Identification and Characterization of Human Iris Pigment Epithelial Cells and Ciliary Pigment Epithelial Cells *In-vitro* for Retinal Stem/Progenitor Properties

SYNOPSIS

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ABSTRACT:

The mammalian central neural retina (CNR) lacks the capability to regenerate, a phenomenon retained by lower vertebrates. However, stem/progenitor cells have been isolated from iris and ciliary pigment epithelial (IPE and CPE) cells of the human eye. The aim of our study was to isolate and characterize the properties of neurospheres and differentiated cellular progeny derived from IPE and CPE cells of human cadaveric eyes. The present study also focuses on the cellular differentiation that involves loss of pluripotency and gain of lineage and cell type-specific characteristics and we show developments from adult ciliary derived stem/progenitor cells to differentiated retinal neurons/glial cells of human cadaveric eyes in vitro using gene expression studies and Methylation studies. We also investigate the influence of the self-assembling peptide nanofibre scaffolds (SAPNs) on the growth, proliferation and retinal neuronal differentiation of the stem/progenitor cells (SCs) derived from the ciliary pigment epithelium (CPE) of human cadaveric eye. The IPE and CPE cells from human cadaver eyes were cultured in the presence of mitogens to generate neurospheres (NS) and the growth characteristics were evaluated. The Neurospheres (NS) were plated under conditions inducing differentiation and their potential was analyzed by RT-PCR, immunocytochemistry, calcium imaging studies and microarray studies using Affymetrix Human GeneChip whole gene 1.0ST arrays to measure the changes involved in the process of differentiation. We also analysed DNA methylation using 1-2µg of (600–1000bp) sonicated genomic DNA and methylation patterns of histone H3K4me3, RNA Pol II and H3K27me3 using 7 mg of chromatin DNA using Agilent SurePrint G3 Human 2x400K Promoter arrays. The effect of SAPNS (RADA16-I, PM), was also analysed on the CPE cells. The CPE cells were cultured encapsulated in the SAPNs in the presence of the mitogens and further differentiated into retinal neuronal cells using differentiation media encapsulated in SAPNs. The IPE and CPE cells can generate NS containing progenitor cells in the presence of mitogens and were capable of producing different retinal cell types under differentiation conditions as demonstrated by RT-PCR and immunocytochemistry. The microarray cluster analyses of the differentially expressed genes show the dynamic changes that occur during the course of IPE and CPE neurospheres differentiating into retinal

progeny. Our results also demonstrate that several promoters including pluripotency and lineage specific genes become DNA methylated in the differentiated population, suggesting that DNA methylation may repress the pluripotency in this population. On the other hand, we detect bivalent modifications that are involved in the process of differentiation of stem/progenitor cells. Therefore, this data suggest a model for studying the epigenetic regulation involved in self-renewal, pluripotency and differentiation potential of ciliary stem/progenitor cells. The entrapped SCs actively expanded and formed clone-like clusters in the scaffolds. Many cells in the cluster were proliferating as revealed by 5-bromo-2-deoxyuridine uptake and could be maintained for up to 6 days and expressed neural progenitor markers like β-III tubulin, Nestin, Pax6 and Musashi1. Upon differentiation of these cells in conditioned medium, the cells exhibited retinal neuronal markers like s-Opsin, rhodopsin and recoverin. Our RT2 profiler PCR array experiments showed selective gene expression, possibly involved in neural stem/progenitor cell adhesion and differentiation. In the present study we have demonstrated the expansion and maintenance of SCs from IPE and CPE of cadaveric eyes. These cells maintain their self-renewal properties and the ability to differentiate along retinal cell lineages and hence could be a practical source of donor cells for ex-vivo stem cell therapy during retinal degeneration. This study gives clues towards the changes that occur during differentiation from NS into retinal progeny. This data therefore suggest a model for studying the dynamic changes in histone group controlled pluripotency and explain the differentiation potential of ciliary stem/progenitor cells. This work presents the first outline of epigenetic modifications in ciliary derived stem/progenitor cells and the progeny that underwent differentiation into retinal neurons/glial cells and shows that specific DNA methylation and histone methylations are extensively involved in gene expression reprogramming during differentiation. These findings also suggest the suitability of the 3D culture system for the proliferation and maintenance of CPE stem/progenitor cells (CPE-NS) and for possible use in ex vivo studies of the small molecule, drug deliveries for retinal diseases and also for use in combination with directed stem/progenitor cell differentiation and ultimately for tissue replacement therapies.

INTRODUCTION:

The retina is a light-sensitive layer at the back of the eye that contains the sensory neurons for vision. It contains many neurons like photoreceptor cells, ganglion cells, horizontal cells, bipolar cells, and amacrine cells surrounded by support cells like müller cells, astrocytes and microglial cells whose terminal output extends to the diencephalon as the optic nerve. Depending on the cell type and degree of damage any damage to retina leads to visual impairments. Retinal degenerative diseases such as retinitis pigmentosa and age related macular degenerations and glaucoma are the leading cause of untreatable blindness characterized by photoreceptor degeneration (T.A. Reh et al., 1998, R. E. MacLaren et al., 2006, Lamba DA et al., 2009, Bartsch U et al., 2008). Till date there is no data or evidence that new neurons can be generated in response to injury or disease. Currently there is no effective treatment available for any potential damage and preventing further damage (Moshiri et al., 2004). A number of promising therapeutic strategies that intend to regenerate and/or replace diseased or damaged retinal tissue are being carried out worldwide (MacLaren et al., 2006). Current treatment strategies are aimed to delay disease progression through gene therapy, growth factor treatment, and anti-angiogenic therapy. An emerging therapeutic idea is cell based therapies that provide a promising approach in restoring and sustaining the retinal function and prevents blindness (Margalit E et al., 2003, Klassen HJ et al., 2004 and MacLaren et al., 2006).

In the recent years, the finding that the adult eye contains a population of cells that have the capacity to differentiate into all the retinal cells types has led to much interest in the prospect of developing cell replacement strategies to repair the injured retina (Tropepe et al., 2000). However, the adult retina does not spontaneously regenerate and retinal stem/progenitor cell transplantation is limited due to non-availability of donors and the low survival rate of grafted cells. Studies have shown that retinal tissue can be replaced and some degree of functional recovery can be obtained following the delivery of Stem/Progenitor cells (SCs) to the subretinal space (Klassen HJ et al., 2004 and MacLaren et al., 2006). SCs, isolated from the adult tissues offer an ideal source that have the properties of self-renewal and the potential to produce large numbers of retinal neurons *in vitro* in response to appropriate cues (Meyera, J. S et al., 2009).

SCOPE AND LIMITATIONS OF THE RESEARCH:

Retinal degenerations and dystrophies, the major causes of genetically inherited blindness, are characterized by the death or degeneration of photoreceptors (rods and/or cones). Approaches to treating this disease include: a) replacing the defective gene; b) introducing a drug or agent that either slows down or stops the premature death of photoreceptors; c) introducing electronic chips; or d) replacing the damaged cells by cellular therapy. Over the past few years a great deal of interest has been generated in using stem cells/progenitors to treat degenerative diseases that afflict different tissues, including retina. The interest is due to the defining properties of stem cells/progenitors, the ability of these cells to self-renew and generate all the basic cell types of the particular tissue to which they belong. In addition, the recent reports of plasticity of adult tissue-specific stem cells/progenitors and directed differentiation of the embryonic stem cells (ESCs) has fuelled the hope for cell and gene therapy using stem cells from heterologous sources. Despite the success of isolation and maintenance of embryonic retinal stem cells/progenitors in culture and demonstration of their differentiation into retinal cells, both in vivo and in vitro, barriers are anticipated that make their clinical use rather impractical. First, these cells are not available in sufficient quantity for clinical use because retinal stem cells/progenitors display limited self-renewal capacity. Second, they do address the important issue of immune-rejection. Thirdly, because of their unlimited self-renewal and high differentiation potential poses the risk of tumour induction after engraftment. Lastly, there is an ethical concern for their clinical use because of the source of these cells is embryonic/fetal tissues. These barriers are significant enough to prompt examination of alternate sources for retinal cells that are renewable and preferably suitable for autologous transplantation, for this addresses not only the extremely important issue of immune rejection but also ethical issues. Recently research is geared to identify non-retinal sources for retinal stem cells/progenitor cells. The present study objectives are aimed to isolate and characterize the stem/progenitor cells from the iris and ciliary body of human cadaveric eyes as a non-retinal source, which would help in better understanding of the molecular mechanisms involved in the dedifferentiation process of these cells into retinal lineage *in vitro*.

OBJECTIVES:

Objective 1:

- To isolate and culture the stem/progenitor cells (SCs) from Iris pigment epithelial cells (IPE) and Ciliary epithelial (CPE) cells from the cadaveric human eyes.
- To characterize the isolated SCs from the IPE and CPE cells, and to investigate the differentiation potential of these SCs *in vitro*.

Objective 2:

- Gene expression analysis of the SCs isolated from the IPE and CPE cells and the terminal differentiated population of these SCs.
- To identify the molecular pathways that governs stem cell differentiation and pluripotency of human IPE cells and CPE cells.

Objective 3:

- To study the functional neuronal property of the neuronal cells obtained from the IPE and CPE derived SCs by analyzing the ability of the differentiated cells to mobilize intracellular calcium in response to glutamate agonists.
- Characterizing the retinal neuronal differentiated cells for activity-dependent vesicle recycling at the presynaptic terminal.

Objective 4:

- Cellular DNA methylation modifications defining the restriction and potential of SCs derived from CPE cells.
- Chromatic modifications defining the differentiation potential of SCs derived from CPE cells.

Objective 5:

- Culturing of the SCs derived from CPE cells in self-assembling peptide nanofiber scaffolds.

- Retinal neuronal differentiation potential of SCs derived from CPE cells in self-assembling peptide nanofiber scaffolds.

METHODS AND RESULTS:

Objective 1:

Proliferative and Self-renewal potential of IPE and CPE derived Neurospheres:

The IPE and CPE cells have the ability to form spheres in the presence of EGF and FGF (mitogens) indicating that a stem/progenitor cell undergoes proliferation to generate a free floating neurospheres containing progenitor cells. This provides an indication for the presence of the SCs in these tissues. To examine the *in vitro* growth characteristics, the IPE and CPE cells were isolated and dissociated into single cells. Neurospheres could be easily derived from single dissociated cells, were visible at 3rd day *in vitro* and the number and size of the neurospheres were assessed at the end of 7th day. Significantly, the number of neurospheres formed from CPE were twofold greater than IPE. Further, we examined the relative response of IPE and CPE cells of human cadaveric eye to different mitogens in generating neurospheres. Cells isolated were cultured in the presence of EGF/FGF2 and the number of neurospheres generated was determined. The number of neurospheres generated by these cells increased significantly in the presence of EGF and FGF compared to the cells cultured in the presence of either EGF/FGF alone, suggesting that the mitogenic effect of FGF2 and EGF are required for the effective culturing of the stem/progenitor cells *in vitro*.

The majority of cells in the IPE neurospheres (~79.9%) and CPE neurospheres (~82.9%) were proliferative indicated by BrdU labeling experiments. Further experiments proved that IPE and CPE derived neurospheres could be maintained by repeated passages. The CPE neurospheres could be maintained for 6 weeks, in contrast to IPE-derived neurospheres which were unable to form any spheres after 5 weeks. This finding suggests that the SCs that initiated the primary sphere underwent many divisions when forming a primary sphere to produce a significant number of self-renewing SCs. In later passages, the number of new spheres dropped dramatically indicating the loss of potency of these cells. Phenotypic characteristics of the neurospheres derived from IPE and CPE cells, showed expression of

the progenitor markers, Nestin, Pax6, Musashi1 and β-III tubulin, which was demonstrated by RT-PCR and Immunolabelling. The morphological characteristics of the neurospheres were analysed and the quantitative characteristics were analyzed.

Differentiation potential of the IPE and CPE

To determine the potential for retinal differentiation, the neurospheres generated from IPE and CPE cells were cultured in conditions that promote retinal differentiation. The characteristics of the Neurospheres and the differentiated cells derived from both the cells were analysed by RT-PCR and immunocytochemistry. The IPE and CPE derived neurospheres showed negative expression when stained for the epithelial marker like pan-cytokeratin indicating the distinct pattern of differentiation. The IPE and CPE derived neurospheres expressed the neural precursor marker Nestin and β III tubulin.

Upon differentiation of the IPE and CPE neurospheres into retinal lineage, these cells strongly expressed the retinal markers Brn3b, Thy1 (Retinal ganglion cells), Chx10 (Bipolar cells), Rhodopsin, Recoverin, s-Opsin, Nrl (Photoreceptors) and Syntaxin1, Calretinin (Amacrine cells and horizontal cells) as demonstrated by the RT-PCR and immunocytochemical analysis. These results suggest that the isolated and expanded IPE and CPE cells can be induced to differentiate into a retinal neuronal direction.

The neurospheres derived from both the IPE and CPE cells are partially or heavily pigmented, upon induced differentiation we found the neurospheres expanded in monolayer cultures with loss of their dense pigmentation and multiple processes with time in culture suggesting that de-differentiation with consequent loss of pigments as described by our morphological and electron microscopy studies. As known previously the melanin synthesis mainly occurs during embryonic development, in which the stage I and II immature unpigmented melanosomes undergo modifications and lead to complete maturation into stage IV pigmented functional melanosomes (Abdouh M and Bernier G, 2006), our results prove that the stem/progenitor stages (NS) and late stages (DC) derived from the pigmented IPE and CPE cells do not retain stage I & II melanosome genes demonstrated by our microarray analysis, indicating that melanin deposition is complete. We therefore assume that pigmentation in IPE and CPE monolayers is lost in culture by melanosome

degeneration over time. Our electron microscopic examinations of the differentiated progeny derived from the IPE and CPE neurospheres also indicate their nonspecific features and the cells are devoid of the immature pigmentation. Therefore our data suggest that a process of differentiation is occurring with the loss of pigmentation in the cell phenotype.

Objective 2:

Gene expression changes in the course of IPE and CPE progenitor cell differentiation:

To understand the underlying mechanism of distinct proliferation and differentiation potential of stem/progenitor cells, we carried out gene expression profiling of these cells in proliferating and differentiating conditions using microarray analysis. Analysis of Average Difference (AD) values for the gene expression in the four conditions (IPE neurospheres, CPE neurospheres, IPE differentiated cells and CPE differentiated cells) revealed similar box plots and Hierarchical Condition Tree, suggesting that further normalization for intersample variation is not required.

The molecular basis for the formation of neurospheres and its further differentiation *in vitro* is not known, it could be mediated by many factors. To analyse the changes occurring in this process, we performed cDNA microarray for samples (Neurospheres and Differentiated cells) derived from IPE and CPE cells. Cells were harvested at two different time points one after 6 days as undifferentiated cells (neurospheres) and the other after induction of differentiation after 21 days (Differentiated). To find relevant differentially expressed genes, all genes with more than two fold estimated differences in expression were considered for further evaluation and this threshold is based on a statistical analysis. The cluster analysis of differentially expressed genes in all the two conditions- IPE neurospheres to differentiated cells and CPE neurospheres to differentiated cells were categorized into various classes according to the Gene ontology classification.

Based on existing literature and databases, we analyzed the genes that play major role in the process of differentiation. Our microarray results indicate that neurospheres after induction of differentiation cease proliferation and exit mitosis. We found decreased expression of the Kinesin family member 23 (Kif23) which is involved in mitosis

(MishimaM et al., 2002). All the genes identified in relation to DNA synthesis and cell cycle progression are downregulated that include the Cyclins B1, D1, D2, and the Cell division cycle protein 20 (Cdc20) which are important factors involved in cell cycle progression. (Malatesta P et al., 2003) (Ulf Guroket et al., 2004) Surprisingly the cell cycle genes are found to be upregulated in the ciliary differentiated cells which indicate these genes are also involved in the coordination of complex neuronal properties including synaptic plasticity, as described by Schmetsdorf S in adult mouse neocortex (SchmetsdorfS et al., 2007). Looking for the genes involved in cell adhesion and defined cell junction structures, we found Cadherin 13 (a glycosylphosphatidyl inositol (GPI)-linked membrane protein that can mediate homophilic cell–cell adhesion, anti-adhesion and a function in cell cycle regulation) (Takeuchi T and Ohtsuki Y, 2001) (Takeuchi T et al., 2000) (Huang ZY et al., 2003) (Ivanov D et al., 2004), plakophilin 2, intercellular adhesion molecule 1, contactin 1&4, and neuronal cell adhesion molecule to be downregulated in the course of differentiation, indicating their expression in undifferentiated neurospheres. Some genes of the cell adhesion group were upregulated, like Neurexin (plays a role in cellular contacts between differentiating cells). Neurexins have been implicated in heterophilic cell–cell adhesion and assembly of presynaptic terminals (Dean C et al., 2003).

Our study also revealed the presence of the ECM proteins that are downregulated in differentiated cells compared with neurospheres which include Chemokine (C-X-C motif) ligand 5 and Chemokine (C-C motif) ligand 20. ECM genes that were upregulated in both the neurospheres and differentiated cells encode the ADAM metallopeptidase with thrombospondin type 1 motif 12, collagen type I, alpha 2, collagen type III, alpha 1, chemokine (C-X-C motif) ligand 6, interleukin 8, interleukin 1alpha, interleukin 1beta, matrix metallopeptidase 1, 3 & 20 and triggering receptor expressed on myeloid cells 1. The increased expression of these inhibitors suggests stabilization of certain ECM components. Among these Tenascin C was found to be highly upregulated in neurospheres than in differentiated cells which are known to be present in the embryonic brain and expressed by glial cells (Malatesta P et al., 2003), it also mediates adhesion of cells (Jones FS and Jones PL, 2000). Thus, our microarray study revealed genes known to play a role in

retinal neurogenesis. It also revealed genes with potentially new relevance for the maintenance, migration, and differentiation of the IPE and CPE derived progenitor cells.

Molecular pathways governing stem cell differentiation and pluripotency:

To understand the molecular mechanisms and pathways involved in the regulation of neural progenitor biogenesis i.e. the reprogramming of the cytokeratin positive epithelial cells to differentiate into Nestin positive neural progenitor cells, we performed literature search on the differentially regulated genes and their downstream targets. The most striking observation is that many genes identified as enriched in Neurosphere cultures are either involved in various aspects of cell-cycle control or represent the downstream targets of cell-cycle regulation and the proliferation and self-renewal activity.

The majority of cell cycle genes that were differentially expressed (70%) were upregulated in neural progenitors, consistent with their self-renewal properties. Specific genes included the cyclins B1, D1, and D2, the cell division cycle 2 homolog, cdc28 protein kinase, and others. These genes play a central role in controlling gene expression at the G1-S phase transition of the cell cycle by orchestrating the expression of genes whose products are required for nucleotide biosynthesis, DNA replication, and cell cycle progression, which also show concomitant changes in their expression. But, it is becoming clear that cell cycle and DNA synthesis control is a fundamental property of neural stem cells that is not simply related to their increased rate of proliferation relative to differentiated cells, but has fundamental implications for cell fate, patterning, and ultimately the evolution of the neural cells *in vitro*. Several genes previously implicated in neurogenesis or neural stem cell fates were found that are involved in the self-renewal, migration and differentiation of neural progenitor cells are controlled by a variety of pleiotropic signal molecules. Members of the family of Notch, Wnt, TGF-β, Hedgehog, and BMP molecules play a crucial role for developmental and repair mechanisms in the adult nervous system.

Objective 3:

Functional Glutamate receptor activity of the differentiated cells:

The functional characterization of the differentiated cells derived from the IPE and CPE neurospheres was determined by analysing the role of NMDA and non-NMDA ionotropic glutamate receptors, using Ca2+ imaging with Fluo-3AM. The samples were treated with of KA 30µM and AMPA 25µM. Increased response in intracellular [Ca2+] in the cells indicated the expression of ionotropic KA/AMPA glutamate receptors which was blocked by 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX 25µM) against AMPA and KA, leading to a decrease in the intracellular [Ca+] flux. The cells were treated with Mg2+-free solution containing glycine (0.1mM) for 2 minutes, followed by the application of NMDA 0.1mM in Mg2+-free glycine (0.1mM) solution for 1 minute. Similar to KA/AMPA, NMDA/Glycine evoked a Ca2+ increase in the cell, as denoted by the region of interest which was blocked by 3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphoricacid (CPP, 10µM) and 5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione (MNQX, 400µM) leading to a decrease in the intracellular [Ca+] flux. The fluorescence, within the marked region of interest, was plotted as a function of time. Thus, consistent with earlier studies, (Sumitra Bhattacharya et al., 2003) the cells express various physiological properties that are stimulated by the differentiating conditions commonly associated with the retinal neurons like the ionotropic glutamate receptors.

Synaptic vesicle recycling- Exocytosis and Endocytosis (FM1-43 unloading profiles in the synaptic terminals of IPE-DC and CPE-DC):

To visualize synaptic vesicle recycling in the IPE-DC and CPE-DC, we first determined the ability of these cells to incorporate the fluorescent styryl dye FM1-43 into synaptic vesicles and to unload this dye after stimulation with 100mM K+. FM1-43fluorescent puncta that were of sufficient quality were initially selected, and the synaptic puncta with unfavourable signal-to-noise ratios were excluded. The stimulation of cells with 1000mM K+ produced different kinetic profiles in terms of the rate and extent of dye unloading, as observed in other K+-stimulated preparations (Klingauf et al., 1998; Mozhayeva et al., 2002). As dye loading and unloading involve the same stimulation protocol (100mM K+, 5 minutes), any

differences in dye loss between synaptic terminals exhibiting strong and weak unloading profiles might reflect distinct capacities for recycling. Thus, consistent with earlier studies on neural cells, the IPE-DC and CPE-DC cells express the synaptic vesicle recycling.

Objective 4:

Correlation of DNA methylation, histone methylation and gene expression profiling in ciliary epithelium derived stem/progenitor cells and retinally differentiated cells:

To define changes in DNA and histone methylation during cellular differentiation process of ciliary epithelium derived SCs, profiling of DNA methylation as well as the presence of histone (H3K4Me3 and H3K27Me3) marks and RNA polymerase II of CPE-NS and CPE-DC was performed. The intensity values of the MeDIP and ChIP-Chip experiments were indicated by the normalized value of log2 ratios between the IP and WCE signals. To define the epigenetically marked genes the total intensity values of the gene body and promoters were considered. The modification intensities of the DNA methylation was very less when compared to the H3K4me3 and H3K27me3 in CPE-NS and CPE-DC as analysed by the significant probes per marked gene.

Further, global gene expression analysis was achieved using Affymetrix human 1.0 ST microarrays. The gene expression levels were indicated by the normalized signal intensities with cut off 6.0 for the lowest and 13.0 for the highest in log2 values based on our previous studies (Srilatha Jasty, et al., 2012). The signal intensities above 13.0 were considered to be active genes while intensities below 6.0 were considered to be silent genes. The mRNA expression values and the RNA polymerase II abundance on promoters were considered to determine the gene activity. Our data show the presence of Pol II at the promoters with mRNA expression in most cases as a predictor of transcript abundance. But for few genes, Pol II is present at the promoter, but no mRNA expression can be seen. This condition may be due to few promoters that are bound by stalled Pol II in human cells, ready to be rapidly produced (Mohn F, et al., 2008) (Guenther, M.G., et al., 2007) (Brookes E and Pombo A 2009). For further analysis we compared the gene list of the promoter microarrays and gene expression microarrays. There were a total of 17135 genes covered in both arrays which were used for analysis. In CPE-NS cells approximately 32%, 30% and 2% of all genes were marked by H3K4me3, H3K27me3 and DNAMe, respectively and in CPE-DC cells

approximately 34%, 31% and 3% of all genes were marked by H3K4me3, H3K27me3 and DNAMe, respectively. We compared all the three methylation modifications with gene activities in CPE-NS and CPE-DC, as it is well known that H3K4me3 is an active mark while both H3K27me3 and DNAMe are marks of silent gene expression (Cedar H and Bergman Y 2009) (Esteller M 2007). Our results show the expression level of the H3K4me3 and H3K27me3 marked gene group was higher compared to the expression level of the DNA methylated gene group, suggesting that DNA methylation alone showed only very low correlation in association with gene repression. The weak correlation between DNA methylation and gene activities was also confirmed by scatter plot analysis, as demonstrated by CPE-NS and CPE-DC cells.

DNA Methylation changes during Retinal neuronal differentiation:

To analyze the methylation changes during differentiation we generated an analogous profile for both the CPE-NS and CPE-DC. The DNA methylation marked gene in CPE-NS (n=544) showed only 30% promoter methylation and 70% Gene body methylation, whereas the DNA methylation marked genes in CPE-DC (n=761) we found 53% promoter methylation and 47% Gene body methylation. CPE-NS and CPE-DC cells show commonly marked genes of n=75, among which 27% were promoter methylation and 73% were of gene body methylation. These results show a gain of DNA methylation during retinal neuronal differentiation of the CPE-NS. Further analysis of the DNA methylation levels in the CPE-NS and the CPE-DC reveals a weak correlation to gene repression which is also confirmed by the scatter plot analysis.

Histone Methylation changes during Retinal neuronal differentiation:

To analyse the Histone methylation changes during differentiation we generated an analogous profile for both the SCs and retinal neuronal differentiation and further compared the Histone modifications with the gene expression levels. Most of the expressed genes in CPE-NS and CPE-DC were marked by H3K4me3 and in contrast the silent genes were marked with H3K27me3 in both CPE-NS and CPE-DC. We identified a large number of genes with only H3K4me3 modification in CPE-NS remained so in the CPE-DC. Other than this 3494 H3K4me3 enriched genes in CPE-NS switched to H3K27me3 (among these

1153 were promoters modifications) or with no modification during differentiation in CPE-DC cells and 4068 genes with H3K4me3 modifications in CPE-NS also showed enrichment for H3K4me3 in CPE-DC (among these 1323 were promoters modifications). This kind of histone methylation changes were associated with the genes mostly involved in neurogenesis and axonogenesis. Consistent with the previous findings in Neural progenitor cells (NSCs) (Mikkelsen TS, et al., 2007) (Matthew J. et al., 2013) (Fu H et al., 2009) our results of CPE-NS also shows H3K4me3 enrichment of the neuronal-specific microtubule protein TUBB3 and transcription factors like POU4f1, BCL11b, SOX10 and LHX4 which are involved in regulation of neuronal differentiation (Arlotta P, et al., 2005) (Lanier J, et al., 2009). Our results also show no H3K4me3 enrichment of certain genes involved in glial and astrocyte lineage markers like transcription factors like TCF4, SOX9, GFAP and AQP4 in CPE-NS consistent with the previous findings (Arlotta P, et al., 2005) (Lanier J, et al., 2009). But the CPE-DC cells show H3K4me3 enrichment of GFAP and AQP4 indicating the differentiation process may also include cells of glial lineage.

Bivalent chromatin states in CPE-NS (Stem/progenitor cells) and CPE-DC (Retinal neuronal differentiated cells):

The Bivalent chromatin signatures at the promoters play a major role in developmental gene regulation for the lineage-specific changes like the activation or the repression during differentiation (Mikkelsen TS, et al., 2007) (Maes J, et al., 2008) (Xu C-R, et al., 2011). To analyse this in the CPE-NS cells and their differentiated progeny, we determined the coincident H3K4me3 and H3K27me3 bivalent peaks across promoters in the array. The promoter regions bound with H3K4me3 alone, H3K27me3 alone and both H3K4me3 and H3K27me3 in both the cellular conditions were determined. In the CPE-NS cells 4595 gene promoters bound to H3K4me3, 4638 gene promoters bound to H3H27me3 and commonly bound include 286 gene promoters. Whereas in the CPE-DC cells 5104 gene promoters bound to H3K4me3, 4880 gene promoters bound to H3H27me3 and commonly bound include 337 gene promoters. This analysis revealed that CPE-DC cells had the highest number of bivalent promoters compared to the CPE-NS cells. In each condition, majority of the bivalent promoters modifications are associated with silent genes. Further analysis on the bivalent gene modifications in CPE-NS, it was found that these are associated with

neurogenesis, tissue morphogenesis and central nervous system development. The Bivalent genes found in the present study were also compared to the previously published results using Bivalent genes database (http://dailab.sysu.edu.cn/bgdb/index.php). Among the 286 gene promoters found in the CPE-NS, 87 gene promoters and among the 337 gene promoters found in the CPE-DC, 109 gene promoters were previously cited.

Degree of Bivalency during retinal neuronal differentiation of CPE-NS:

We further analysed the resolution of promoter bivalency upon retinal neuronal differentiation as it is associated with cellular potential. Among the 286 bivalent promoters in CPE-NS, 148 promoters resolved to monovalency or became unmodified upon differentiation. As predicted, resolution to monovalent H3K4me3 was associated with gene expression, while resolution to monovalent H3K27me3 was associated with gene repression. Moreover, the bivalent genes that resolved to monovalent H3K4me3 upon differentiation were largely members of GO categories associated with neuronal development. Although the majority of bivalent promoters in the CPE-NS resolved to monovalency during the differentiation process, only 8 of them remained bivalent even after differentiation which include LRRK1 (mediates early differentiation of human neural stem cells), MRAS, NOM1 (involves in embryonic stem cell plasticity), NPY1R, PCDH7 (Neuronal cell adhesion), PLEC1, TTPAL, WNT10a (involves in early differentiation of neural stem cell and embryonic stem cells).

Objective 5:

Characteristics of SCs expanded in SAPNs:

The CPE cells encapsulated in SAPNs were capable of forming neurospheres in the presence of EGF and FGF supplemented media. Further to analyse the progenitor properties of the neurospheres, qRT- PCR and Immunofluorescence analysis for progenitor markers were performed and the neurosphere showed expression of the Neural precursor markers Nestin, β-III tubulin, Pax6 and Musashi1. Upon induction of the dissociated neurospheres

in the differentiation medium, the cells could differentiate in the SAPNs. Our results demonstrate that CPE-NS encapsulated in SAPNs have the potential to differentiate into retinal cell lineage similar to the normal culture conditions. Apart from the dissociated cells the neurospheres were left encapsulated in the SAPNs to analyse the migration patterns and differentiation capacity, after day 5 the neurosphere medium was replaced with differentiation medium and the cells could migrate out of the neurospheres. Images were taken using a light microscope at 6, 10 and 14 days to determine the extent of migration.

Quantification of Cell proliferation (BrdU labelling indices):

To quantify cell proliferation (BrdU incorporation), the CPE-NS encapsulated in SAPNs were cultured in neurosphere medium for 7days and the cells were incubated with BrdU for 4 h before being processed for BrdU immunocytochemistry. We analysed the proliferation index at different concentrations of the SAPNs by quantifying the expression of BrdU positive cells in terms of the total cell number. The percentage of proliferating cells varied in different concentrations of SAPNs. The concentration of the SAPNs showing similar proliferation index compared to cells cultured without SAPNs was maintained throughout the study. Increasing concentrations of the SAPNs show a decrease in the percentage of proliferating cells, indicating the increased concentration of SAPNs could not maintain the stem/progenitor properties of the generated neurospheres. Results are presented as mean ± Standard deviation of five independent experiments performed with cells encapsulated in various concentrations of SAPNs.

Differentiation potential of the SCs in SAPNs:

To evaluate the capacity of SCs to generate differentiated progeny, after 6 days in SAPNs with neurosphere medium the CPE-NS were dissociated and cultured in differentiation medium encapsulated in the SAPNs for 14 days. The differentiation in the SAPNs could be achieved in the conditioned medium showing neuronal/glial morphology. Upon differentiation of the CPE-NS neurospheres into retinal lineage, these cells strongly expressed the retinal markers Rhodopsin, Recoverin, s-Opsin, Nrl and Crx as demonstrated by the qRT-PCR and Immunocytochemical analysis.

Gene expression analysis of the CPE-NS and CPE-DC encapsulated in the SAPNs:

To understand the effects of the SAPNs on differention potential of CPE derived SCs, we carried out a quantitative Real Time-PCR using an RT2Profiler^{TM} plate. The plate contained a set of primers for 84 genes expressed for the stem cell signalling molecules for CPE-NS and 84 genes expressed for extracellular matrix and cell adhesion molecules for CPE-DC. We analyzed the gene expression profiles of the CPE-NS and CPE-DC that were cultured encapsulated in SAPNs (Test) and cultured without SAPNs (Control). All the experiments were performed in biological triplicates. The gene expression changes in the cells cultured in SAPNs were analyzed when compared to cells cultured without SAPNs.

When analyzing the stem cell signaling molecules, among the 84 genes that were analyzed the cells cultured in the presence of SAPNs resulted in significant upregulation of SMAD7 which is known to regulate neural stem/progenitor cell proliferation in a TGF-β and BMP-independent manner (Monika Krampert, et. al. 2010) and TGFβ auxiliary co-receptor endoglin is also upregulated and is known to express in neural crest stem cells in vivo (Maria L. et. al. 2007) the other TGFβ pathway genes like LTBP2, EP300, SMAD4, TGFBRAP1, SP1, SMAD2, CREBBP, SMAD3, SMAD5, RBL2, TGFBR3, BMPR1B were significantly downregulated indicating cell proliferation in TGF-β and BMP-independent manner. The other significant downregulation of the Wnt pathway related genes like FZD3, FZD7, NFATC3, LRP6, VANGL2, NFAT5, FZD4, FZD2; Notch pathway genes like PSEN2, NOTCH4, FGF pathway genes like FGFR4, FGFR1; Pluripotency genes like LIFR and Hedgehog Pathway like GLI3, PTCH1 when compared to the cells cultured without puramatrix.

Further analyzing the extracellular matrix and cell adhesion genes, among the 84 genes that were analyzed the cells cultured in the presence of SAPNs resulted in significant upregulation of TIMP1, MMP2, MMP3, MMP9, MMP12, CD44, SPARC, ICAM1, ITGA7, ITGA5. The increased expression of the ECM Protease inhibitors like TIMP1 suggests a stabilization of certain ECM components of differentiating cells and they are known to be a mediator of neuronal migration by regulating axon guidance and process outgrowth. (Jaworski DM & Fager N. 2000) The increased expression of the MMPs helps in the

adhesion migration and elongation of the neuronal cells in the SAPNs (Sarig-Nadir O & Seliktar D. 2010). The CD44 expression is known to be present in neural progenitor cells as well as in neurons. (Naruse M, et. al. 2013) The upregulation in the ECM genes indicated better 3D environment for the cell survival and migration. The Downregulation of COL12A1, ITGA1, ITGA4, ITGA6, NCAM1, ITGA8, SPG7, CLEC3B, CTNND2, SPP1, ITGB2, and VTN indicated there are changes occurring during the differentiation process in the cells-cell interactions and cells- matrix interactions that are yet to be studied.

DISCUSSION:

In the present study we have described the isolation and in vitro characterization of the IPE and CPE derived stem/progenitor cells and their differentiation potential. Here we have shown that a small number of cells in the iris and ciliary body regions of the adult human eye yielded cells that are capable of significant expansion in presence of the mitogens EGF/FGF-2 indicating the presence of stem/progenitor cells in the IPE and CPE of human cadaveric eye. These results are consistent with previous studies in adult mice, rats, porcine and human (Ahmad I, et al., 2000) (Tropepe V, et al., 2000) (Yuji Inoue, et al., 2005) (Kohno R, et al., 2006). In all these instances proliferation occurred on stimulation with epidermal and fibroblast growth factors. The neurosphere assay demonstrates the presence of the proliferating cells that are capable of forming spheres which is known to be the characteristic of the neural stem/progenitor cells. Individual neurospheres from each of these regions were similar in size, indicating that they have comparable expansion potential when grown as neurospheres.

We analysed the effect of the growth factors (mitogens) and demonstrated that FGF2 and EGF promoted cell division among progenitors in both the tissues. A small number of sphere colonies were generated in the absence of either of the exogenous growth factors, the colony formation ability was much facilitated by the additions of FGF2 and EGF together. Thus, progenitor cells isolated from these tissues are partly dependent on exogenous growth factors, which is in variance with stem/progenitor cells in mice (Bartsch U, et al., 2008) and rabbits (Moe MC, et al., 2009). In the present study we show the

presence of not more than 1-2% of proliferating cells in the IPE and CPE cells of human postmortem eyes as demonstrated by the sphere suspension assay.

When analyzing the growth rate of IPE and CPE cells in the presence of growth factors EGF and FGF we show that only a small population of the propagated cells in the sphere can proliferate after passage and the cells become senescent after the fifth or sixth passage in vitro suggesting that these cells have relatively limited proliferative capacity. Our results are consistent with previous studies on animal models (Ahmad I, et al., 2000) (Brenda L. K. Coles, et al., 2004) (Das AV, et al., 2004) (Dean C, et al., 2003). Our data are compatible with the hypothesis that soon after dissociation, most cells in neurospheres divide to produce daughter cells, where after few passages, only one fifth have proliferative capacity, as judged by our self-renewal assay and BrdU labeling assay. The potential advantage of the reduced proliferative capacity is it may decrease the chance of tumorigenesis after transplantation.

Our results also demonstrate the expression of progenitor markers ABCG2, Nestin and Musashi1 by the neurospheres generated from both the regions indicating that these tissues have the stem/progenitor cells. Our results were consistent with the previous studies using mice (Lamba DA, et al., 2009), rats (Bartsch U, et al., 2008), rabbits (Moe MC, et al., 2009), and porcine (Ahmad I, et al., 2000) ocular tissues. In addition to expression of general progenitor components, IPE and CPE derived stem/progenitor cells express homeobox genes and transcription factors that are prominent in normal retinal development (Jones FS and Jones PL 2000), including Chx10 which plays an important role in normal eye development (Larysa Pevny and Mahendra S. Rao 2003) (Ilyas Singec, et al., 2006), Pax6 expressed in brain and retina during development and plays an important role in the regulation of cell proliferation and the determination of neuronal fate (Hangxiu Xu, et al., 2007) and Sox2 as previously reported (Ahmad I, et al., 2000) (Tropepe V, et al., 2000) (Das AV, et al., 2004) (Margit Burmeister, et al., 1996) (Warren N, et al., 1999). The expression of these markers can be taken as evidence that IPE and CPE derived SCs are committed to develop into retinal cells.

In our present study we also show that the NS derived from IPE and CPE cells could be differentiated into retinal progeny in presence of BDNF, Retinoic acid and 10% FBS when incubated for 21 days. Our RT-PCR and immunolabelling data on IPE and CPE differentiated cells revealed the expression of the markers typical for mature retinal cells, including markers of photoreceptors, horizontal cells, amacrine cells, or ganglion cells. The differentiated progeny show the expression of the photoreceptor markers indicating the retinal potential of these cells *in vitro*. We report here differentiation potential of the IPE and CPE derived NS into retinal cells in the absence of growth factor stimulation and in the presence of the retinal differentiation conditions like addition of BDNF and Retinoic acid. These conditions allowed the transition toward cells expressing molecules seen in adult retina, such as neurofilament protein, β-III tubulin, s-opsin, and Rhodopsin.

In the present study we have also shown that the proliferating neurospheres generated from the IPE and CPE cells of adult human cadaveric eyes show some properties of the retinal progenitor cells. These proliferating cells could differentiate into a mixed population of the retinal cells and epithelial cells. This is an important finding, as there are no studies that have thoroughly addressed the question whether IPE and CPE neurospheres derived from the adult human IPE and CPE cells could differentiate into a mixed population of cells with both epithelial and neural properties. Our data thus support previous reports to a certain degree that the neurospheres generated from both the regions retain the pigments and some of the epithelial markers as described previously (Hangxiu Xu, et al., 2007) (Moe MC, et al., 2009) (Frøen RC, et al., 2011). Our microarray data shows the expression of the reprogramming factors Oct-4, Sox-2, c-Myc and Klf-4, as described by Kohno (Kohno et al., 2006). The neurospheres undergo differentiation by a new and distinct mechanism of reprogramming from the epithelia-like IPE and CPE derived cells to neural cells with the expression of retinal cell markers. Though the neurospheres derived from both the IPE and CPE cells are partially or heavily pigmented, upon induced differentiation we found the neurospheres expanded in monolayer cultures with loss of their dense pigmentation and multiple processes with time in culture suggesting that de-differentiation with consequent loss of pigments as described by our morphological and electron microscopy studies and our results are also consistent with previous studies (Gualdoni S. et. al. 2010). As known

previously the melanin synthesis mainly occurs during embryonic development, in which the stage I and II immature unpigmented melanosomes undergo modifications and lead to complete maturation into stage IV pigmented functional melanosomes (Gualdoni S. et. al. 2010), our results prove that the stem/progenitor stages (NS) and late stages (DC) derived from the pigmented IPE and CPE cells do not retain stage I & II melanosome genes demonstrated by our microarray analysis, indicating that melanin deposition is complete. We therefore assume that pigmentation in IPE and CPE monolayers is lost in culture by melanosome degeneration over time. Our electron microscopic examinations of the differentiated progeny derived from the IPE and CPE neurospheres also indicate their high intracellular activity, nonspecific features and the cells are devoid of the immature pigmentation as reported previously (Kohno et al., 2006). Therefore our data suggest that a process of differentiation is occurring with the loss of pigmentation in the cell phenotype.

In addition to the cell type specific marker expression (demonstrated by our RT-PCR studies) and other characteristics of the differentiated cells, we also analysed the functional maturation of the retinal neurons in the differentiated conditions at lower density with calcium imaging techniques. Our calcium imaging studies have shown that a subset of cells with distinct neuron-like morphology differentiated from adult IPE and CPE neurospheres, respond to NMDA, AMPA and Kainic acid stimulation with an increase in cytoplasmic Ca2+ levels. These transient changes in the Ca2+ levels and the presence of the glutamate receptors are important for regulating neuronal differentiation, transmitter selection, and axonal targeting in neurons, moreover calcium signals regulate multiple cellular processes such as synaptic transmission. Using calcium imaging studies, we observed that the neuron-like cells produced transient increases in the cytoplasmic Ca2+ levels in response to the application of the stimulation with the receptor agonists. These evoked an increase in the cytosolic Ca2+ levels. The increase in Ca2+ could be caused by a direct influx of extracellular Ca2+ through glutamate-induced ion channels. We also show the decreased Ca2+ influx upon addition of the receptor inhibitors. Our calcium imaging experiments extend the previous studies and demonstrate that the IPE and CPE differentiated cells were capable of differentiating into cells that had many of the basic functional properties of retinal neurons in our defined culture system.

One of the very important features of tissue-specific stem cells is their ability to differentiate into different lineages which requires interplay between extrinsic cues and cell intrinsic programs, which include the epigenetic modifications. Gene expression studies allow characterization of SCs, but this does not explain the cell fate or potential that occurs as cell becomes lineage restricted during the process of differentiation. In this context, the epigenetic profiling of the SCs provide us a precious information about the potency of the SCs, as they mark the genes that have the potential to be expressed and lead to changes in gene expression. Till date several studies have addressed the epigenetic makeup of the ESCs and the role of epigenetic mechanisms in their self-renewal and differentiation (Mikkelsen TS, et al., 2007) (Lee TI, et al., 2006) (Pasini D, et al., 2007) (Ren X, et al., 2008). Significantly, very less information is known regarding the epigenetic modifications like DNA methylation and Histone methylation during the cell fate determination of the ciliary body derived SCs. Moreover, previously the concurrent presence of H3K4me3 and H3K27me3 (bivalency) at the promoters of many lineage-specific genes has been interpreted as an epigenetic signature of pluripotency in ESCs (Bernstein BE, et al., 2006) (Mikkelsen TS, et al., 2007). In the present study, we provide first whole genome profiling of DNA and histone methylation during the retinal neuronal differentiation of the ciliary pigment epithelial derived SCs. Our work would describe the epigenetic signature of CPE-NS, retinal neuronal potential that is dynamically regulated during their differentiation.

The whole genome profiling of DNA and histone methylation in the present study showed consistent relationships between promoter modifications and gene expression patterns concerning many genes that are known to play important role in the process of differentiation of CPE-NS to CPE-DC cells. Therefore it appears that epigenetic modifications are involved in the coordination of entire gene expression programs during this process. On considering the entire results in the study most of the genes are modified by at least two of the DNA or H3K4 or H3K27 methylation marks and the complete modification signal intensities of active and repressive marks was associated with the modification of gene activities. The transcriptional intensity of the genes with bivalent modifications rely on the intensities of the active and repressive marks. DNA methylation is known as a repressive mark of the gene activity, however this may vary in several aspects

of the gene activity as described in previous studies (Bernstein BE, et al., 2006) (Mikkelsen TS, et al., 2007). In the present study we found that DNA methylation alone is not a strong silencing mark, but it acts as a strong silence mark without the concurrent H3K4me3 modification and DNA methylation associates very less to the promoter repression. In the process of CPE-NS differentiation, we found that DNA methylation has less significant effect than histone methylation. Upon analysing the DNA methylation data we found that the number of significant DNAMe marked genes was very less when compared to the H3K4me3 and H3K27me3 in both CPE-NS and CPE-DC (Matthew J. et al., 2013). Therefore, there was no significant correlation between DNA methylation modification and altered gene expression in both SCs and differentiation state, consistent with the previous results the weak correlation between DNA methylation and gene silencing was also seen in ESCs (Mohn F, et al., 2008).

In the present study, we also found that most of the active gene promoters are marked by H3K4me3, while only a minority of silent genes were marked by H3K27me3 in CPE-NS, indicating that maintenance of gene silencing in CPE-NS is largely carried out by polycomb independent mechanisms, consistent with the previous results in NSCs (Matthew J. et al., 2013). However, studies in ESCs have shown that maintenance of gene silencing is carried out by H3K27me3 and DNA methylation is predominantly involved in differentiation of ESCs to NSCs, whereas little change in DNA methylation occurs during subsequent differentiation of NSCs to neurons (Mohn F, et al., 2008). During the process of differentiation, CPE-NS cells give rise to a mixed population of cells, majority of them include neuronal cells and minority of other cell phenotypes also, as demonstrated in our previous data (Srilatha Jasty, et al., 2012). Additionally evidence show that bivalent modifications provides a map of neuronal developmental potential and regulation of neuronal identity, we find that bivalency is not restricted to the promoters of genes regulating only for the neuronal phenotypes. Our results also marks the promoters of non-neural markers such as COL1A1, GATA6, EOMES etc., in CPE-NS, which are bivalent in NSCs and ESCs. These bivalent genes mostly resolve upon differentiation. Consistent with the previous studies on ESCs our results show that bivalency in CPE-NS is particularly related to neurogenesis (Matthew J. et al., 2013) (Mikkelsen TS, et al., 2007) (Mohn F, et

al., 2008) thus indicating that the bivalency marks regulatory genes required for the differentiation of the CPE-NS cells into neuronal phenotypes *in vitro* there by indicating the plasticity of these cells.

On comparing the bivalency data produced in our study with the earlier studies on epigenetic changes during neuronal differentiation, we report that most of the gene undergo resolution upon differentiation, but they reported little resolution of bivalent marked genes (Mohn F, et al., 2008) (Matthew J. et al., 2013). The causes for this dissimilarity is unknown, but this may be due to the differences in the SCs production, culturing protocols, differentiation protocols and the purity of the cell populations as well as the different epigenetic marks used in the studies, it may also show differences in the acquisition, loss, and retention of the bivalent modifications in different neuronal cell populations. Thus, it would appear that in CPE-NS, developmental genes are bivalently marked, reflecting their developmental potential (Conti L, et al., 2005), and that upon differentiation this bivalency resolves with acquisition of a neuronal fate. Although most of the bivalently modified promoters resolve during the process of differentiation, a specific group of genes retain bivalency the majority of bivalent promoters resolve during differentiation, a distinct group of promoters either retain or gain bivalency. Therefore, our data support the opinion that limitations as well as alterations of bivalency occurs at different stages of development and differentiation. The changes in epigenetic signatures occur before any subsequent gain of gene expression underlining the ability of the epigenetics to provide a read-out of developmental potential (Matthew J. et al., 2013).

We had characterized the CPE-NS and CPE-DC grown without the SAPNs, (Srilatha Jasty, et. al. 2012) when cultured in sphere formation media containing FGF2 and EGF. The CPE cells formed spheres that differentiated into retinal neuronal cells upon induction with differentiation media containing BDNF and Retinoic acid on poly-D-lysine and laminin coated dishes. The differentiated cells expressed retinal neuronal morphology and markers. (Srilatha Jasty, et. al. 2012) In the present study, we have also used a recently introduced group of biomaterial, self-assembling peptides, which are based on 16 residue peptide (RADA 16-I) that self-assembles into antiparallel β-sheets at physiologic pH, that could

form nanofibers that mimic the architecture of the Extracellular matrix by providing anchorage, mechanical buffering, aiding intercellular communications and cell migrations that would help in the regeneration of tissues in three dimensional construct. (Thonhoff JR, et al. 2008) (Taraballi F, et al. 2009) (Carla Cunha, et. al. 2011)

We used SAPNs as scaffolds to grow the CPE-NS and assessed their survival, proliferation and differentiation after encapsulation. The CPE cells encapsulated in SAPNs were capable of forming neurospheres in the presence of EGF and FGF supplemented media. Further to analyze the progenitor properties of the neurospheres, qRT- PCR and Immunofluorescence analysis for progenitor markers were performed and the neurosphere showed expression of the neural precursor markers. Upon induction of the dissociated neurospheres in the differentiation medium, the cells could differentiate in the SAPNs. Our results demonstrate that CPE-NS encapsulated in SAPNs have the potential to differentiate into retinal cell lineage similar to the normal culture conditions (Srilatha Jasty, et al., 2012). The CPE-NS express many genes associated with retinal neuronal SCs, after stimulation with FGF2 and EGF growth factors, the generated spheres are morphologically similar to the neurospheres as described in the previous studies (Deleyrolle LP & Reynolds BA. 2009). The cultured CPE-NS expressed many genes typically associated with retinal neuronal SCs, including Nestin, Pax6, βIII tubulin and Musashi1 consistent with normal culture conditions (Srilatha Jasty, et. al. 2012), which demonstrates that the SAPN scaffolds could maintain the stemness. Apart from the dissociated cells the neurospheres were left encapsulated in the SAPNs to analyze the migration patterns and differentiation capacity, after day 5 the neurosphere medium was replaced with differentiation medium and the cells could migrate out of the neurospheres. As mentioned in the previous studies (Thonhoff JR, et al., 2008) (Semino CE, et al., 2003) (Zaman MH, et al., 2006) we have used lower concentration of SAPNs to provide adequate pore size, allow medium infusion and lower the toxicity levels due to release of harmful or acidic byproducts during degradation, and our results support the previous studies (Thonhoff JR, et al., 2008).

We analyzed the proliferation index at different concentrations of the SAPNs by quantifying the expression of BrdU positive cells in terms of the total cell number. Increasing concentrations of the SAPNs show a decrease in the percentage of proliferating cells,

indicating the increased concentration of SAPNs could not maintain the stem/progenitor properties of the generated neurospheres. To evaluate the capacity of SCs to generate differentiated progeny, after 6 days in SAPNs with neurosphere medium the CPE-NS were dissociated and cultured in differentiation medium encapsulated in the SAPNs for 14 days. Upon differentiation of the CPE-NS neurospheres into retinal lineage, these cells strongly expressed the retinal markers Rhodopsin, Recoverin, s-Opsin, Nrl and Crx as demonstrated by the qRT-PCR and Immunocytochemical analysis. These results suggest that the isolated and expanded CPE cells can be induced to differentiate into a retinal neuronal direction even when encapsulated in SAPNs as demonstrated in normal culture conditions (Srilatha Jasty, et. al. 2012).

To understand the effects of the SAPNs on differention potential of CPE derived SCs, we carried out a quantitative Real Time-PCR using an $RT^2Profiler^{TM}$ plate. We analyzed the gene expression profiles of the CPE-NS and CPE-DC that were cultured encapsulated in SAPNs (Test) and cultured without SAPNs (Control). When analyzing the stem cell signaling molecules, among the 84 genes that were analyzed the cells cultured in the presence of SAPNs resulted in significant upregulation of SMAD7 which is known to regulate neural stem/progenitor cell proliferation in a TGF-β and BMP-independent manner (Monika Krampert, et. al. 2010) and TGFβ auxiliary co-receptor endoglin is also upregulated and is known to express in neural crest stem cells in vivo (Maria L. et. al. 2007). Further analyzing the extracellular matrix and cell adhesion genes, among the 84 genes that were analyzed the cells cultured in the presence of SAPNs resulted in significant upregulation of Extracellular matrix proteins. The increased expression of the ECM Protease inhibitors suggests a stabilization of certain ECM components of differentiating cells and they are known to be a mediator of neuronal migration by regulating axon guidance and process outgrowth. (Jaworski DM & Fager N. 2000) The upregulation in the ECM genes indicated better 3D environment for the cell survival and migration.

The transition of multipotent IPE and CPE Stem/progenitor cells into differentiated retinal cells involve complex gene expression changes of which only few are known to date. To learn more about significant changes, we compared the gene expression of neurosphere cells from the IPE and CPE with cells after induction of differentiation using BDNF and Retinoic

acid. Previously, no studies have been reported to assess the changes occurring during the course of differentiation of the IPE and CPE neurosphere cells using microarray. Our analysis has identified many new candidate molecules and confirmed several others that are likely to play important roles during the maintenance and differentiation of the IPE and CPE neurospheres. Thus, our data could serve as a basis for future analyses of the molecular and cellular characteristics of adult IPE and CPE derived stem/progenitor cells and their differentiation potential. Moreover, further studies on this models may provide subsequent stages of epigenetic modifications that will provide queues for stage specific retinal neuronal development and lead it to therapeutic applications. Our data on SAPNs support that these scaffolds could be used as a potential source for SCs culturing and as a 3D cell culture model. These SAPNs are commercially available, easily synthesized and purified with a low batch-to-batch variability on a large scale, highly biocompatible, biodegradable and could provide a temporary niche for cell-replacement strategies without any cell damage and modifying the cellular characteristics. These characteristics allow SAPN scaffolds to be the potential source for tissue engineering strategies and to meet the clinical application standards in the near future. This culture system also provides a matrix for the in vitro experimental validation of the novel drugs and the recent advancements of small molecule technology on the SC cultures.

CONCLUSION:

In conclusion, the differentiated cells derived from the neurosphere cultures do not show proliferation, and self-renewal potential and gene expression studies, whereas the neurospheres are highly proliferative and could with stand for 5 generations indicating the self-renewal potential. The gene expression studies on both the iris and ciliary neurospheres show that the self-renewal and proliferative genes to be highly expressing, indicating them to have more potential for transplantation. The molecular pathway like WNT, NOTCH and TGF-β signaling show the cells to be in progenitor state and differentiation along the neural cell lineage. Moreover, the neurospheres derived from both the tissues show more similar characteristics and the neuroshperes show the pigmentation genes like Palmdelphin, Tyrosinase, Rpe65 and MitfD and MitfA to be down-regulated indicating the cells are capable of differentiating. Our *in vitro* live cell imaging studies on the differentiated cells

derived from the ciliary and iris neurospheres with Fluo-3AM and FM-143 in calcium flux prove that the differentiating conditions promote the expression of various physiological properties commonly associated with retinal neurons. The genome-wide DNA methylation and Histone methylation profiles generated will further provide an understanding of the epigenetic control of SCs isolated from the pigmented ciliary epithelial cells. These data will provide an important framework for understanding the epigenetic mechanisms of lineage commitment throughout the process of differentiation. Our study provides the basic *in vitro* molecular and functional characteristics of the stem/progenitors isolated from the iris and ciliary pigment epithelium indicating them to be best for the transplantation studies. Moreover, using these results, we can further attempt to reprogram a more specified lineage directed cells towards a more restricted retinal neuronal phenotype using a combination of transcription factors, thought to play an essential role in retinal neuron development. Although our results demonstrate the presence of retinal neurogenesis, the advancement in these results would shape help further reprogramming methods with the ambition of engineering desired pure population of cellular subtypes for therapeutic applications. Our data also support that SAPNs could be used as a potential source for SCs culturing and as a 3D cell culture model. These SAPNs are commercially available, easily synthesized and purified with a low batch-to-batch variability on a large scale, highly biocompatible, biodegradable and could provide a temporary niche for cell-replacement strategies without any cell damage and modifying the cellular characteristics. These characteristics allow SAPN scaffolds to be the potential source for tissue engineering strategies and to meet the clinical application standards in the near future. This culture system also provides a matrix for the *in vitro* experimental validation of the novel drugs and the recent advancements of small molecule technology on the SC cultures. Further studies for the integration of the neurosphere cells into the retina can be studied in animal models by labelling the neurosphere cells with fluorescence tag and injecting them through the sub retinal space using these kind of scaffolds reducing the cell damage.

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