debris is also toxic to the oligodendrocytes leading to wide-spread death of oligodendrocytes in the periphery of the injury location[11,12,13]. Demyelination of axons in the white matter leave the longitudinally traversing axons intact but they

lose their function due to loss of the insulating myelin sheath around their axons. With the progressing secondary injury, the axons in the white matter may remain intact but become non-functional due to demyelination. Although the extent to which this occurs in humans is controversial, remyelination of these demyelinated axons suggests a potential strategy for improving neurological function after SCI.

OLIGODENDROCYTES AND THEIR SIGNIFICANCE IN SCI:

Oligodendrocytes are essential for the production of the lipid-enriched myelin sheath that wraps around axons and aids in saltatory conduction in neurons. During embryonic development, the early oligodendrocyte progenitor cells are first found to emerge in the Ventricular Zone/Sub-Ventricular Zone (VZ/SVZ) of the fetal forebrain[5]. The spinal Oligodendrocyte Projenitor Cells (OPCs) arise from motor neuron precursor cells which develop in the ventral ventricular zone and undergo neurogenic/gliogenic switch to produce the OPCs. There is evidence to suggest that the OPCs that are generated during embryonic development from the motor neuron precursor cells travel large distances through the spinal cord before undergoing terminal differentiation to form mature oligodendrocytes[6](Fig 2). Another wave of OPCs are generated from the dorsal spinal cord and contribute 10-15% of the total oligodendrocytes present in the cord. The OPCs arising from the ventral ventricular zone contribute the maximum percentage of the total oligodendrocyte population of the spinal cord[7]. These mature oligodendrocytes then specifically choose neurons with axonal diameters greater than 0.2µm and ensheath the axon with myelin. A single mature oligodendrocyte can cause the myelination of several axons[7].

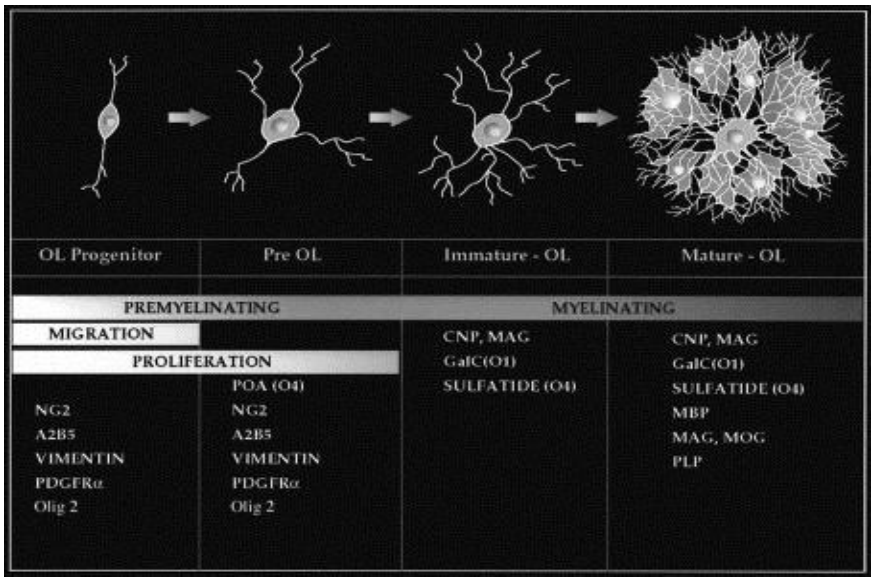

Figure 2. Oligodendrocyte Differentiation with specifying markers for each stage of differentiation. [4]

Some OPCs that migrate from the embryonic point of origin are believed to reside in the brain and spinal cord without undergoing terminal differentiation. It can be hypothesized that these resident OPCs are involved in the oligodendrocyte turn-over in the central nervous system and also in minor injury repair. OPCs retain their proliferative capacity and migrate to regions requiring myelination before undergoing terminal differentiation. These characteristics of OPCs make them an attractive target for cellular therapy in demyelinating diseases and SCI. In SCI, there is progressive demyelination of axons in the white matter during secondary injury. It appears that the resident OPCs are unable to mount a sufficient response to repair this damage. Therefore transplantation of OPCs could potentially repair this damage and restore some functionality previously lost.

There is evidence that transplantation of OPCs does result in functional improvement in a rodent model of SCI[9]. While remyelination may contribute to this improvement, there is also evidence that there are other mechanisms involved[9,27] ,

mainly because of the early improvement identified. The transplantation of OPCs in the rat optic chiasm model displayed an improvement in the P1 wave-latency, which is a determinant of the functionality of the neurons, within the first week of transplantation[27]. OPCs produced from human Embryonic Stem cells also express neurotrophic factors which may be involved in inhibiting axonal dieback and assist in regeneration of transected neurons[28]. Transplanted OPCs are also found to interact with and modulate the gene expression pattern of resident phagocytic cells and decrease the inflammatory response in the injury site[29]. OPCs are believed to have other paracrine effects around the injury site which helps decrease the pathology of the injury and provide a favorable environment for regeneration.

HUMAN EMBRYONIC STEM CELL DERIVED OPCs

Human Embryonic Stem (ES) cells are derived from the inner cell mass in the blastocyst stage of a developing embryo. These cells are pluripotent in nature and can be maintained in culture indefinitely. Under defined growth conditions, these cells can be differentiated into the cells of ectodermal, mesodermal and endodermal lineages. In studies by other groups, these cells were subjected to specific growth conditions in order to direct them towards the oligodendrocyte lineage (Fig 3). This protocol involved the use of a Glial Restrictive Medium (GRM) and Retinoic Acid[8]. During embryonic development, Retinoic Acid is responsible for triggering human ES cells towards forming spinal progenitors[6]. The protocol used to make OPCs from human ES cells was refined and optimized and a high yield of OPCs was obtained and characterized by immunostaining and quantification of immune stained cells (Fig 4)[9].

The ability to produce myelin by these OPCs derived from human ES cells was confirmed by injecting these cells into the T9 level of the spinal cord in shiverer mice (shi/shi) which have a homozygous mutation in the Myelin Basic Protein (MBP) gene and lack natural myelination. The evidence of myelination surrounding the needle tract was confirmed by immunostaining sections of the mouse spinal cord for MBP[9].

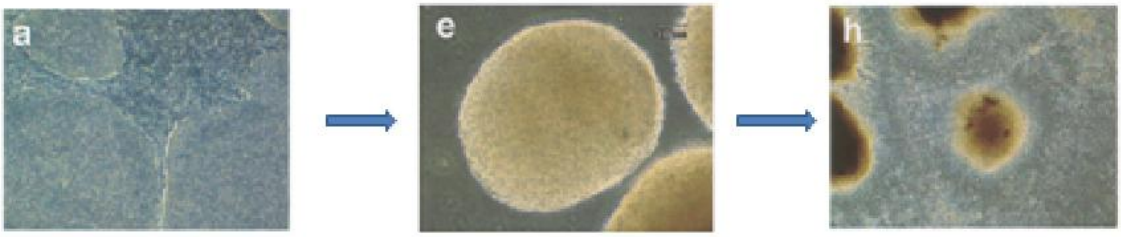

Figure 3. Differentiation of hES cells to OPCs through the formation of aggregates as adapted from Nistor et al. (2005). The human ES cells growing on matrigel (a) was aggregated on a low-attachment plate (e). The aggregates are plated on matrigel after differentiation (h)[9].

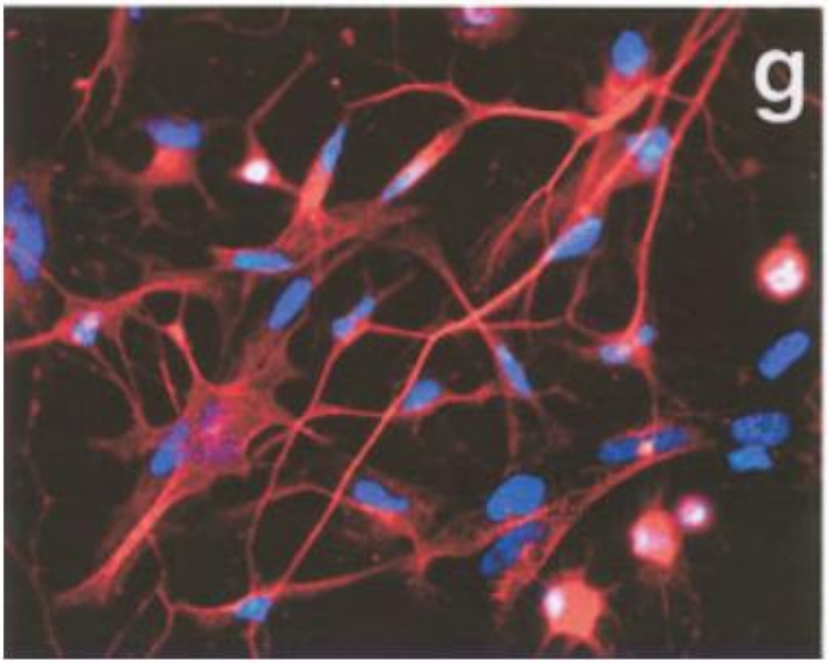

Figure 4. OPCs made from ES cells plated on an adherent substrate stained for PDGFRα (red) and DAPI (blue) [9].

The human OPCs produced from human ES cells were patented by the pharmaceutical company, Geron and labeled as GRNOPC1. These cells were approved by the Food and Drug Administration (FDA) for a Phase I clinical trial on patients with complete subacute spinal cord injury after pre-clinical testing on rodent spinal cord injuries in 2010[30]. The trial was suspended due to financial concerns but the follow-up on patients who received the OPC injection is ongoing. The results of this clinical trial have not been published. The data from the pre-clinical studies demonstrate a proof of principle of using OPCs as a strategy in the treatment of human spinal cord injury.

INDUCED PLURIPOTENT STEM CELLS (iPSCs)

The reprogramming of adult human somatic cells to a pluripotent state was first reported in 2007 following experiments that showed the successful reprogramming of adult mouse somatic cells to a pluripotent stage using specific transcription factors. Oct4, Sox2, Klf4 and c-Myc were identified to be the four defined factors required to reprogram adult human dermal fibroblast into a pluripotent state[10]. The successful formation of the iPSCs was confirmed with the help of immunocytochemistry for pluripotency markers and RT-PCR data for several pluripotency and differentiation markers. The pluripotent capacity of these cells was determined by forming embryoid bodies from them and confirming differentiation of these cells into all three germ layers by immunostaining for markers specific for each germ-layer such as Vimentin, Desmin and GFAP[10].

These iPSCs are essentially advantageous in a clinical setting for cellular transplantation. Patient specific iPSCs can be obtained and differentiated to a specific lineage and injected back into the patient for therapy. The use of patient-specific cells

theoretically decreases the risk of immune rejection of the transplanted cells considerably and the antigen compatibility may help in increasing the integrative capacity of the transplanted cells into the host system. However certain disadvantages are the potential for teratogenicity, and the time taken after SCI to both culture the iPSCs and to subsequently differentiate them into OPCs. To date, there are no clinical trials approved by the FDA for the use of iPSCs in humans.

iPSCs are generated by the insertion of reprogramming factors within a somatic cell with the help of integrating viral vectors. The disadvantage of using such vectors is the integration of the viral genome with the host cell genome. This may lead to the expression of viral genes and proteins in the pluripotent cells which may elicit an adverse reaction in a human host during transplantation. The need for alternative delivery methods for the reprogramming factors into the cell is necessary to reduce the risk of adverse side-effects during a clinical trial. Human iPSCs have been generated with the help of Sendai Virus as a vehicle[31]. The sendai virus is a non-integrating RNA virus whose genome gets translated into protein in the cytoplasm (Fig. 5). The viral proteins are cleared from the cells in subsequent cycles of host cell replication thus eliminating the risk of an immune reaction. Sendai virus is non-tumorigenic and non-pathogenic to humans. Sendai virus membrane fusion with host cells occurs efficiently with low species and cell specificity. These properties of the sendai virus make them an advantageous vehicle for delivering foreign DNA into human cells. These iPSCs theoretically present a lesser risk during clinical transplantation than other iPSCs produced using integrating viruses.

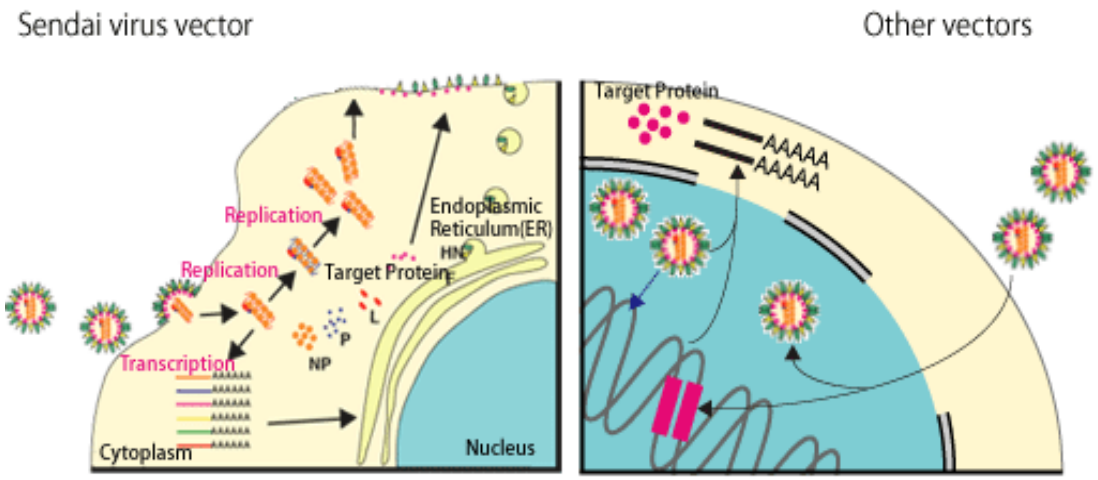

Figure 5. Comparison of life-cycle of the Sendai virus and integrating viral vectors[32].

To date, our laboratory has created mouse OPCs from mouse fibroblasts for the investigation of their use in a rodent model of SCI. The next logical step is to further this work to human cells. Therefore, the aim of my thesis work is to differentiate human iPSCs into OPCs which may be utilized for the treatment of spinal cord injuries. Owing to the similarity between human Embryonic Stem cells and induced Pluripotent Stem cells, adopting the Keirstead protocol to differentiate iPSCs to OPCs was the baseline for this thesis.

MATERIALS AND METHODS

MAINTAINING INDUCED PLURIPOTENT STEM CELL LINE

Human iPSCs (made using sendai virus) were kindly provided by Lucas Greder on irradiated Mouse Embryonic Fibroblasts (MEFs) (provided by Joseph Dalton). The iPSCs produced using sendai virus displayed a lack of transgene expression by passage 8. Absence of viral protein in the iPSC colonies was confirmed using western blots. The iPSCs generated using the sendai virus showed a similar global gene expression profile to iPSCs genererated using a lentiviral vector.[33] These iPSCs were supplemented with pluripotent stem cell specific medium consisting of Dulbecco's Modified Eagle's Medium:F12 (DMEM : F12) (Gibco), 20% Knock-out Serum Replacement (KSR) (Gibco), 1% Non-Essential Amino Acids (NEAA) (Gibco), 2mM L-Glutamine (Gibco), 0.1mM β-Mercaptoethanol (Gibco), 1X Penicillin-Streptomycin (Pen-Strp) (Gibco) and 10ng/ml basal Fibroblast Growth Factor (bFGF) (R&D Systems). Mouse Embryonic Fibroblasts (MEFs) were plated on gelatin coated plastic 6-well plate (BD Falcon) at a density of 200,000 cells per well such that they form a uniform monolayer. The iPSCs were passaged using 1ml of Collagenase IV (1mg/ml) (Gibco) per well and scraped with a cell scraper (BD Falcon) after 10 minutes incubation at 37˚C. The collagenase was diluted with medium and centrifuged at 750 rpm for 5 minutes. The pellet was re-suspended in medium and centrifuged to remove all traces of collagenase. The pellet was re-suspended once again in medium and placed in a new plate of feeders. The cells were regularly passaged within 6-8 days when the iPSC colonies were large enough but

did not make contact with one another. The cells were split in a 1:2 to 1:3 ration to maintain confluency.

PREPARATION OF GLIAL RESTRICTIVE MEDIUM (GRM)

The supplements utilized in preparing the Glial Restrictive medium were reconstituted and aliquoted as follows

S.No.	SUPPLEMENT	COMPANY	RECONSTITUTION	STOCK CONCENTRATION	ALIQUOT
1.	B27	Gibco	-	50X	1ml
2.	Insulin	R&D Systems	100 m l glacial acetic acid, rest with WET*	10mg/ml	1ml
3.	Progesterone	R&D Systems	1 ml of Ethanol then rest with KO-DMEM#	63µg/ml	1ml
4.	Putrescine	R&D Systems	KO-DMEM#	10mg/ml	1ml
5.	Sodium Selenite	R&D Systems	WET*	50µg/ml	1ml
6.	Transferrin	Sigma	KO-DMEM#	50mg/ml	20µl
7.	T3	R&D Systems	Add 1 ml of 1 N Sodium Hydroxide then KO-DMEM#	40µg/ml	1ml

Table 1. Components of Glial Restrictive Medium (GRM)
*Water for Embryo Transfer, #Knock-Out Dulbecco's Modified Eagle's Medium (Gibco)

GRM was prepared with Dulbecco's Modified Eagle's Medium:F12 (DMEM:F12) supplemented with B-27, 10µg/ml Insulin, 63ng/ml Progesterone,

10µg/ml Putrescine, 50ng/ml Sodium Selenite, 50µg/ml transferrin, and 40ng/ml T3. The medium was then filtered and the tube was covered with aluminium foil to avoid the break-down of B27.

The GRM consists of several components that are involved in aiding glial differentiation of the pluripotent cells such as insulin, T3 (triiodo thyronine, a thyroid hormone), transferrin and selenium ions. Insulin and Insulin-like Growth Factor (IGF) are known to be involved in oligodendrocyte development and proliferation[20]. Thyroid hormone is known to aid in the differentiation of mature oligodendrocytes from precursor cells. Thyroid hormone also promotes proliferation of cells in a pathway involving the mitogen Platelet-Derived Growth Factor (PDGF)[20]. Insulin, Transferrin and Selenium ions are part of a specialized medium known as ITSF medium which is known to promote migration of cells during neural crest development[20]. Epidermal Growth Factor (EGF) receptor and its signaling is identified to promote proliferation of uncommitted glial cells that are $Olig2^+$, $NG2^+$, $PDGFR\alpha^+$ and $Ki67^+$ [21]. Retinoic Acid (RA) is a caudalizing factor commonly utilized in several differentiation protocols to generate ventral spinal progenitors in glial restricted cells[14,18]. Therefore, GRM was supplemented with EGF and RA to promote OPC differentiation.

KEIRSTEAD DIFFERENTIATION PROTOCOL[14]

The iPSCs were treated with 1ml of Collagenase IV (1mg/ml) per well for 10 minutes. The collagenase was then removed and the cells were washed with PBS. 2ml of Transition Medium which was made of a 1:1 ratio of GRM and Mouse Embryonic Feeder (MEF) Conditioned iPSC medium supplemented with 4ng/ml of bFGF was then added to the cells. The cells were dissociated with a cell scraper and the large clumps

were broken up by pipetting. They were then placed in an ultra low-attachment 6-well plate (Costar). The next day (Day 2) the medium was changed to transition medium supplemented with 4ng/ml bFGF, 20ng/ml EGF (R&D Systems) and 10µM RA (R&D Systems). From Day 3 to 10 the aggregates were supplemented with GRM containing 20ng/ml EGF and 10µM RA with a change of medium everyday. From Day 11 until Day 28 the culture was fed with GRM supplemented with 20ng/ml EGF with a change of medium every other day. Matrigel (BD Biosciences) was diluted 1:10 in DMEM:F12 using chilled pipettes and centrifuge tubes and 1ml was placed in each well of a 6-well plate and left at room temperature for 24 hours. After Day 28, the aggregates were placed in the matrigel coated plate.

MANIPULATIONS OF THE KEIRSTEAD PROTOCOL

To improve viability of iPSC aggregates, the Keirstead protocol was modified in several ways. The aggregation step utilized in the Keirstead protocol was changed to the protocol utilized to make Embryoid Bodies (EBs) from iPSCs[14] which involves treating the iPSCs with Collagenase IV (1mg/ml) and washing twice with ES medium and centrifuging at 750rpm for 5 minutes. This protocol is similar to the one utilized to passage iPSCs with the difference being that in the final step, the pellet is reconstituted in transition medium supplemented with 4ng/ml bFGF and the cells are placed in a low-attachment plate. The aggregation step was further changed according to STEMDiff™ Neural Induction Kit from human Pluripotent Stem Cells (hPSCs) where the iPSCs are treated with Collagenase IV (1mg/ml) and the cells are diluted 1:10 in pluripotent cell specific medium and centrifuged at 750 rpm for 5 minutes. The transition of the iPSC aggregates to GRM utilized in the Keirstead Protocol was manipulated such that

initially the aggregates were placed in pluripotent cell specific medium lacking KSR according to the STEMDiff™ Neural Induction Kit from human Pluripotent Stem Cells (hPSCs). This step was further changed to place the aggregates in MEF Conditioned iPSC Medium for the initial 2 days. The initial concentration of bFGF was increased to 10ng/ml. The transition to GRM was further modified to a gradual transition that involves placing the aggregates in 25, 50 and 75% GRM mixed in MEF Conditioned Medium with a decreasing concentration of bFGF. The addition of Retinoic Acid to the medium was postponed to Day 6 when the aggregates were placed in 100% GRM. The aggregates were treated with GRM supplemented with 20ng/ml of EGF for 7 days and plated down on Matrigel (Diluted 1:25 in DMEM:F12). All the media utilized for the differentiation protocol was supplemented with Penicillin-Streptomycin to avoid contamination.

AGGREGATION	• Centrifuged at 750 RPM for 5 mins. • Pellet washed twice with iPS Medium – Small size, Low number and Viability of Aggregates. • Diluted 1:10 in iPS medium with less pipetting.
AGGREGATION MEDIUM	• Placed in iPS medium without KSR – Loss of viability • Placed in Feeder Conditioned Medium.
TRANSITION	• Transitioned to GRM with a 1:1 GRM and Conditioned medium intermediate – Loss of Viability in Aggregates • Transitioned slowly to GRM with 25, 50 and 75% intermediates. • Addition of bFGF, RA and EGF at Day 3 of Protocol – Loss of Viability. • Postponed addition of RA and EGF to Day 6 and gradual decrease in bFGF concentration
CHANGE IN CONSTITUENTS	• 10ng/ml of bFGF is utilized • 1X Penicillin and Streptomycin utilized to avoid contamination

Table 2. Summary of Modifications to the Keirstead protocol[14].

FINAL DIFFERENTIATION PROTOCOL

A schematic of the final Differentiation protocol is represented in Figure 6. The iPSCs growing on irradiated MEFs were washed with PBS (CellGro) and treated with collagenase IV (1mg/ml) (1ml/Well) for 10 minutes at 37˚C. The cells were then scraped using a cell scraper and diluted 1:10 in iPSC medium. The cells were centrifuged at 750 rpm for 5 minutes. The pellet was reconstituted in MEF Conditioned iPSC medium supplemented with antibiotics and placed in an ultra low-attachment plate. The medium was changed to fresh conditioned medium on Day 2. The cells were then treated to 25%, 50% and 75% Glial Restrictive Medium made with conditioned medium and supplemented with antibiotics with a decreasing concentration of bFGF (7.5ng/ml, 5ng/ml and 2.5ng/ml) on consecutive days till Day 5. On Day 6, the cells were provided with GRM supplemented with 20ng/ml EGF and 10µM RA and the medium is changed everyday for the next 7 days. On Day 13, the aggregates were given GRM supplemented with 20ng/ml of EGF with a change of medium every alternate day. The aggregates were broken up on Day 1 and Day 2. On Day 20, the aggregates were plated onto matrigel (diluted 1:25 in DMEM:F12).

PASSAGING OPCs PLATED ON MATRIGEL

The OPCs growing out of the aggregates on matrigel were supplemented with GRM containing 20ng/ml of EGF. The medium was changed every other day. The well is washed with PBS and treated with warm 0.05% Trypsin-EDTA (HyClone) (500µl per well of a 6-well plate and 300µl per well of a 12-well plate) for 7 minutes at 37˚C. The cells were then observed under the microscope for detachment of the cells from the substrate and the plate was tapped from the bottom and sides to dislodge all the cells

from the matrigel. The trypsin-EDTA cell suspension was then diluted 1:10 with DMEM:F12 medium and centrifuged at 750 rpm for 5 minutes. The pellet was reconstituted with fresh GRM containing 20ng/ml of EGF and distributed in a new 12-well plate at a ratio of 1:2 or 1:3 wells to maintain the density required by OPCs for proliferation. The medium was changed every other day to fresh GRM with 20ng/ml of EGF to aid in the proliferation of OPCs. The OPCs were passaged at regular intervals when the cell density reaches the desired confluence. The OPCs were passaged onto matrigel diluted at a ratio of 1:25 in DMEM:F12.

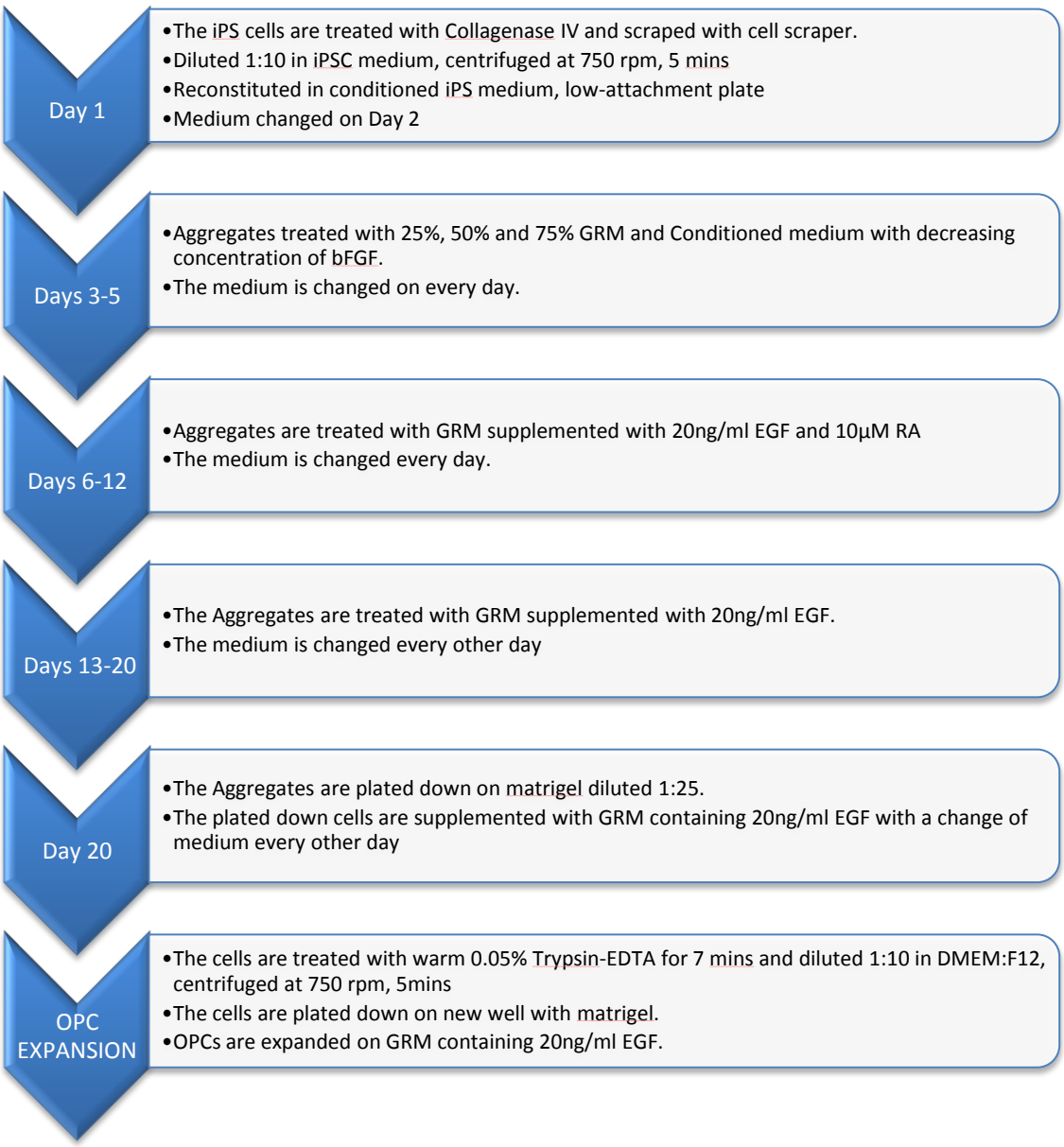

Figure 6. Flow diagram of the differentiation protocol currently employed.

IMMUNOHISTOCHEMISTRY

Initially aggregates plated on matrigel were fixed by removing the medium from the well and adding 1ml of formalin (Protocol) per well of a 6-well plate followed by 20 minutes incubation at room temperature. After 20 minutes, the formalin was removed and the well was washed twice with PBS. Blocking buffer was prepared by adding 1% Bovine Serum Albumin (Sigma) and 0.1% Tween-20 (Biorad) to PBS and filtering. Permeabilizing buffer was prepared by adding Tween-20 to the blocking buffer such that it has a 1% Tween-20 concentration. The PBS was removed from the cells and 500µl of permeabilizing buffer was added to the well and incubated for 10 minutes at room temperature. After incubation period, the permeabilizing buffer was removed and washed 3 times with wash buffer (PBS with 0.1% Tween-20) for 2 minutes each. The cells were then treated with 1ml of blocking buffer per well and incubated for 30 minutes at room temperature and after incubation they were washed 3 times with wash buffer (PBS with 0.1% Tween-20) for 2 minutes each. The primary and secondary antibody treatments were performed according to Table 3. The cells were also stained with DAPI by diluting DAPI 1:500 in PBS. The cells will be treated with the secondary antibody alone as a form of negative control.

S.No.	Primary Antibody	Company	Dilution	Secondary Antibody	Company	Dilution
1.	Mouse Anti β-Tubulin Isotype III	Sigma	1:1000	Alexa Fluoro 488 Rabbit Anti-Mouse IgG	Invitrogen	1:500
2.	Rabbit X Olig 2 Polyclonal Antibody	Millipore	1:250	Alexa Fluoro 555 Donkey Anti-Rabbit IgG	Invitrogen	1:500
3.	Goat Anti hOlig 2	R&D Systems	1:100	Alexa Fluoro 555 Donkey Anti-Goat IgG	Invitrogen	1:500
4.	Rabbit Anti NG 2	Millipore	1:200	Alexa Fluoro 488 Goat Anti Rabbit IgG	Invitrogen	1:500

Table 3. Antibodies used for immunostaining and their dilutions.

RESULTS

AGGREGATION OF iPSCs

The aggregation step employed in the Keirstead protocol[14] involved treating the pluripotent stem cells with collagenase and placing them in Transition Medium followed by dissociating them with a cell scraper. In the Keirstead paper[14], since the ES cells were maintained and passaged on matrigel, the aggregation method utilized would provide uniform aggregates. In contrast, the iPSCs utilized for this project were maintained and passaged on MEF feeder cell layers (Fig 7A) which may inhibit uniform aggregate formation due to the presence of feeder debris. This was overcome by diluting the cells with iPSC medium and centrifuging at 750 RPM for 5 minutes. This resulted in depletion of feeder cell debris and formation of uniform aggregates. The aggregating cells are placed on a plate rocker overnight inside the incubator such that the cells collect together and ensure the formation of uniform aggregates.

TRANSITION TO GLIAL RESTRICTIVE MEDIUM

A difference in the differentiation capability of human iPSCs and ES cells has been observed by several groups[15,16]. The neural differentiation capacity of different pluripotent lines shows significant variation indicating a possible difference in pluripotency and epigenetic signals between ES cells and iPSCs[15,16]. In these studies the differentiation of each pluripotent cells line was evaluated by their ability to form PAX 6^{+} adherent neural rosettes. Differences such as those reported in these papers may provide an explanation for the difference in the ability of aggregates formed from ES cells and iPSCs to survive in 1:1 Glial Restrictive Medium (GRM) and MEF conditioned medium. The iPSCs used in this study exhibited a low survival capacity

when subjected to the transition medium (1:1 GRM and conditioned medium) immediately upon aggregation. The survival of iPSC aggregates was initially determined by studying their morphology. In these experiments, aggregates of dead cells appeared very dark with irregular shapes and did not display any growth. Therefore to increase the survival rate of our iPSCs following aggregation, they were placed in MEF conditioned medium for the first 2 days with a change of medium on both days. The aggregates appeared yellow and had a more regular spherical morphology (Fig 7B, 7C).

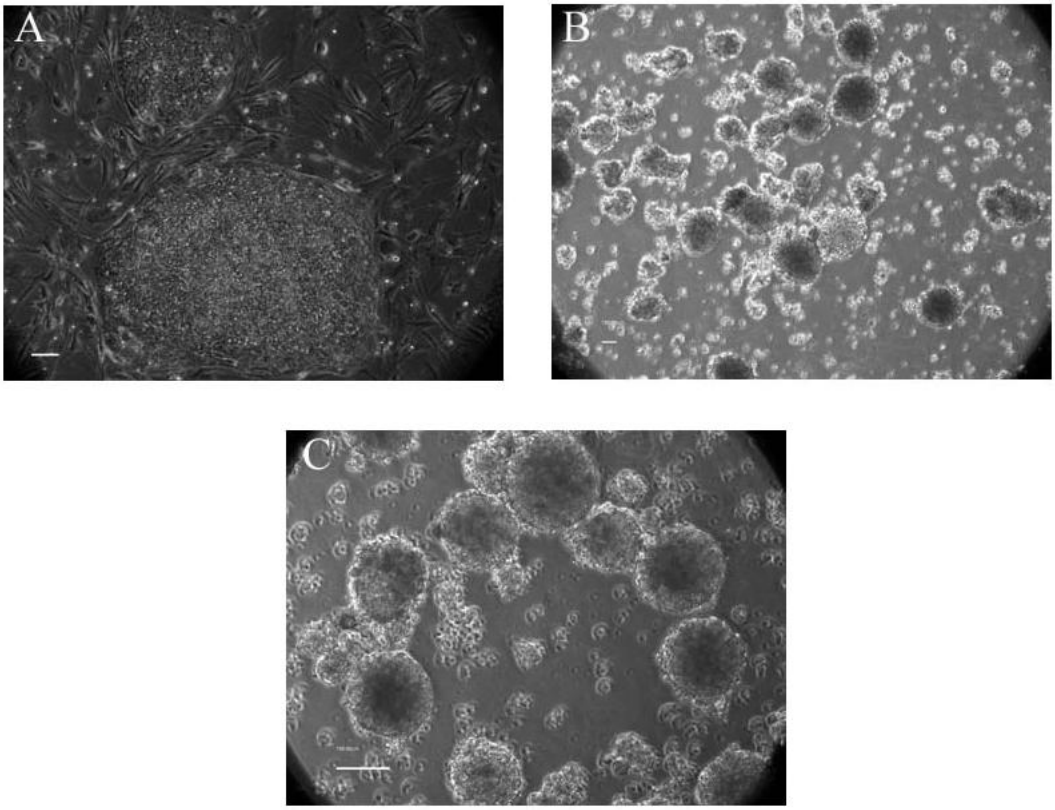

Figure 7. Initial Aggregation of iPS Cells. iPS cell colonies growing on MEFs (A), Day 2 Aggregates on conditioned medium 10X (B) and Day 2 Aggregates 20X (C)

Subjecting the aggregates to GRM on Day 3, according to the Keirstead protocol[14], rapidly decreased their viability. A concentration of 10µM Retinoic Acid was utilized in this project at early stages (Day 2) of transition into GRM according to the Keirstead protocol[14] which is 100 fold greater than the concentration normally utilized for neural differentiation[19,25] and this may cause loss of viability in the aggregates. Introducing RA and EGF on Day 6 lead to glial differentiation of aggregates that were better adapted to the GRM.

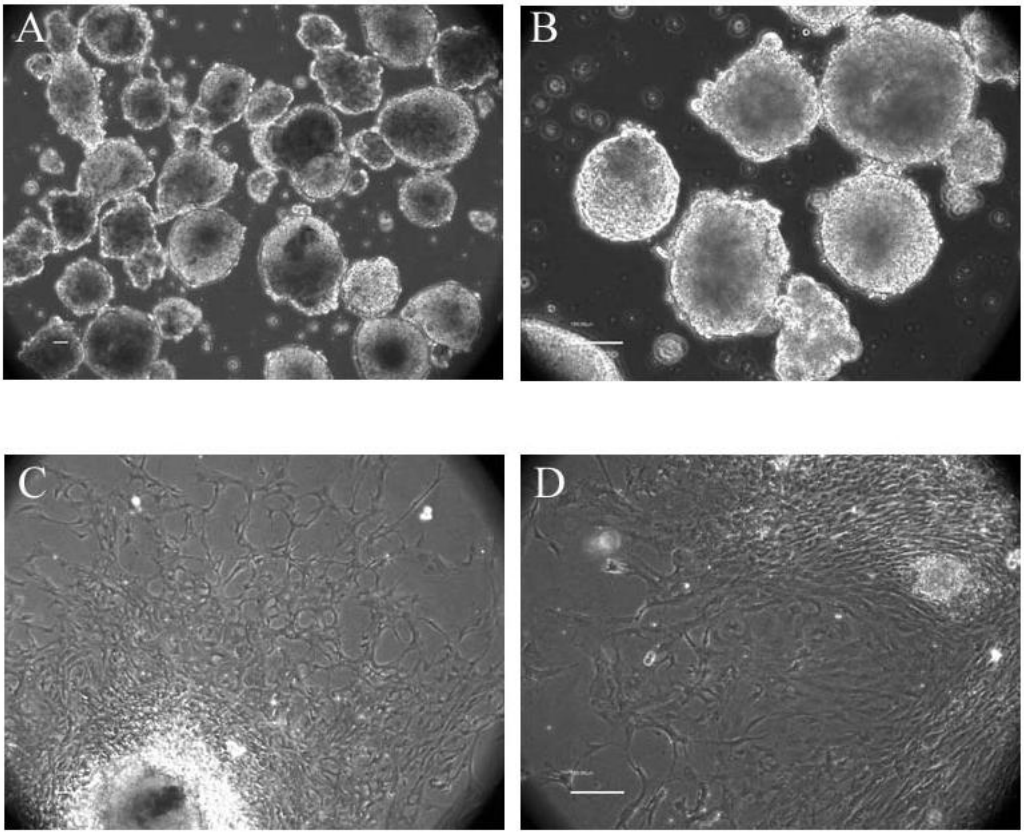

Figure 8. Aggregates on Day 6 and plated on matrigel after Day 20. Day 6 Aggregates 10X (A) and 20X (B). Aggregates plated on matrigel after Day 20 10X (C) and 20X (D)

DIFFERENTIATION AND PLATING OF AGGREGATES

The aggregates were placed in GRM supplemented with EGF and RA from day 6 to day 12 after transition and expanded further in GRM with EGF alone from day 13

to day 20 (Fig 8A, 8B). After expansion, the aggregates were plated onto matrigel at day 20. Initially, the aggregates were plated and expanded in matrigel diltuted 1:10 in DMEM:F12 according to the Keirstead protocol[14]. However the dilution was changed to 1:25 in DMEM:F12 due to the common usage of this dilution in other projects in our laboratory and used to coat 6-well and 12-well plates resulting in a uniform matrigel coating. Aggregates plated on this diluted matrigel adhered well to the plate and proliferated (Fig 8C, 8D).

In the experiments conducted in our laboratory on mouse iPSC derived OPCs, the cells were treated with TrpLE, plated and expanded on poly-ornithine[22]. Poly-ornithine is routinely utilized for the selection and expansion of neural cell types. When aggregates formed from human iPSCs were placed in poly-ornithine coated plates for long periods of time (4 weeks), they did not display any adherence. Treating the human iPSC generated aggregates with TrpLE before plating onto poly-ornithine, following more closely the mouse iPSC to OPC protocol, decreased the viability of the aggregates. Therefore the aggregates were plated on matrigel, and the cells from within migrated onto the matrigel and exhibited typical OPC morphology (Fig 8C, 8D).

The OPCs growing out of the aggregates on matrigel were passaged using 0.05% Trypsin-EDTA as described in the Keirstead Protocol[14]. The trypsin was neutralized using an anti-trypsin solution in the previously established Keirstead's protocol. In this project, the cells were treated with 0.05% trypsin-EDTA at 37˚C for 7 mins following which the plate was tapped well to dislodge the cells. The cell suspension in trypsin was then diluted 1:10 in DMEM:F12. The cells were collected by centrifugation and redistributed on matrigel coated wells in GRM containing 20ng/ml

EGF. The medium was changed every other day to expand the OPCs. These cells were plated on wells of a 12-well plate to maintain the density of cells per well (Fig 9A, 9B). OPCs require an appropriate density and contact with one another in order to expand and inhibit terminal differentiation to oligodendrocytes. The cells were passaged regularly for further expansion. Human OPCs are reported to not expand more than 4-5 passages after which they undergo terminal differentiation.

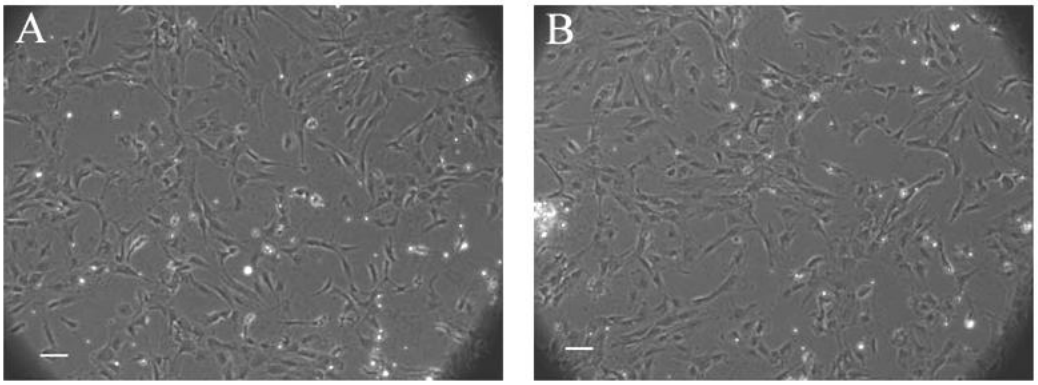

Figure 9. A and B are representative wells of OPCs passaged on matrigel in a 12-well plate.

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The aggregates plated on matrigel were fixed using formalin, permeabilized using tween-20 detergent and blocked using a mixture of Tween-20 and Bovine Serum Albumin. OPCs can be characterized by the co-expression of Olig2 and NG2. Olig2 expression in OPCs is nuclear whereas NG2, which is a chondroitin sulfate proteoglycan, is found on the surface of OPCs. The cells were immunostained using a polyclonal rabbit Olig2 that has been tested previously on mouse OPCs obtained in our laboratory. More than 80% of the cells were positive for Olig2 expression. Nuclear localization of Olig2 was confirmed using DAPI staining of the nucleus and merging the images (Fig 10).

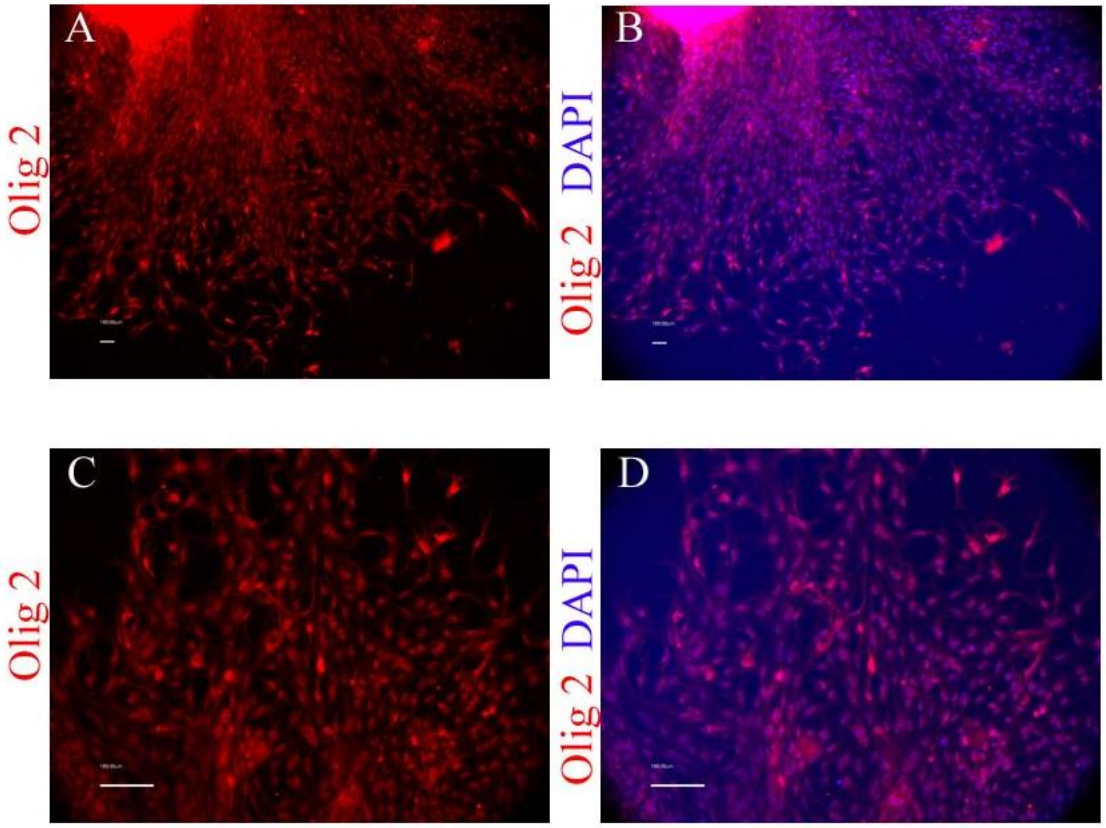

Figure 10. Olig2 staining of aggregates plated on matrigel. 10X Olig2 (A) with DAPI merge (B). 20X Olig2 (C) with DAPI merge (D)

Some aggregates exhibited neuronal axons growing out on the matrigel and this was confirmed by staining the cells for β III tubulin which detects axonal fibres with specificity. The well was also stained for Olig2. The Olig2 positive cell bodies were not stained by β III tubulin (Fig 11). The secondary antibody utilized for staining β III tubulin was a rabbit anti-mouse antibody and the primary utilized for Olig2 was also a rabbit antibody. Therefore, the anti-rabbit secondary antibody utilized for Olig2 also bound to the secondary antibody utilized for β III tubulin.

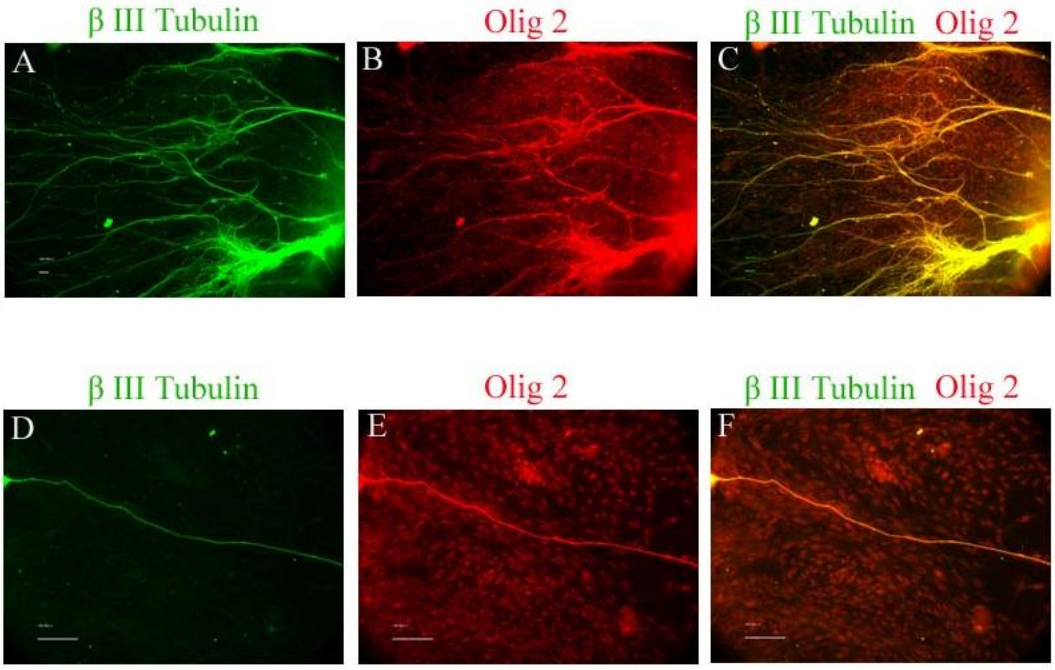

Figure 11. Merge of β III tubulin and Olig 2 staining. β III tubulin stain of aggregate plated on matrigel (10X - A) (20X – D), Olig 2 stain of the same aggregate (10X - B) (20X – E), merged image (10X - C) (20X – F)

NG2 immunostaining was performed on the fixed cells above treated with an anti-Olig2 antibody to look for double positive cells. However, the NG2 immunostain showed negative results on the fixed cells. Being a surface proteoglycan, it was assumed that perhaps treating the cells with detergent (Tween 20) may lead to loss of detectable NG2 epitope on the surface of the cells. In order to preserve the NG2 proteoglycan the cells were subjected to live staining of NG2. Rabbit anti-NG2 was utilized as a primary antibody overnight. Live staining showed positive results for the presence of NG2 on the surface of the cells (Fig 12).

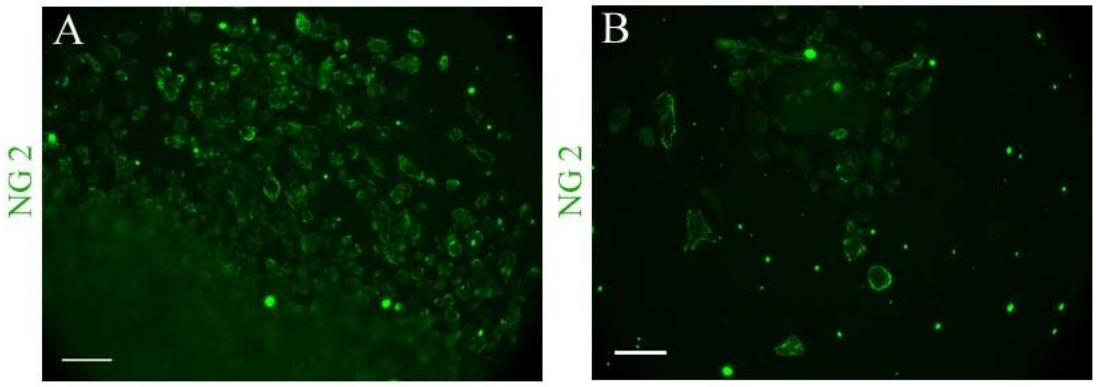

Figure 12. NG2 Live Staining. Field 1 (A) and Field 2 (B)

NG2 staining appears as a ring around the cell in several cases, displaying the expected membrane localization of NG2 in OPCs. The cells appeared distorted in morphology as they were placed in PBS overnight. The NG2 and the Olig2 antibodies are both prepared from rabbits therefore, they could not be co-stained to detect the presence of double positive cells. Therefore, an appropriate step forward is to directly conjugate the primary antibody with the fluorophore and utilizing this to look for double positive cells. Conjugating the primary antibody with the fluorophore would decrease the intensity of fluorescence obtained but clearly display double positive cells with no cross-reaction.

DISCUSSION

The similarity between iPSCs and ES cells motivated the use of the Keirstead protocol[14] in the differentiation of iPSCs to OPCs. The overall concept of the protocol was successful in differentiating the iPSCs to OPCs with modification at each stage to better suit the survival of the aggregates made from iPSCs. The OPCs generated from the iPSCs were primarily confirmed using immunostaining for Olig2 and NG2. The OPCs produced using this protocol, were expanded on matrigel in GRM supplemented with EGF and passaged for further expansion.

The modifications to the Keirstead protocol were aimed at producing viable and uniform aggregates from the iPSCs. Washing the iPSCs with pluripotency specific medium during the aggregation stage of the protocol decreased the feeder debris and lead to the production of more uniform aggregates. Placing the aggregates in MEF feeder conditioned medium for the initial 2 days of the protocol aids in forming stable aggregates that may be treated to GRM. A gradual transition to GRM with 25%, 50% and 75% intermediates helps in acclimatizing the aggregates to the drastic change in medium composition.

The presence of Retinoic acid in this differentiation protocol plays a vital role in producing spinal progenitors but retinoic acid proved to be toxic in the early stages of transition to GRM. It was hypothesized that the high concentration of retinoic acid (10μM) at the early stages of aggregation and differentiation lead to increased cell death. Postponing the use of retinoic acid to day 6 of the differentiation protocol aided in increasing the viability of the aggregates.

Immunostaining performed for the detection of NG2 displayed a distorted morphology of the cells as they were placed in PBS overnight. Lack of medium supplementation lead to loss of morphology of the cells. The primary antibodies of Olig2 and NG2 that were previously tested on mouse cells were made from rabbit. Therefore double staining for Olig2 and NG2 was made impossible. These are certain significant aspects of this project that need attention in order to obtain better results.

The OPCs generated with this method should be further characterized using immunostaining for other OPC specific markers such as PDGFRα, A2B5[24] and mRNA expression analysis must be performed using RT-PCR to confirm the expression of Olig2, NG2, PDGFRα and A2B5. RT-PCR data for the decrease in pluripotency marker expression such as Oct4, Nanog and SSEA-4 and increase in glial specific markers such as Pax6, Sox10, Olig2 and PDGFRα at each stage of differentiation can also provide further confirmation of OPC generation from iPSCs. Immunostaining for Olig2, NG2 or Olig2, PDGFRα double positive cells would give positive identification of OPCs present in the dish[23]. The cells can be analyzed using flow cytometry to identify the ratio of Olig2 positive cells to overall number of cells in order to determine the efficiency of the differentiation protocol in use. The initial number of iPSCs utilized to make the aggregates would give an overall efficiency of OPC production.

Functional testing of the OPCs produced can be performed by injecting a purified quantity of these cells into the brain/spinal cord of shiverer mice and staining the sections for MBP[26]. These cells can be further characterized by plating on laminin coated wells which would induce terminal differentiation of OPCs to oligodendrocytes. The oligodendrocytes produced should exhibit the typical multi-polar morphology and

can be functionally tested *in vitro* by co-culturing them with human motor neurons and identifying their myelination capacity. These mature oligodendrocytes can also be immunostained for GalC, MBP and PLP[24].

The use of these OPCs in cellular therapy for spinal cord injury holds great promise as patient specific iPSCs can be used as a source of OPCs and this reduces the risk of immune-rejection by the patient. As a pre-clinical testing process, spinal cord injury can be induced in a rodent model using an impactor which would mimic the contusion injury observed in humans. These OPCs can then be injected in the area surrounding the injury and the change in functionality of the paralysed portion of these animals can be recorded with the help of BBB scores[25]. The animals should also be sacrificed and sections of the spinal cord immunostained to reveal the changes induced by these OPCs and identify the target locations towards which they migrate.

It is clear that differentiated cell lines can be generated from iPSCs using differentiation protocols utilized for ES cells, however these protocols are likely to required certain modifications to better fit the project goals.

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