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

**THESIS**

Submitted in partial fulfillment

of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**


by

**SRILATHA JASTY**

**2008PHXF019P**

Under the supervision of

**Dr. S. KRISHNAKUMAR**

**BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE**

**PILANI (RAJASTHAN) INDIA**


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# CERTIFICATE

This is to certify that the thesis entitled **"Identification and Characterization of Human Iris Pigment Epithelial Cells and Ciliary Pigment Epithelial Cells In-vitro for Retinal Stem/Progenitor Properties."** submitted by **Ms. Srilatha Jasty, ID. No. 2008PHXF019P** for award of Ph.D. Degree of the Institute embodies original work done by her under my supervision.

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# ACKNOWLEDGEMENTS

Completing my Doctoral degree is undoubtedly the most challenging activity of my life. This thesis is the end of my journey in obtaining my Doctoral degree. I have not travelled alone in this journey; the best and worst moments have been shared with many people and been complete with the support and encouragement of my well-wishers, my friends, colleagues and various institutions. At the end of my thesis I would like to thank all those who made this thesis possible and an unforgettable experience for me, it is also a pleasant task to express my thanks to all those who contributed in many ways to the success of this study.

At First my head bows down to the Creator.

At this moment of accomplishment, my sincere thanks to my supervisor **Dr.S.Krishnakumar**, Head, L&T Department of Ocular Pathology and Nanotechnology. This work would not have been possible without his guidance, support and encouragement. Under his guidance I successfully overcame many difficulties and learned a lot. I thank him for giving me the fellowship to work in his project, contributing with good ideas and valuable comments on the work.

I am also extremely obligated to **Prof. H.N.Madhavan**, President, Vision Research Foundation, Director of VIBS, for his support and encouragement.

I would like to thank Padmabhushan **Dr.S.S.Badrinath**, Chairman Emeritus, and **Dr.S.B.Vasanthi Badrinath**, Director, Laboratory Services, and **Dr.S.Baskaran**, Chairman, Sankara Nethralaya, Chennai, India for providing me the opportunity to work in this esteemed institution and to pursue my doctoral programme.

I thank **Dr.Ronnie George**, Director of Research, Vision Research Foundation, **Dr.Tarun Sharma**, Honorary Secretary and Treasurer, Vision Research Foundation, **Dr.T.S.Surendran**, Vice-Chairman, Medical Research Foundation for their support.

I take this opportunity to sincerely acknowledge the **Department of Biotechnology, Indian council of medical Research, International Stem Cell Corporation** and **Sumathi Chitra Charitable trust** for providing financial assistance in the form of

Senior Research Fellowship which supported me to perform my work comfortably. I would also like to thank Genotypic technologies pvt. Ltd. Bangalore for Microarray technology and **Dr.Puspha Visvanadhan** of Cancer Institute, Adyar, Chennai for the Transmission Electron Microscopic analysis.

My sincere thanks to **Prof.J.Biswas,** Director, Ocular Pathology Department, **Prof.K.N.Sulochana,** Head and **Prof.Angayarkanni**, Reader, **Dr.K.Coral**, Lecturer, **Dr.SR.Bharathi Devi**, PDF of Biochemistry and Cell Biology Department, **Dr.A.J.Pandian**, Head, **Dr.N.Soumittra, Dr.S.Sripriya**, Lecturer of ONGC Department of Genetics and Molecular Biology, **Dr.V.Uma Shankar**, Head of Bioinformatics Department, **Dr.Lilly Therese**, Head & Professor, **Dr.J.Malathi,** Reader, **Dr.B.Mahalakshmi**, Lecturer of L & T Microbiology Research Centre including SAIL Molecular Microbiology, **Dr.Doreen Gracious**, consultant, clinical lab for their kind words, support and encouragement during my research work.

I sincerely thank **Prof.L.K.Maheswari**, Vice Chancellor and Director, BITS, Pilani for giving me the great opportunity to do my thesis under the esteemed university. I thank **Dr.Ashish Kumar Das**, Dean, Research and Consultancy Division, BITS, Pilani, **Dr.Ravi Prakash**, Past Dean, Research and Consultancy Division, BITS, Pilani, **Prof.Sundar**, Dean Distance Learning Programme division, **Dr.Sanjay D.Pohekar**, **Dr.Deshmukh** and **Dr.Dinesh Kumar**, Convenor, PhD Monitoring, BITS, Pilani.

I extend my sincere thanks to **Mr.S.Narayan**, Manager, Vision Research foundation and **Mr.N.Sivakumar**, Academic Officer for their support. I thank **Mr.Thiyagarajan** Head, Medical records Department and all his colleagues for their support in giving me patient’s files.

I thank **Mr.Senthil kumar**, Secoratory-Chairman Emeritus, **Ms.Manjula, Ms.Asha, Mr.Arun kumar** from Office, Vision Research foundation, **Ms.Rekha**, Secoratory-President, **Ms.Parvathi**, Secoratory-Department of Biochemistry for their help and encouragement in my work.

This list is incomplete without mentioning **Dr.Sudha Balasubramanian**, Senior Research Scientist at Stempeutics Research Pvt. Ltd. Bangalore, who was the source of inspiration for me in my early days and who taught me all the basics in molecular biology and cell biology, you are the best person I ever met. I would also like to thank

**Dr.Gunisha Pasricha** in my early days, who guided me in learning about project, standardizing of primary cultures.

I would also like to thank **Dr.G.Suganeswari,** Vitro-Retinal clinician, Medical Research Foundation for teaching me dissection of the eye ball, which helped me a lot throughout my study. I thank **Dr.Nivedita Chatterjee** for helping me in trouble shooting for calcium imaging techniques during my fellowship. I would also like to specially mention **Dr.V.Ram Prasad** for teaching me Affymetrix microarray technology at Vision Research Foundation.

I am indebted to my many Colleagues for providing a stimulating and fun filled environment at lab. I would like to thank specially **Ms.Vanitha** for her timely help in getting proper retinal sections and many other histopathological related works, I would like to thank **Mr.Purushothaman**, technician, **Ms.Chitra**, Secoratory for their help in histopathology. I would also like to thank **Ms.T.Seethalakshmi, Ms.P.Vandhana, Mr.Gopinath Krishnan, Mr.Job Sudhakar, Ms.K.Moutushy Mitra, Mr.Mallikarjuna Kandalam, Ms.Sasirekha Krishnan, Ms.V.Nalini,Ms. Uma** (Ex-Secoratory), **Ms.Saranya Suriyanarayanan**, **Ms.Vidya Gopakumar, Ms.S.Charanya, Mr.Madhu Beta, Mr.Ravikanth Danda, Ms.Jaisy Samuel, Ms.Deepa, Ms.Sushma Kalmodia, Ms.Sangeetha, Ms.Meenalakshmi, Ms.Nithya, Ms.Kalaivani, Ms.Jayashree, Ms.Srinithi, Mr.Prasana kumar, Mr.Jagadesh, Mr.Goutham, Mr.Narayan, Ms.Preethi, Ms.Yamuna, Ms.Rajalakshmi**, **Ms.Rajeswari, Ms.Tamil, Ms.Mary and Ms.Sukanya**.

I wish to extend my thanks to all the colleagues from the Departments of Microbiology, Biochemistry, Genetics and Molecular Biology and Clinical Laboratory for their support and help.

My special thanks to **Mr.Anadurai**, Lab Attendant for helping me in acquiring, products from the communication department and for all the postal related jobs.

I would like to specially mention about my friend **Ms.Vidhya Srinivasan** who was with me all the time from my masters sharing the best and worst moments, you're the one I would never forget. I enjoyed the days spend at your house with your family, making me one among you. I will never forget the ice creams, Chocolates, speechless arguments we had. There are no words in this world to mention about you. A special

thanks to **Mr.Manigandan Srinivasan**, **Ms.Sunitha Srinivasan, Ms.Radha Srinivasan, Master Narayana and Mr.Akshay Chandra Dekha** and your others family members.

I would also like to mention about my friends **Ms.Kirthika Dinesh** and **Ms.Mahalakshmi** for their support and encouragement all the time. I would also like to specially mention about my junior **Ms.Gomathy Iyer Narayanan**, who helped me a lot at times of need, with her constant support and encouragement all the time.

I would like to extend my thanks to my roommates **Ms. AV. Sai Jyothi**, **Ms.Praveena**, **Ms.Vijayalakshmi, Ms.Namitha** for their support and sharing all the good and bad times. I enjoyed a lot with you people. Thanks a lot for your suggestions at work Jyothi akka.

I would like to express my gratitude to my Parents **Mr.Venkateswara Rao Jasty** and **Ms.Sandhya Lakshmi Jasty**, they not only gave life and a much cherished childhood, but also taught me to love and instilled great values that I live by today. Their unfailing support and encouragement in the most desperate circumstances and constant emphasis on good education were drivers that got me this far. Father you inspire me a lot, I have learnt a lot from you.

I would like to express my gratitude to my dearest brother **Mr. Srikanth Babu Jasty** for his constant love and support. Without you these many years would not be so enjoyable. I would never forget all the phone calls made to you during my stay in Chennai, you taught me all the Technical (Hardware and Software) related queries all the time. Thanks for bearing with me. I would also like to express my appreciation to my sister-in-law **Ms.Kanaka Sri** for helping me in the needful situations.

I would like to express my thankfulness to my grandmother **Ms.Anasuyamma Jasty**, Aunt **Ms.Sunitha Jasty**, Uncle **Ms.Srinivasa Rao Jasty**, brothers **Naresh Jasty** and **Sasikiran Jasty** for their unconditional love, affection and timely help in times of need.

I would like to express my gratefulness and my love to my mother-in-law **Late Shri.Seetaramamma Nadimpalli** and my father-in-law **Mr.Nageswara Rao Nadimpalli** for their understanding towards my studies and career.

I would like to thank my dear husband **Mr.Rajesh Nadimpalli** and my son **Master Chaitya Nadimpalli** for their unconditional love, support, concern and their great encouragement throughout my studies.

I also want to thank all my other relatives and extended families for their help and support during my study.

**Srilatha Jasty**

***Dedicated to my Father***

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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 $RT^2$ 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.

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# ABBREVIATIONS

ADVASEP 7 - Sulfobutylated derivative of beta-cyclodextrin

AMD/ARMD – Age-related macular degeneration

AP-5 - D (−)-2-Amino-5-phosphonopentanoic acid

ASCs – Adult stem cells

ATP - Mg-adenosine triphosphate

BDNF - Brain derived neurotrophic factor

BM – Bruch's membrane

BMSCs – Bone marrow stem/progenitor cells

BrdU – 5-bromo-2'-deoxy-uridine

CaCl2 - Calcium Chloride

cDNA – Complemantary Deoxy ribose nuclic acid

CE – Ciliary epithelium

CNQX - 6-cyano- 7-nitroquinoxaline-2, 3-dione

CNS – Central nervous system

CNV – Chroidal neovascularization

CPE – Ciliary pigment epithelium

CPE-DC – Ciliary pigment epithelium derived differentiated cells

CPE-NS – Ciliary pigment epithelium derived neurospheres/SCs

CSCs – Corneal stem/progenitor cells

DAPI - 4', 6-diamidino-2-phenylindole

DMEM – Dolbeco's minimum essential medium

DMEM/F12 – Dolbeco's minimum essential medium/

DNA – Deoxy ribose nucleic acid

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

EGTA - Ethylene glycol tetraacetic acid

ESCs – Embryonic stem cells

FGF - Basic Fibroblast growth factor

FITC - Fluorescein isothiocyanate

FSCs – Fetal stem cells

GCL – Ganglion cell layer

GTP - Guanosine triphosphate

H2O – Water

HBSS – Hanks balanced salt solution

HEPES - Hydroxyethyl piperazineethane sulfonic acid

HEPES-KOH - Hydroxyethyl piperazineethane sulfonic acid – Potassium Hydroxide

IE – Iris epithelium

ILL – Inner limiting layer

INL – Inner nuclear layer

IPE- Iris pigment epithelium

IPE-DC – Iris pigment epithelium derived differentiated cells

IPE-NS – Iris pigment epithelium derived neurospheres/SCs

IPL – Inner Plexiform layer

iPSC – Induced pluripotent cells

KCl - Potassium chloride

KH2PO4 - Potassium Phosphate monobasic

MgCl2 - Magnesium chloride

MgSO4 - Magnesium Sulfate

MNQX - 5, 7-dinitroquinoxoline-2, 3-dione

NA – Numerical aperture

Na2HPO4 Sodium Phosphate dibasic

NaCl2 - Sodium Chloride

NaCl2 - Sodium chloride

NaHCO3 - Sodium Bicarbonate

NSCs – Neuronal stem/progenitor cells

OLL – Outer limiting layer

ONL – Outer nuclear layer

OPL – Outer plexiform layer

PBS – Phosphate buffered saline

PI – Propidium Iodide

RA - Retionic acid

RNA – Ribose nucleic acid

RP – Retinitis pigmentosa

RPCs – Retinal stem/progenitor cells

RPE – Retinal pigment epithelium

RT-PCR – Reverse transcriptase polymerase chain reaction

SAPNs – Self Assembling peptide nanofibre scaffolds

SCs – Stem/progenitor cells

TRITC - Tetramethylrhodamine isothiocyanate

ß-ME – ß Mercaptoethanol

# CHAPTER 1: GENERAL INTRODUCTION AND REVIEW OF LITERATURE

## 1.1. General introduction:

The human eye is a conscious sense organ that reacts to light allowing vision and has several purposes. Through evolution, when compared to rodents which rely heavily on auditory and olfactory information, human beings have developed a large neocortex with a great percentage of it dedicated to visual information processing and storage (Kandel et al., 2000, Schoenemann, 2006). Therefore, vision is an exceptionally important aspect of human life.

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. 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).

### 1.2. Overview of eye development:

The process of eye development in humans is of great complexity. At the end of gestation, the three layers of embryo become more evident. These layers include ectoderm, mesoderm and endoderm, among this endoderm which is the inner most layer of the embryo do not participate in the formation of the eyes.

The ectoderm which is the outer most layer proliferates successively to form neural plate, neural groove, neural tube and subsequently the formation of neural crest *(Figure 1.1)*. Forebrain is developed from the anterior most part of the neural tube. At this stage a small optic pit becomes evident, one on each side of the forebrain *(Stage 1, Figure 1.1)*. This optic pit starts pouching leading to the formation of optic vesicle and has a bulbous shape with a narrow, the optic vesicle enlarges and its vortex touches the inner side of the surface ectoderm *(Stage 2, Figure 1.1)*.

The space surrounding the optic vesicles is filled with the middle layer para-axial mesoderm. The lens plate is developed at spot where the optic vesicle touches the surface ectoderm and gets separated from the surface ectoderm *(Stage 3, Figure 1.1)*. After touching the surface ectoderm the optic vesicle continues to grow and its surface bows inside to form a depression called optic cup that has two layers *(Stage 4, Figure 1.1)*. Later on the mesoderm finds its access inside the optic cup to form the vasculature of the eye and the axons of ganglion cells will come out to form the optic nerve *(Stage 5-6, Figure 1.1)* (Duke Elder. S et al., 1964, MannIda et al., 1964, Barber A.N et al., 1955, Kozart D.M. et al., 1977, Vaughan D et al., 1980, Hamming Nancy et al., 1987, Buffam F.V. et al., 1993, Nema H.V. et al., 1991).

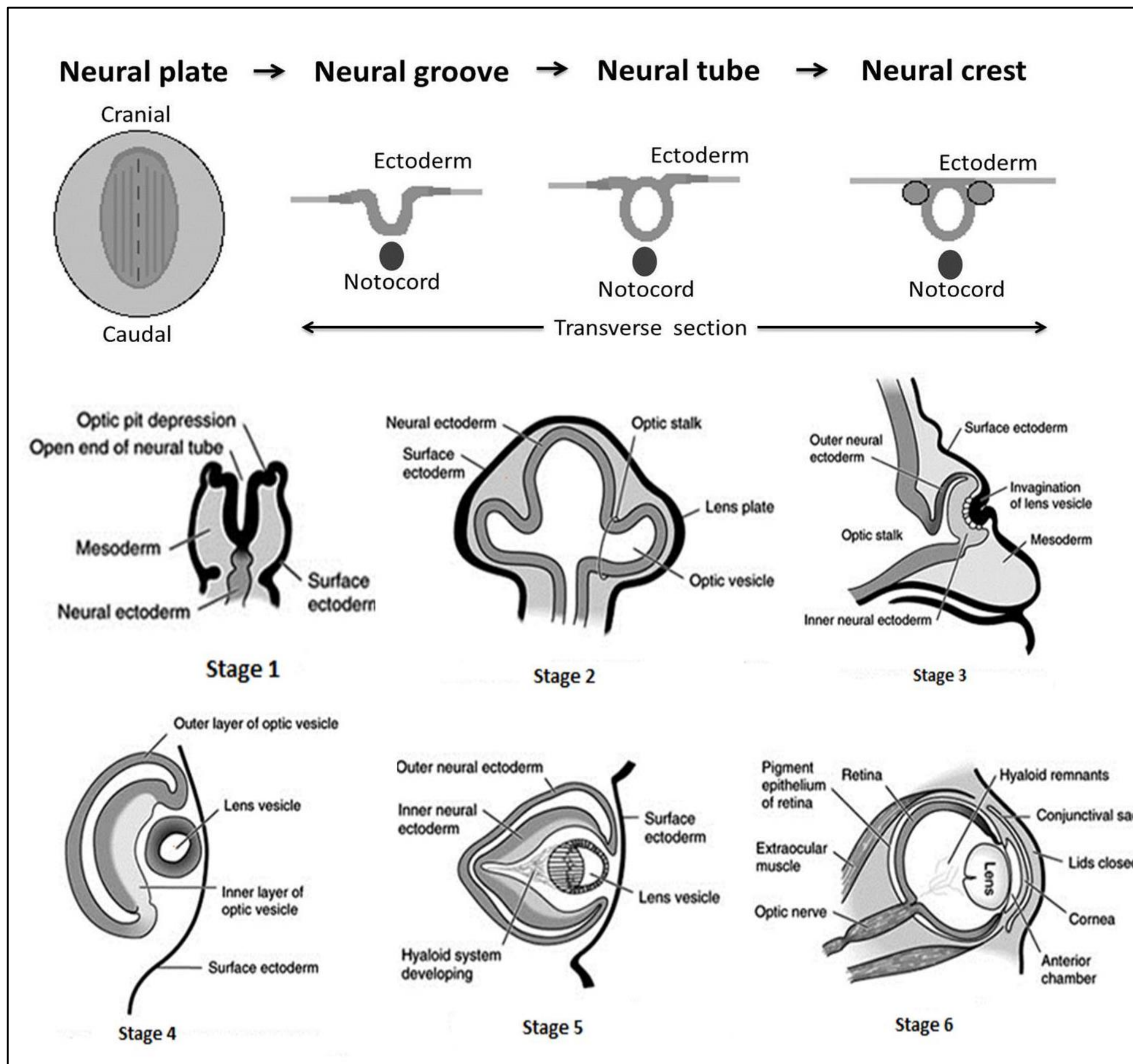



*Figure1.1: Various stages in the embryologic development of ocular structures. Adapted from Paul Riordan-Eva. Anatomy and Embryology of eye 2002-2003.*

## 1.3. Embryonic development of the Retina:

During eye development, the retina is derived from the neural tube from both the layers of optic cup. The inner wall of the optic cup gives rise to the retina. The inner layer gives rise to nine layers of sensory retina while the outer layer that remains single layered gives rise to pigment epithelium. The space between the two layers is very large in early stage of development that gradually shrinks to a potential space at the time of complete development of retina. The Retinal pigment epithelium separates neural retina from the choroidal vessels (Saha et al., 1992, Graw et al., 1996), it also plays a critical role in the maintenance of photoreceptors, reduces backscattering of light that enters the eye renewing photopigments, and phagocytosis.

## 1.4. Anatomy of the adult retina:

The retina or neural portion of the eye is a multicellular component that is actually part of the CNS and is peripherally located which is the approachable part of the brain. It is extensively studied due to ease of accessibility, simplicity of its neural circuit and crucial role in vision. The retina has ten layers and is responsible for gathering light focused upon it by the cornea and lens. The primary layers of the retina from outermost surface to innermost surface.

- ❖ Retinal Pigment epithelium (RPE) - supporting cells for the neural portion of the retina (photo pigment regeneration, blood) it is also dark with melanin which decreases light scatter within the eye.
- ❖ Rod and cone layer/Layer of photoreceptor cells - contains the outer segments and inner segments of the rod and cone photoreceptors.
- ❖ Outer limiting layer (OLL)
- ❖ Outer Nuclear Layer (ONL) - cell bodies of rods & cones
- ❖ Outer Plexiform Layer (OPL) - rod and cone axons, horizontal cell dendrites, bipolar dendrites
- ❖ Inner Nuclear Layer (INL) - Nuclei of horizontal, bipolar and amacrine cells
- ❖ Inner Plexiform Layer (IPL) - axons of bipolars (and amacrines), dendrites of ganglion cells
- ❖ Layer of Ganglion cells (GCL) - Nuclei of the ganglion cells and displaced amacrine cells
- ❖ Layer of optic nerve fibers - fibers from ganglion cells traversing the retina to leave the eyeball at the optic disk.
- ❖ Inner limiting layer (ILL)

Consistent with its position as a part of the CNS, the retina comprises complex neural circuitry that converts the graded electrical activity of photoreceptors into action potentials that travel to the brain via axons in the optic nerve. The retina comprises only five types of neurons - photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells, and these are arranged in a manner that has been less difficult to unravel. The cell bodies and processes of these neurons are stacked in five alternating layers as mentioned above, with the cell bodies located in the inner nuclear, outer nuclear, and ganglion cell layers, and the processes and synaptic contacts located in the inner plexiform and outer plexiform layers (Purves D et al., 2001) *(Figure 1.2)*.

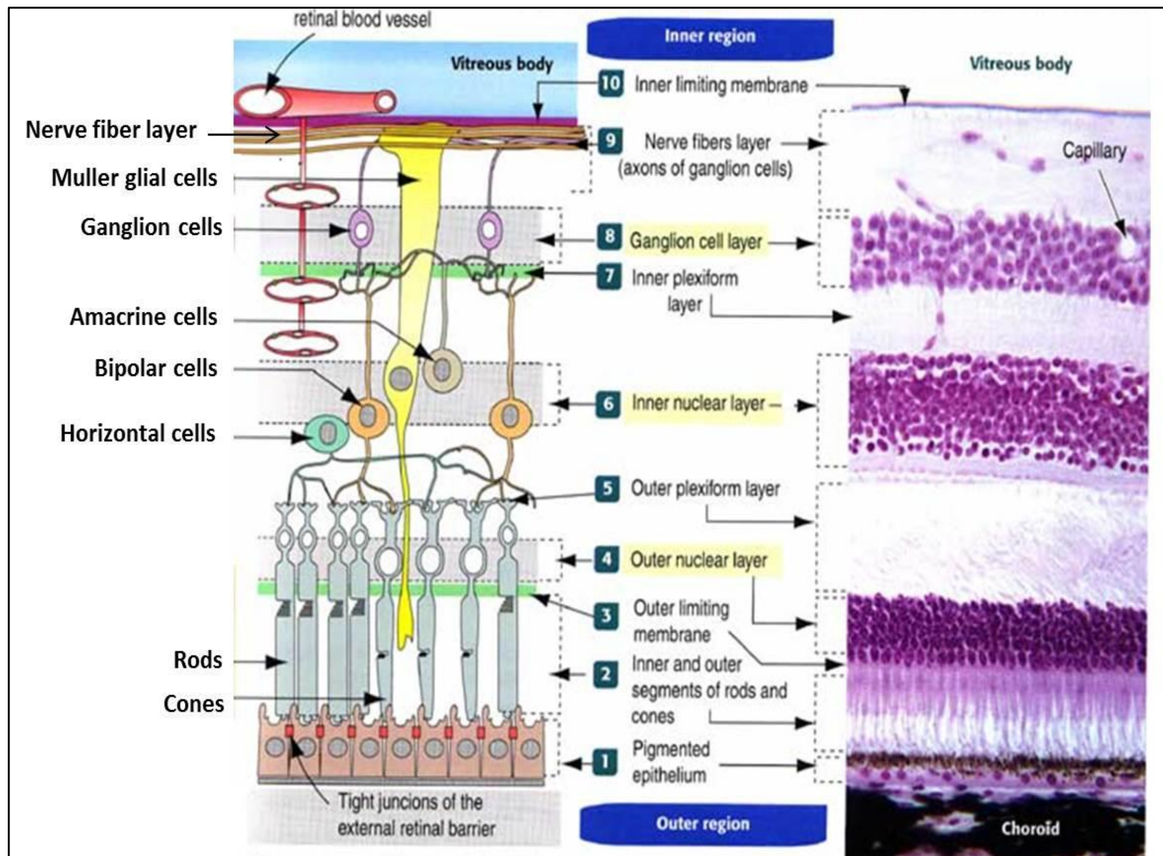



*Figure 1.2: Anatomy of the adult retina. (Modified and Adapted from Gray, Henry. Anatomy of the Human Body. Philadelphia: Lea &Febiger, 1918; Bartleby.com, 2000.20$^{th}$ edition)*

### 1.4.1. Cells in Retina:

The Retina consists of five types of neurons: photoreceptors, bipolar cells, ganglion cells, horizontal cells, amacrine cells and three basic types of glial cell Muller cells, astroglia and microglia.

a. ***Photoreceptors:*** Photoreceptors are specialized type of neurons that convert light (visible electromagnetic radiation) into signals that can stimulate biological processes (Phototransduction). The two types photoreceptor cells are rods and cones that contribute information used by the visual system. Cones function well in bright light and are adapted to detect fine detail, central and colors vision. Rods functions well in dim light and are responsible for peripheral vision. Detailed Comparison of human rod and cone cells is mentioned in table (Kandel E. R. et al., 2000) *(Table 1.1)*

| Rods | Cones |
|---|---|
| Used for vision under low light conditions (Scotopic vision) | Used for vision under high light conditions (Photopic vision) |
| Very light sensitive; sensitive to scattered light | Not very light sensitive; sensitive to only direct light |
| Loss causes night blindness | Loss causes legal blindness |
| Low visual acuity | High visual acuity; better spatial resolution |
| Not present in fovea | Concentrated in fovea |
| Slow response to light, stimuli added over time | Fast response to light, can perceive more rapid changes in stimuli |
| Have more pigment than cones, so can detect lower light levels | Have less pigment than rods, require more light to detect images |
| Stacks of membrane-enclosed disks are unattached to cell membrane directly | Disks are attached to outer membrane |
| About 120 million rods distributed around the retina | About 6 million cones distributed in each retina |
| One type of photosensitive pigment | Three types of photosensitive pigment |
| Confer achromatic vision | Confer color vision |

*Table 1.1: Comparison of human rod and cone cells.* Adapted from *Kandel, E. R. et al 2000.*

***b. Bipolar cells:*** Bipolar cells are interneurons that are round or oval in shape and are located in the inner nuclear layer. These cells contain a cell body between two dendritic ends with two axons and the axons contain vesicles and mitochondria which allows better shaping of the structure. They transfer information from the outer plexiform cells to the inner retina. Their dendrites receive information from photoreceptors and horizontal cells and pass it onto the ganglion cells and amacrine cells through their axons. The bipolar cells are

divided into rod bipolar cells (receive input from rod cells) and cone bipolar cells (receive input from cone cells).

c. ***Horizontal cells:*** The horizontal cells are located in the outer part of the inner nuclear layer along with retinal bipolar cells and amacrine cells and possess somewhat flattened cell bodies with their dendrites divided into numerous branches interconnecting neurons in the outer plexiform layer of the retina. These cells synapse on bipolar cells and retinal photoreceptors. These cells are responsible for allowing eyes to adjust to see well under both bright and dim light.

d. ***Amacrine cells:*** The amacrine cells are interneurons lie in the inner part of the inner nuclear layer and lack axons (Santiago RamónyCajal, 1892). They are the second synaptic retinal layer that influences retinal signal processing at contact between the bipolar and ganglion cells. There are about 40 different subtypes of amacrine cells. They are classified based on the dendritic properties of tree size, branching style, the width of their receptive field and location within the strata of the inner plexiform layer. Functionally, they are responsible for retinal image processing particularly by adjusting image brightness and by combining sequential activation of neurons and detecting motion.

e. ***Ganglion cells:*** Ganglion cells are type of neurons located near the inner surface of the retina and are the final output neurons. These cells vary significantly in terms of their size, connections, and responses to visual stimulation. Ganglion cells receive visual information from bipolar cells and amacrine cells in their dendrites and transmit it to the brain throughout their axon to the brain. Based on their projections and functions, these cells are divided into 5 classes: Parasol, Midget, Bistratified, Photosensitive ganglion cells/melanopsin ganglion cells and Ganglion cells projecting to the Superior Colliculus for eye movements/saccades.

f. ***Müller cells:*** Müller cells are the major glial element of the retina. These cells run from the OLL to the ILL. Cell bodies of these cells are located in the INL and the processes of these cells are projected irregularly from the ILL to the bases of the rods and cones where they form a row of junctional complexes in OLL. They provide architectural support and metabolic support to retina, play a

central role in the homeostatic regulation of the retina and maintain low synaptic levels of neurotransmitters.

g. ***Microglia cells:*** Microglia are specialized macrophages capable of phagocytosis. These cells protect neurons in the retina and are required for neuronal homeostasis and innate immune defence. These cells comprise approximately 15% of the total CNS.

h. ***Astroglial cells:*** These cells are also called as Astrocytes and are most abundant type of glial cells. These cells have a characteristic morphology of a flattened cell body and numerous projections that anchor neurons to their blood supply. They regulate the external chemical environment of neurons by removing excisions, notably potassium and recycling neurotransmitters released during synaptic transmission. There are generally two types of astrocytes, protoplasmic and fibrous. They are similar in function but distinct in morphology and distribution. These cells are restricted to the nerve fiber layer of the retina. The astrocytes have varied morphology from symmetrical stellate at peripheral retinal to extremely elongated at the optic nerve head (Schitzer, 1988).

i. ***Retinal Pigment Epithelium:*** The retinal pigment epithelium (RPE) is a monolayer of pigmented cells forming a part of the blood/retina barrier, and is attached to the choroid, a layer filled with blood vessels. These cells, closely interacts with photoreceptors in the maintenance of visual function. RPE is mainly involved in the phagocytosis of the outer segment of photoreceptor cells and it is also involved in the visual cycle of retinal (Vitamin A cycle). This layer transports ions, water, and metabolic end products from the subretinal space to the blood and transports nutrients such as glucose, retinol, and fatty acids from the blood to photoreceptors. Moreover these cells are capable of secreting a variety of growth factors and help in the maintenance of structural integrity of choriocapillaris endothelium and photoreceptors.

## 1.5. Retinal Degenerative diseases:

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).

### 1.5.1. Retinitis Pigmentosa:

Retinitis pigmentosa (RP) is a rare inherited, neurodegenerative disease denoting to a group of genetic disorder in which the light-sensitive retina of the eye slowly and progressively degenerates leading to blindness by affecting the visual system. Eventually resulting in blindness. There are approximately 200 different genetic mutations leading to RP. The major form of RP includes cone-rod dystrophy, where the mutation affects the RPE and cone photoreceptors and rod-cone dystrophy, where cell death occurs in rod photoreceptors. The symptoms of RP are variable, though most patients experience initial symptoms of night blindness and progressive peripheral visual field loss causing tunnel vision before they finally reach blindness (Hartong et al., 2006). In many cases, RP progresses involving central visual field.

### 1.5.2. Age related macular degeneration:

Macular degeneration or Age related macular degeneration (AMD or ARMD) is a condition that usually affects among age 50 and older adults resulting in loss of vision in the macula (center of the visual field) because of damage to the retina (Paulus T.V.M. de Jong, 2006) (Jonathan C. Horton, 2004) and the part of the eye needed for sharp, central vision, which lets us see objects that are straight ahead. The clinical symptoms of macular degeneration, includes blurriness, dark areas or distortion in central vision, and permanent loss of central vision. It usually does not affect side, or peripheral vision. Macular degenerations are categorized into two types.

#### a. Dry, or atrophic, macular degeneration:

It is also called as non-neovascular macular degeneration. This condition is caused by aging and thinning of the tissues of the macula. In this kind of macular degeneration a tiny yellow or white pieces of fatty protein called drusen form under the retina between the Bruch’s membrane (BM) and the RPE a condition called polygenic complex disease. Eventually, the macula becomes thinner and stops working properly. The vision loss usually gradual in dry ARMD, if unnoticed/not monitored properly this can change into the more damaging wet (exudative) macular degeneration (Tan JS, et al., 2008). About 85 to 90 percent of AMD patients are diagnosed with dry AMD.

**b. Wet, or exudative, macular degeneration**

Wet macular degeneration occurs when abnormal blood vessels begin to grow underneath the retina. This type of blood vessel growth is called choroidal neovascularization (CNV) as these vessels grow from the choroid. It is also called as neovascular macular degeneration. These new blood vessels are very fragile, leading to blood and protein leakage under the macula causing irreversible damage to the photoreceptors. The loss of vision from this form of macular degeneration may be faster and more noticeable than that from dry macular degeneration (Paulus T.V.M. de Jong, 2006). In about 10 percent of AMD patients, dry AMD progresses to the more advanced and damaging wet degeneration.

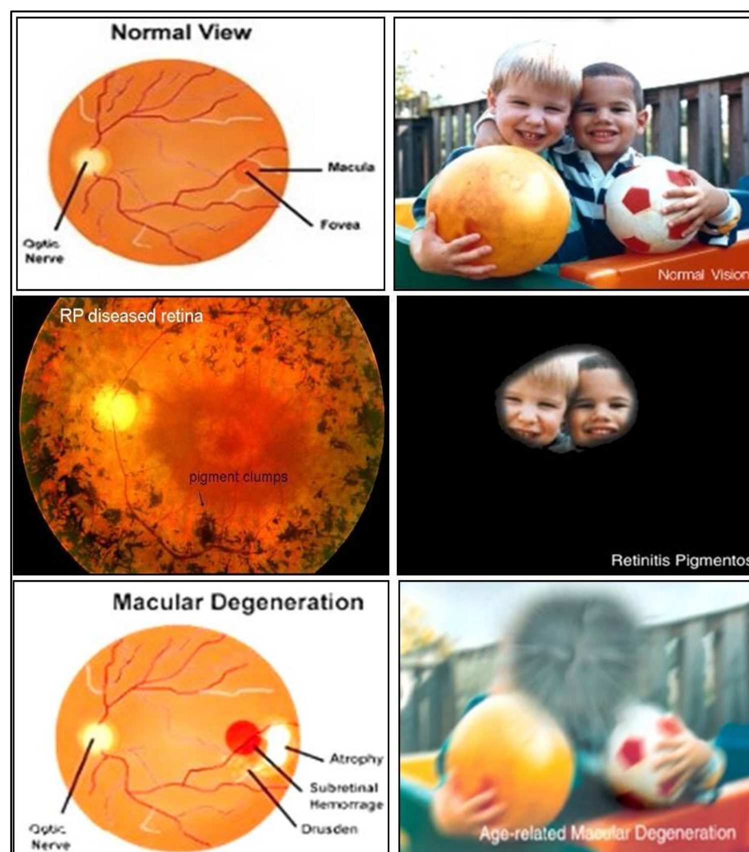



*Figure 1.3: Representative images of the fundus and visual field showing a) Normal vision b) Retinitis pigmentosa and c) Age-related macular degeneration. Modified and adapted from http://www.nei.nih.gov.*

Approaches for treating these degenerative diseases include: a) replacing the defective gene; or b) introducing a drug or agent that either slows down or stops the premature death of photoreceptors; or c) introducing electronic chips; or d) replacing damaged

cells by cellular therapy. As knowledge relating to stem cells has increased over the last two decades, attempts have been made to translate this research into clinical practice.

### 1.6. Stem cells:

Stem cells are the undifferentiated cells that are found in every organ and tissues in the body. These are capable of self-renewing to produce undifferentiated progenies that give rise to several lineages of differentiated cell types and some of these undifferentiated cells are stem cells again. Stem cell are also known as special kind of cells with unique capacity to self-renew that is uncommitted and remains uncommitted, until it receives specific queues to develop into a specialized cell. Their self-renewal capacity and proliferative capacity along with the ability to become specialized cell types make stem cells inimitable ***(Figure 1.4)*** (Mike Boulton et al., 2004).

Firstly, in 1981, researchers were capable of isolating this category of pluripotent stem cells from early human embryos and grow them in culture (Weiss et al., 2005). Few years since this innovation, evidence has emerged that these stem cells are capable of becoming almost all of the specialized cells of the body. Thus, may have the potential to generate replacement cells for wide range of tissues and organs, such as the heart, the pancreas, nervous system and other cells. Consequently, this category of human stem cell holds the promise of being able to repair or replace cells or tissues that are damaged or destroyed by many of our most devastating diseases and disabilities. At about the same time a flurry of new information was emerging about a class of stem cells that have been in clinical use for years so called adult stem cells. Adult stem cell is an undifferentiated cell that is found in a differentiated (specialized) tissue in the adult. It can yield the specialized cell types of the tissue from which it originated. During the past decade, scientists discovered adult stem cells in tissues that were previously not thought to contain them, such as the blood, brain, liver, etc. More recently, they reported that adult stem cells from one type of tissue are capable of developing into cell types that are characteristic of other tissues.

It is because of these characteristics that stem cells are a prime target of applied research that seeks to treat degenerative diseases by cell replacement therapies. Any disease caused by tissue degeneration can be a potential candidate for stem cell

therapies, including Parkinson's and Alzheimer's disease, stroke, spinal cord injury, heart diseases, burns, and many more. However to realize the clinical potential of stem cells, it is crucial to have a deeper insight into the mechanisms regulating stem cell self-renewal and their ability to produce the correct cell type at the appropriate time and location in correct numbers (Mike Boulton et al., 2004).

### 1.7. Characteristics of Stem cells:

Stem cells are unique cells that differ from other kinds of cells in the body. They are found small sub-set of population constituting 0.5% to 10% of total body cells. Regardless of their source they stand out to be special kind of cells due to the three general properties. *(Figure 1.4)*

- Uncommitted/Unspecialized
- High Proliferation and Self-renewal capacity
- Differentiation capacity

These characteristics can be elaborated as follows:

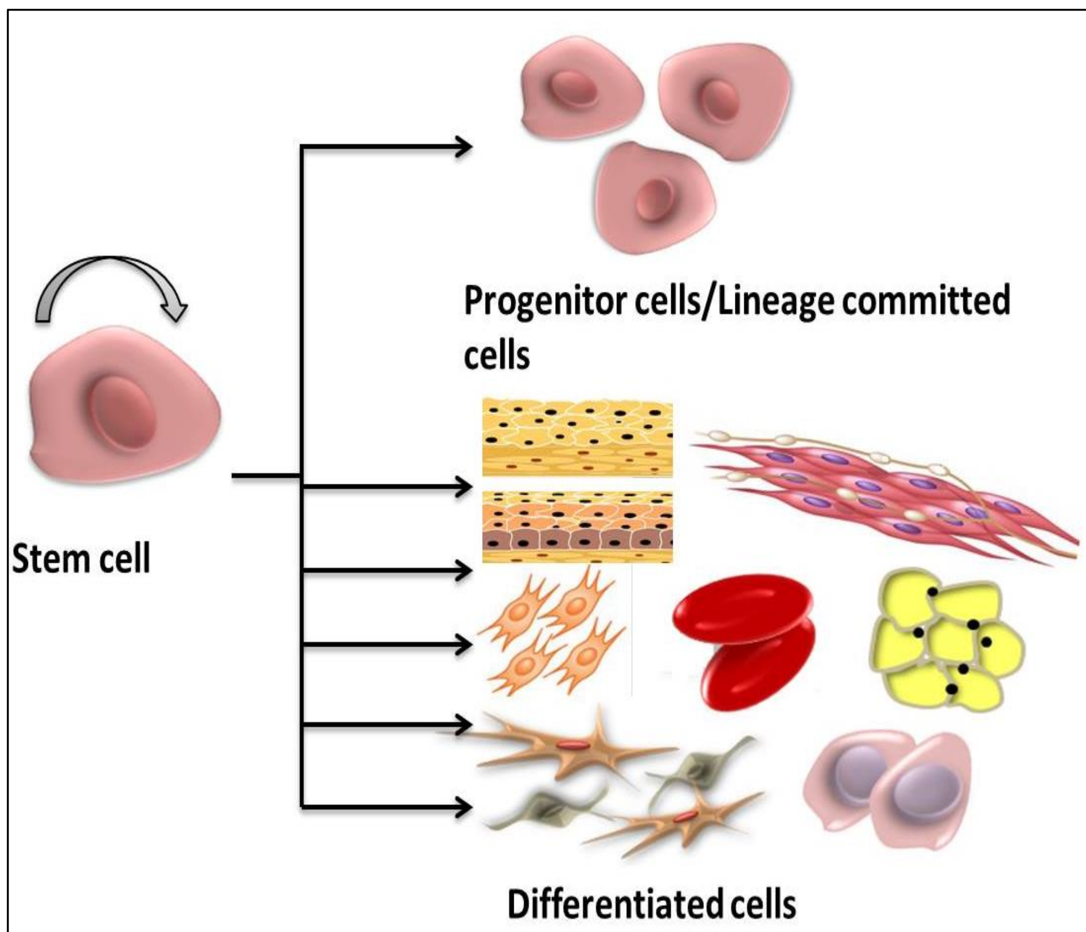



*Figure 1.4: A schematic representations of the characteristics of a stem cell.*

### 1.7.1. Uncommitted/Unspecialized:

One of the important characteristics of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. Stem cells are 'uncommitted' cells, capable of dividing to make more stem cells or progenitor cells. Under appropriate conditions they produce the different types of specialized cells that make up the tissues and organs of the body.

### 1.7.2. High Proliferation and Self-renewal capacity:

The ability of a starting population of stem cells to divide in a fashion which helps them maintains their population; the resulting cells continue to be unspecialized. Like the parent stem cells, these cells are said to be capable of long-term self-renewal and proliferation.

### 1.7.3. Differentiation capacity:

When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step which includes the internal signals controlled by a cell's genes, the external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighbouring cells, and certain molecules in the microenvironment.

## 1.8. General hypotheses of stem cells:

### 1.8.1. Classification of Stem cell by Potency:

All stem cells can be classified into four different categories based on their potency or the extent to which they can differentiate *(Figure 1.5)*. They are a) Totipotent, b) Pluripotent, c) Multipotent, d) Unipotent

**a.** ***Totipotent:*** These stem cells are capable of forming a complete organism i.e., can differentiate into embryonic andextra-embryonic cell types. These cells are produced from the fusion of an egg and sperm cell. For example for totipotent cells are the fertilized egg and the first four divisions of the embryo are also totipotent. Totipotent stem cells are capable of forming somatic SCs and primitive germ-line stem cells (Weissman IL, 2004).

b. ***Pluripotent:*** These stem cells can differentiate into nearly all cells, i.e. capable of forming all 200 different types of tissues. With the potential to make any differentiated cell in the body these are known as true stem cells (Horie M et al., 2011). Embryonic Stem Cells come under this category. For example pluripotent cells include cells derived from any of the three germ layers (Shamblott MJ et al., 1998).

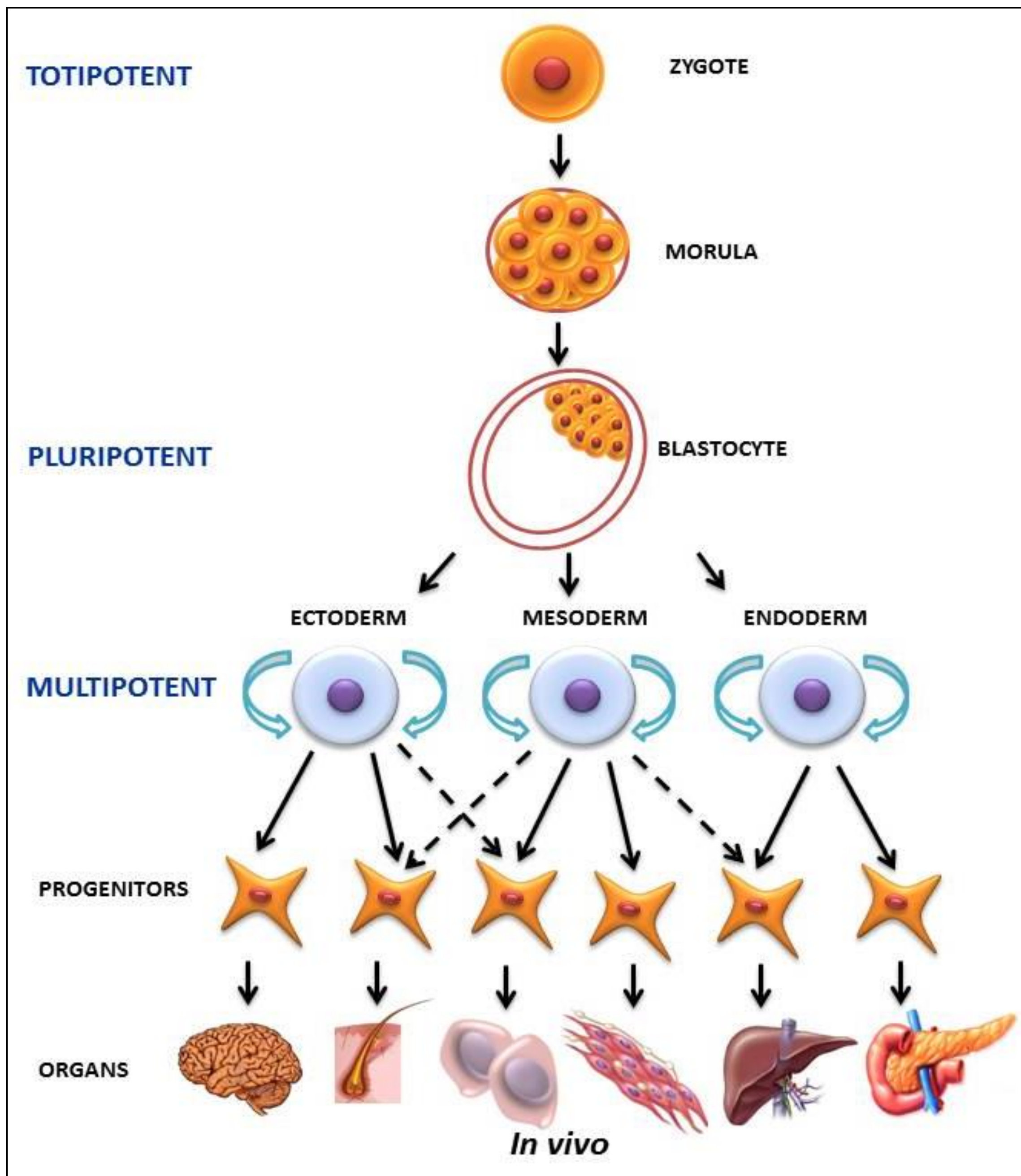



*Figure 1.5: Path from totipotency to organogenesis. Modified from (Wobus and Boheler 2005)*

**c.** ***Multipotent:*** These stem cells are capable of forming many types of specialized cells beyond the barriers of germinal layers i.e., can differentiate into a number of cells, but only those of a closely related family. For example, the bone marrow mesenchymal stem cells, haematopoietic stem cells and adipose tissue is a source of multipotent stem cells (Zuk PA et al., 2002 and Quintana AM et al., 2011).

**d.** ***Unipotent:*** These stem cells are capable of producing only into a single type of specialized cell, their own, but have the property of self-renewal and proliferation, which distinguishes them from other cells. For example muscle stem cells, prostrate stem cells and most epithelial tissues (Blanpain C et al., 2007).

### 1.8.2. Classification of Stem cells by Source:

**a.** ***Embryonic stem cells:*** Embryonic stem cells (ESCs) are capable of differentiating into any kind of cells of the body (A Hutchins R, 2007). In the early embryonic stage the cells are totipotent and possess the two important characteristics like self-renewal and pluripotency (Kang L et al., 2010). ESC lines are referred to as immortal due to their ability to self-renew over many generations. ESCs may have great potential in forming the basis of long term cell therapies, but issues regarding their safety need to be addressed (Patel R and Lomax G, 2011) (Khoo MLM et al., 2011). Derivation of human pluripotent ESC lines and recent advances in ESC biology open a new window for autologous stem cell based therapies in degenerative diseases (Ostrowska A et al., 2011) (KokkinakiM et al., 2011) (Rippon HJ and Bishop AE, 2004).

**b.** ***Fetal stem cells:*** Fetal stem cells (FSCs) are the primitive cell types found in the organs of fetus. These SCs can be isolated from fetal blood and bone marrow as well as from other fetal tissue (Donoghue K and Fisk NM, 2004). The developing baby is referred to as a fetus from approximately 10 weeks of gestation. FSCs are tissue specific and generate mature cell types within the particular tissue or organ in which they are found. Fetuses are rich sources of hematopoietic stem cells (HSCs) (Guillot PV et al., 2006).

**c.** ***Umbilical cord blood stem cells:*** The Umbilical cord blood contains some stem cells that are genetically identical to the newborn. These cells are capable of differentiating into certain, but not all, cell types and are multipotent stem cells

(Lee OK et al., 2004). The Umblical cord blood stem cells are often banked for future use of stem cell therapy. The discovery that stem cells can be obtained from umbilical cord blood instead of the more controversial source of embryonic SC's, has renewed interest on the new, exciting therapeutic potentials of this technology (Rajab KE and Sequeira RP, 2009).

**d.** ***Pluripotent stem cells:*** Induced pluripotent Stem cells (iPSC) are powerful method for creating patient- and disease-specific cell lines for research, as useful tools for drug development as well as in transplantation medicine. iPSC are created by inducing the specialized cells to express genes that are normally present in ESCs and that control cell functions. ESCs and iPSC share many characteristics, including the ability to become the cells of all organs and tissues, but they are not identical. iPSC are derived from somatic cells, epigenetically reprogrammed with protein transcription factors to lose tissue-specific features and gain pluripotency (Roelen BA et al., 2011). Similar to hESCs, they can theoretically differentiate into any type of cells (Li J et al., 2011). The concept of iPSC remains an important area of focus for future research and has serious implications for the stem cell cancer theory (Mangum R and Nakano I, 2011).

***e.*** ***Adult stem cells:*** Adult stem cells (ASCs) have been isolated from several tissue sources, including the CNS, bone marrow, retina and skeletal muscle (Toma JG et al., 2001). Adult stem cell refers to any cell which is found in a developed organism that has two properties that is the ability to divide and create another cell like itself and also divide and create a cell more differentiated than itself. ASCs are mostly lineage-restricted i.e., multipotent and are generally referred to by their tissue origin. Adult or somatic stem cells exist throughout the body after embryonic development and are found inside of different types of tissue. These stem cells have been found in tissues such as the brain, bone marrow, blood, blood vessels, skeletal muscles, skin, and the liver. These include mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, dental pulp stem cell, etc. The ASCs mostly undergo rapid proliferation when it needs to regenerate the specialized tissues (Ponnusamy MP, 2010).

They remain in a quiescent or non-dividing state for years until activated by disease or tissue injury. It is generally thought that adult stem cells are limited in their ability to differentiate based on their tissue of origin, but there is some evidence to suggest that they can differentiate to become other cell types. ASCs, exhibit a certain degree of developmental plasticity that enables them to differentiate across boundaries of lineage, tissue and germ layers (Valarmathi MT and Fuseler JW, 2011). The use of adult stem cells isolated from patients could solve immunological problems associated to cell transplant (Gioviale MC et al., 2011).

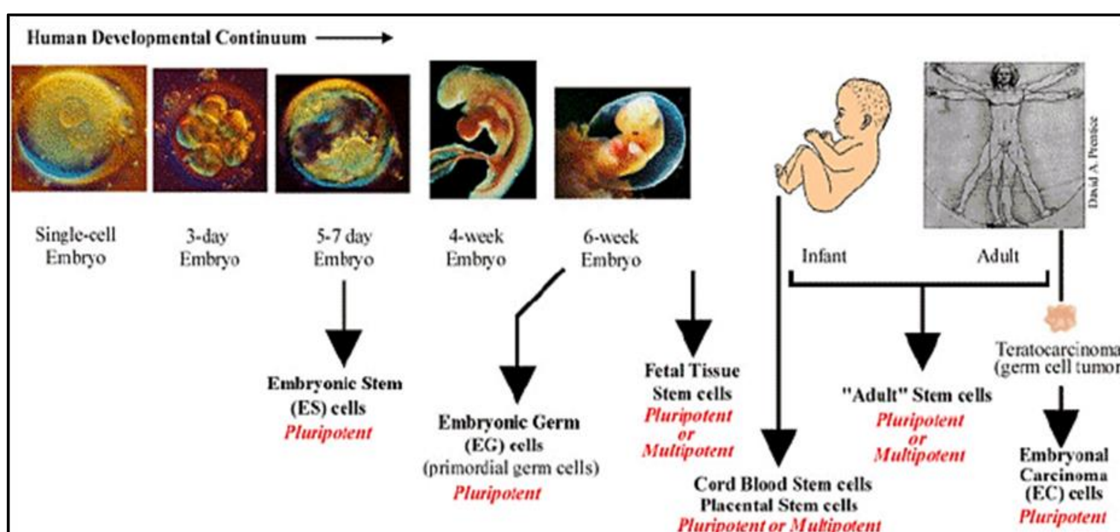



*Figure 1.6: Classification of Stem cells based on source and potency. Adapted from http://www.stemcellresearch.org/images.*

## 1.9. Retinal stem/progenitor cells:

SCs have been receiving considerable attention as potential tools for the effective restorative treatment of retinal degenerative diseases. An understanding of the relationship between normal development of human eye and human Retinal stem/progenitor cells (RPCs) and also the ability to culture cells *in vitro* would provide a promising step towards the treatment of these disorders. RPCs have been isolated and characterized from the embryonic and neonatal retina and can be maintained *in vitro* (Scott Schmitt et al., 2009). These multipotent embryonic and fetal cells are fated to develop into the adult retina. Because of their ontogenetic role, the developmental population of RPCs must have the potential to differentiate into each of the six neuronal cell types (ganglion, horizontal, amacrine, cone, rod, or bipolar cells) or one

glial cell type (Müller cell) present in the mature neural retina (Scott Schmitt et al., 2009). RPCs have been isolated from both neonate and adult retina from a number of different species like mouse (MacLaren RE et al., 2006), rat (Chacko DM et al., 2000, Yang P et al., 2002, Yang J et al., 2006), pig (Gu P et al., 2007, Klassen H et al., 2007) and human (Kelley MW et al., 1995, Yang P et al., 2002a, Klassen H et al., 2004, Carter DA et al., 2007, Aftab U et al., 2009). As retina is the inner most layer at the posterior segment and it is an inaccessible source of SCs isolation and transplantation for replacing the damaged cells is not possible. Hence there is a need to identify an alternative source for SCs.

## 1.10. Alternative sources for Retinal neurons:

### 1.10.1. Embryonic stem cells (ESCs):

ESCs have the ability to differentiate into precursors of defined lineages and then into corresponding somatic cells. This makes them an ideal model in the study of embryonic development and lineage determination, which is predicted to be valid for cell transplantation therapy. Till date, much effort has been made to differentiate ESCs into RPCs, and then into retinal neurons that can be used to promote recovery from some retinal degenerative diseases through cell transplantation. Many approaches have been made for the differentiation of the ESCs into retinal cells. (Ahmad I et al., 2001, Ahmad I et al., 1999, Ahmad I et al., 2004) Therefore, advances in the understanding as to how ESCs differentiate should provide answers for reprogramming of stem cells from adult tissues.

### 1.10.2. Non ocular sources:

**a.** ***Brain:*** Given the fact that retina is a part of the CNS it could be a best source for the availability of the neuronal stem/progenitor cells (NSCs), which could easily integrate into the retina. The first extra ocular heterologous SCs examined for retinal potential was from the brain (Takahashi M, et al., 1998). The NSCs isolated from the brain were transplanted in the rat models with retinal dystrophy and those with ischemic or mechanical injuries to evaluate their retinal potential and integration within the host retina. The grafted NSCs could integrate within different retinal lamina and remarkable morphological adaptation to different retinal cell types, but they did not express retinal cell specific markers, suggesting their inability to differentiate along retinal lineage

(Nishida A et al., 2000, Kurimoto Y et al., 2001). These studies emphasized the inherent regional limitation on the plasticity of tissues, even of similar origin. Recently, SCs derived from neonatal mouse brain were transplanted in the eyes and unlike other studies these cells not only incorporated into host retina, but some of these cells expressed retinal markers like Protein kinase C and Recoverin (Van Hoffelen SJ et al., 2003). The causes for differentiation of these cells along the retinal lineage could be the progenitor cells were more plastic or the host contained cues that are favourable for retinal differentiation or both.

**b.** ***Bone Marrow cells:*** Bone marrow stem/progenitor cells (BMSCs) have been reported to be highly plastic in terms of generating cells across the germ layer limitations (Wagers AJ et al., 2004, Camargo FD et al., 2004). There are many studies showing the transplantation of whole bone marrow or enriched SCs has shown phenotypic transition into those of hepatocytes (Lagasse E et al., 2000), skeletal muscles cells (Ferrari G et al., 1998), neurons (Mezey E et al., 2000, Brazelton TR et al., 2000) and cardiac cells (Jakson KA et al., 2001). Due to their plasticity, renewable nature and the potential of autologous transplantation that address the issue of immune rejection, the bone marrow cells have been regarded as an ideal source for stem cell therapy. Recent studies on the BMSCs indicate that these cells could be differentiated along the retinal lineage *in vitro* and *in vivo* (Kicic A et al., 2003). In this and similar kind of study a significant proportion of BMSCs were observed to have differentiated along the photoreceptor lineage as demonstrated by the Immunocytochemical, reverse transcriptase-polymerase chain reaction and western blotting analysis. In addition subretinal transplantation of these cells showed that these cells can be incorporated within the host retina and a subset of them expressed photoreceptor-specific marker (Kicic A et al., 2003, Tomita M et al., 2002).

### 1.10.3. Ocular sources:

**a.** ***Cornea:*** Cornea is a regenerative ocular tissue and the SCs reside in the basal layer of the limbus (Cotsarelis G et al., 1989, Pellegrini G et al., 1999, Schermer A et al., 1986). Based on the fact that corneal stem/progenitor cells (CSCs) are derived from the embryonic ectoderm similar to the NSCs whose

neural fate is the default pathway (Weinstein DC et al., 1999), it was assumed that limbal SCs might display imperceptible neural potential when removed from their niche (Zhao X et al., 2002a). The hypothesis was tested by exposing these cells to mitogens; they could form neurospheres similar to that of NSCs. The cells from the neurospheres when exposed to neuronal differentiation conditions differentiated into neuronal and glial lineage as demonstrated by the expression of neuron and glial specific markers (Zhao X et al., 2002a). However these cells failed to demonstrate the functional properties characteristic of neurons, emphasizing the importance of rigorous characterization of neural potential of heterogeneous stem cells that should include evaluating differentiation on multiple criteria like morphology, expression of biochemical and molecular markers and function. A recent study demonstrate the retinal potential of the cornea derived NSCs when transplanted in the eyes with retinal injuries, they integrated in the retina and a small subset of transplanted cells expressed retina specific markers (Chacko DM et al., 2003).

b. **Ocular melanocytes:** Melanocytes colonise the ocular tissue. Based on the fact that melanocytes are derived from neural crest cells, they have been evaluated as a candidate for an alternate source for NSCs (Arsenijevic Y et al., 2003). Ocular melanocytes obtained by scraping human sclera and choroid and cultured in the presence of EGF proliferated and some of them expressed neural ectodermal stem cell marker nestin. When these cells were exposed to FGF2/BDNF, a subset of them exposed neuron and glial specific markers suggesting their neural potential *in vitro*. Though these cells have been proposed as alternate sources for NSCs for cell therapy purpose in the retina, their potential to differentiate into retinal cells *in vivo* and *in vitro* has not been reported (Arsenijevic Y et al., 2003).

c. ***Retinal pigment epithelium:*** The definite and earliest example of generation of retinal cells from heterogeneous sources is the trans-differentiation of the RPE cells into retinal neurons (Raymond PA et al., 2000, Zhao S et al., 1997, Del Rio-Tsonis K et al., 2003). The RPE cells, derived from the outer layer of the developing optic cup consist of the non-neural pigment cells that support the structure and function of the retina. The trans-differentiation of RPE cells in

rats has been demonstrated *in vitro* when exposed to FGF2 and differentiation along the retinal ganglion cells and photoreceptor lineages (Zhao S et al., 1995). But such trans-differentiation potential is limited to a narrow window of embryonic development and not observed beyond the embryonic day 15. Due to the in accessibility of the tissue and the lack of plasticity in adult these cells may not be acquiescent for cell therapy. However, more information regarding the underlying mechanisms of trans-differentiation emerge, these cells may be used for treating the degenerative disorders.

**d.** ***Ciliary epithelium:*** The margin of the optic neuroepithelium gives rise to ciliary epithelium (CE), between the perspective retinal pigment epithelium and retina. The CE in adults regulates the accommodation and flow of aqueous humor. A rare population of mitotically quiescent cells were detected in the ciliary pigment epithelium (CPE) of adult rats (Ahmad I et al., 2000), which proliferate extensively when removed from their niche and cultured in the presence of mitogens.

These proliferating cells could differentiate along the neuronal and glial lineages (Ahmad I et al., 2000, Tropepe V et al., 2000). Similar population of cells were identified in CPE of adult mice, rats, rabbits, porcine and humans (Ahmad I et al., 2000, Tropepe V et al., 2000, Yuji Inoue, et al., 2005, MacNeil A, et al., 2007, Fischer, A.J et al., 2003, Brenda L. K. Coles, et al., 2004, Moe MC et al., 2009). The SCs derived from the CE display retinal potential; they express retinal progenitor markers and differentiate into retinal cells when provided/exposed to conductive environment (Ahmad I et al., 2000, Tropepe V et al., 2000). The ability of CE-SCs to differentiate along the retinal lineages and self-renew suggests that these cells may represent a precursor to that of RPCs and can be a good source for cell therapy.

**e.** ***Iris:*** As CE, iris is another neuroepithelial derivative. Due to the common origin of the iris and retina, the potential for autologous transplantation and the ease with which the tissue could be obtained for clinical use would be the reason for considering it by researches for isolating the SCs from this tissue (HarutaM et al., 2001). When the iris tissue was cultured as explant in the presence of mitogens, cells migrated out of the explants, proliferated and

formed a monolayer. Later upon exposure to the condition that promoted retinal differentiation, they did not express retinal markers, but they expressed neurofilament demonstrating their neural potential.

However, when Crx- a regulator of photoreceptor differentiation was over expressed, these cells began to express immunoreactives representing photoreceptor specific markers suggesting that these mitogen treated iris cells can be induced to differentiate along the retinal lineage. Recent years the SCs properties of these cells have been extensively studied from the Iris epithelium (IE) of chicken (Maki Asami et al., 2007), adult rodents and primates (Tadamichi Akagi et al., 2005), non-human mammals (Masatoshi Haruta et al., 2001) and also from humans (Frøen RC et al., 2011). Although their functions and roles *in vivo* remain unknown, the SCs proliferate *in vitro* and differentiate into cells expressing markers of certain mature retinal neurons raising the possibility that these cells can be used as a potential cell source in transplantation therapy for retinal diseases.

## 1.11. Characterization of the Iris and ciliary pigment epithelium derived SCs and the retinal differentiated cells:

Stem cell research requires cellular and molecular tools to confirm pluripotency or differentiation potential to help determine the utility of cells in downstream experiments. This varies from analysing morphological characteristics, proliferation, marker studies, gene expression profiles, or epigenetic profiles.

### 1.11.1. Marker studies:

SCs markers or the lineage specific markers allow for the identification and isolation of these cells in different types of tissues and cell populations. To date, no factor has been identified that can exclusively mark all or tissue-specific stem cell populations or distinguish between stem and progenitor cells. Several stem cell populations from different tissues may be identified by similar markers, but these may also be expressed in lineage-restricted progenitors of other populations, or other non-proliferative cell types. As for the ciliary derived SCs, the expression levels and combination of a number of these markers need to be investigated for identifying a SCs. Table 1.2

summarises the markers to date for SCs characterisation and Table 1.3 summarizes the markers to data for the retinal cell types.

| **Sl. No.** | **Identification factor** | **Symbol** |
|---|---|---|
| 1 | ATP-binding cassette sub-family G member 2 | ABCG2 |
| 2 | Beta III tubulin | TUJ |
| 3 | Musashi homolog 1 | MSI1 |
| 4 | Nanog homeobox | Nanog |
| 5 | Nestin | NES |
| 6 | Notch homolog 1 | Notch 1 |
| 7 | Paired box gene 6 | PAX 6 |
| 8 | SRY (sex determining region Y)-box 2 | SOX 2 |
| 9 | Glial fibrillar acidic protein | GFAP |
| 10 | ELAV-like protein 4 | HuD |
| 11 | Feminizing Locus on X-3, or Neuronal Nuclei | NeuN |
| 12 | Peripherin | PRPH |
| 13 | Neurogenin-2 | NEUROG2 |
| 14 | P-Cadherin | CDH3 |

*Table 1.2: Summary of commonly used markers for the identification of ciliary body derived retinal SCs.*

<table>
<tr><th>Sl. No.</th><th>Identification factors</th><th>Symbol</th><th>Type of cell</th></tr>
<tr><td>1</td><td>Cone-rod homeobox</td><td>CRX</td><td rowspan="4">Rod Photoreceptors</td></tr>
<tr><td>2</td><td>Neural retina leucine zipper</td><td>NRL</td></tr>
<tr><td>3</td><td>Rhodopsin</td><td>RHO</td></tr>
<tr><td>4</td><td>Recoverin</td><td>RCVRN</td></tr>
<tr><td>5</td><td>s-Opsin</td><td>OPN1SW</td><td rowspan="4">Cone Photoreceptors</td></tr>
<tr><td>6</td><td>Guanine nucleotide binding protein</td><td>Gnat2</td></tr>
<tr><td>7</td><td>Retinal Homobox</td><td>RX</td></tr>
<tr><td>8</td><td>Visual system homeobox 2</td><td>Chx-10</td></tr>
<tr><td>9</td><td>Protein kinase C</td><td>PKC</td><td rowspan="2">Bipolar cells</td></tr>
<tr><td>10</td><td>Metabolotropic glutamate receptor</td><td>mGluR6</td></tr>
</table>

<table>
<tr><td>11</td><td>POU Class 4 Homeobox 2</td><td>Brn3b</td><td rowspan="3">Ganglion cells</td></tr>
<tr><td>12</td><td>Microtubule-associated protein 2</td><td>MAP-2</td></tr>
<tr><td>13</td><td>Thy-1 cell surface antigen</td><td>Thy1</td></tr>
<tr><td>14</td><td>apolipoprotein E</td><td>ApoE</td><td rowspan="3">Müller glial</td></tr>
<tr><td>15</td><td>Glutamine Synthetase</td><td>GS</td></tr>
<tr><td>16</td><td>Vimentin</td><td>VIM</td></tr>
<tr><td>17</td><td>Hereditary prostate cancer 1</td><td>HPC1</td><td rowspan="3">Amacrine cells</td></tr>
<tr><td>18</td><td>Syntaxin 1</td><td>SYN-1</td></tr>
<tr><td rowspan="2">19</td><td rowspan="2">Calbindin</td><td rowspan="2">CALB1</td></tr>
<tr><td rowspan="2">Horizontal cells</td></tr>
<tr><td>20</td><td>Neural filament 165</td><td>NF165</td></tr>
</table>

*Table 1.3: Summary of commonly used markers for identification of retinal cell phenotypes.*

**1.11.2. Gene expression profiles:**

The *in vitro* differentiation of SCs includes various steps like cessation of proliferation, decreased self-renewal capacity and their actual differentiation into different cell types. To learn more about the SCs properties and the terminally differentiated population gene expression analysis using cDNA microarrays would provide good insights. Recent advances in cDNA microarray technology allow large-scale screening of gene expression in SCs and the terminally differentiated cells from a wide range of tissues leading to the discovery of new stem-cell markers (Geschwind et al., 2001; Ivanova et al., 2002; Ramalho-Santos et al., 2002). However, a detailed description of genes constitutively expressed in human neurospheres is not currently available.

**1.11.3. Epigenetic profiles:**

Emerging evidence suggests that the initiation and maintenance of changes in gene expression that are associated with stem cell differentiation involve the action of a unique epigenetic program (Jiang Y et al., 2002). Epigenetics is the study of genomic properties that influence phenotype without directly involving genotype (DNA sequence). Although differentiated cells and their precursors are genetically identical, the differentiated cells have radically different expression profiles and highly specialized functions. Increasing evidence also suggests that the multilineage

differentiation ability of SCs is brought about by the potential for expression of developmentally regulated transcription factors and of lineage-specification genes. This suggests that epigenetic mechanisms must play a pivotal role in the differentiation process. Major epigenetic mechanisms include covalent DNA and chromatin modifications as well as small noncoding RNA-mediated pre- and post-transcriptional gene regulations.

***DNA Methylation:*** DNA methylation consists in the addition of a methyl group to the 5 position of a cytosine in a cytosine-phosphate guanine (CpG) dinucleotide *(Figure 1.7A)*. DNA methylation is a hallmark of long term silencing *(Figure 1.7B)* CpG methylation is symmetrical (it occurs on both DNA strands) and targets isolated CpGs, clustered CpGs, or even clustered CpGs within a CpG island. A CpG island is defined as a sequence in which the observed/expected C frequency is greater than 0.6 with a GC dinucleotide content greater than 50%. CpG islands are often protected from methylation, enabling constitutive expression of these genes (Laird PW 2005).

The methyl groups create target sites for methyl-binding proteins that induce transcriptional repression by recruiting co-repressors such as histone deacetylases (Nan X et al., 1998). So DNA methylation largely contributes to gene silencing (Hoffman AR and Hu JF 2006) (Klose RJ and Bird AP 2006) and as such it is essential for development (Morgan HD et al., 2005) (Young LE and Beaujean N 2004) (Mann JR 2001) (Razin A and Shemer R 1995), X chromosome inactivation (Hellman A and Chess A 2007), and genomic imprinting (Tremblay KD et al., 1995) (Reik W et al., 1990) (Sapienza C et al., 1987) (Reik W et al., 1987). The relationship between DNA methylation and gene expression is complex and recent evidence based on genome-wide CpG methylation profiling highlights promoter CpG content as a component of this complexity (Jones PA and Takai D 2001).

*In vitro* differentiation of ESCs and embryonal carcinoma (EC) cells also correlates with changes in DNA methylation notably on the promoter of developmentally regulated genes expressed in pluripotent ESCs such as the transcription factors OCT4 and NANOG (Weber M et al., 2007) (Deb-Rinker P et al., 2005) (Dahl JA and Collas P 2007) (Freberg CT et al., 2007). However, to date, only sporadic indications of CpG methylation changes have been reported during differentiation of SCs (Boquest AC et al., 2007) (Noer A et al., 2006).

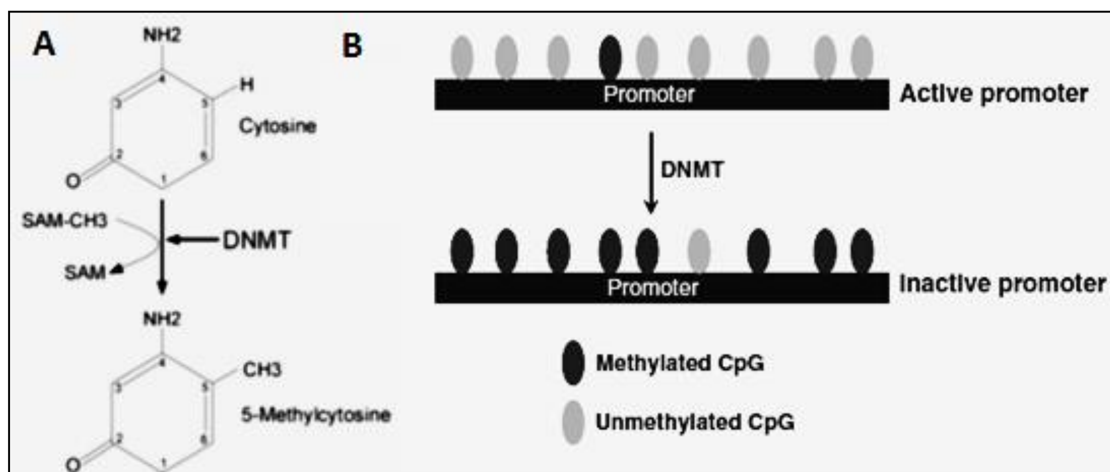



*Figure 1.7: Principles of DNA methylation. (A) Mechanism of DNA methylation. (B) Textbook view of the relationship between DNA methylation and gene expression.*

DNA methylation is catalysed by DNA methyltransferases (DNMTs) (Laird PW 2005). In humans, the process of DNA methylation is carried out by three enzymes, DNA methyltransferases 1, 3A and 3B (DNMT1, DNMT3A, DNMT3B). It is thought that DNMT3A and DNMT3B are the de novo methyltransferases that set up DNA methylation patterns to the daughter strands during DNA replication (Chen and Li, 2004). DNA methylation is known to regulate single gene expression in the differentiation process and in most cases expressed genes are demethylated at promoter regions (Roloff and Nuber, 2005). For example astrocytic differentiation of forebrain progenitor is activated following demethylation of a promoter region of the astrocytic gene GFAP (Takizawa et al., 2001).

***Histone modifications:*** Chromatin is essential for DNA organization and it also presents a barrier to any DNA templated event, which can be improved by remodelling processes. Due to the inherent difficulty to distinguish between sequence –independent self-propagation of epigenetic states and a re-establishment after cell division mediated by sequence dependent recruitment of enzymatic activities, most chromatin modifications are termed epigenetic without knowing the molecular mechanisms that mediate propagation (Bird, 2007).

***Post-translational modifications of core histones:*** DNA-bound histone proteins contain over 60 sites which are subject to posttranslational modifications (PTMs) such as acetylation, methylation, ubiquitination, phosphorylation, sumoylation and others (Kouzarides, 2007). These modifications mostly take place at the N-terminal tails of

histone H3 and H4, which protrude out of the nucleosomes cores *(Figure 1.8)*. As a result the PTMs are accessible to non-histone proteins and present a way to specifically recruit non-histone protein complexes to chromatin. A second possible regulatory role of PTMs is direct interference with binding of DNA around nucleosomes via altering the electric charge of histones. The histone demethylases indicated that PTMs are much more dynamic (Shi et al., 2004). Although many of these PTMs are considered to be epigenetic and are thought to be inherited during mitotic cell divisions, so far the propagation mechanisms are unknown for all of these modifications.

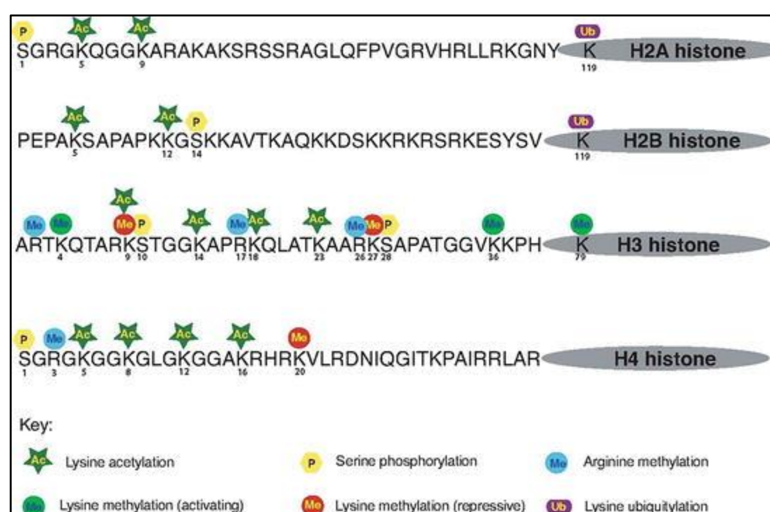



*Figure 1.8: Overview of known sites for post-translational modifications on the N- and C-terminal tails of canonical histones. ph: phosphorylation, ac: acetylation, me: methylation, ub1: monoubiquitination. Adapted from Khatuna Gagnidze and Donald W. Pfaff 2013.*

Methylation of histones can either occur at lysine or arginine residues *(Figure 1.7)*. The differential methylation states present another level of regulatory potential which indeed appears to be exploited. Several lysines display diverging functions and localization in the genome depending on their methylation state (Barski et al., 2007; Peters and Schubeler, 2005). Arginine methylation is performed by protein arginine methyltransferases (PRMTs) and is antagonized by PADI4 (Klose et al., 2006; Zhang and Reinberg, 2001). Lysine methylation is carried out by specific lysine methyltransferases (KTMs), which all contain a conserved SET-domain, with the exception of Dot1/KTM4 (Zhang and Reinberg, 2001). Lysine methylation can be removed by two distinct classes of histone demethylases (KDMs): the LSD1 enzyme and the JmjC protein family (Klose et al., 2006).

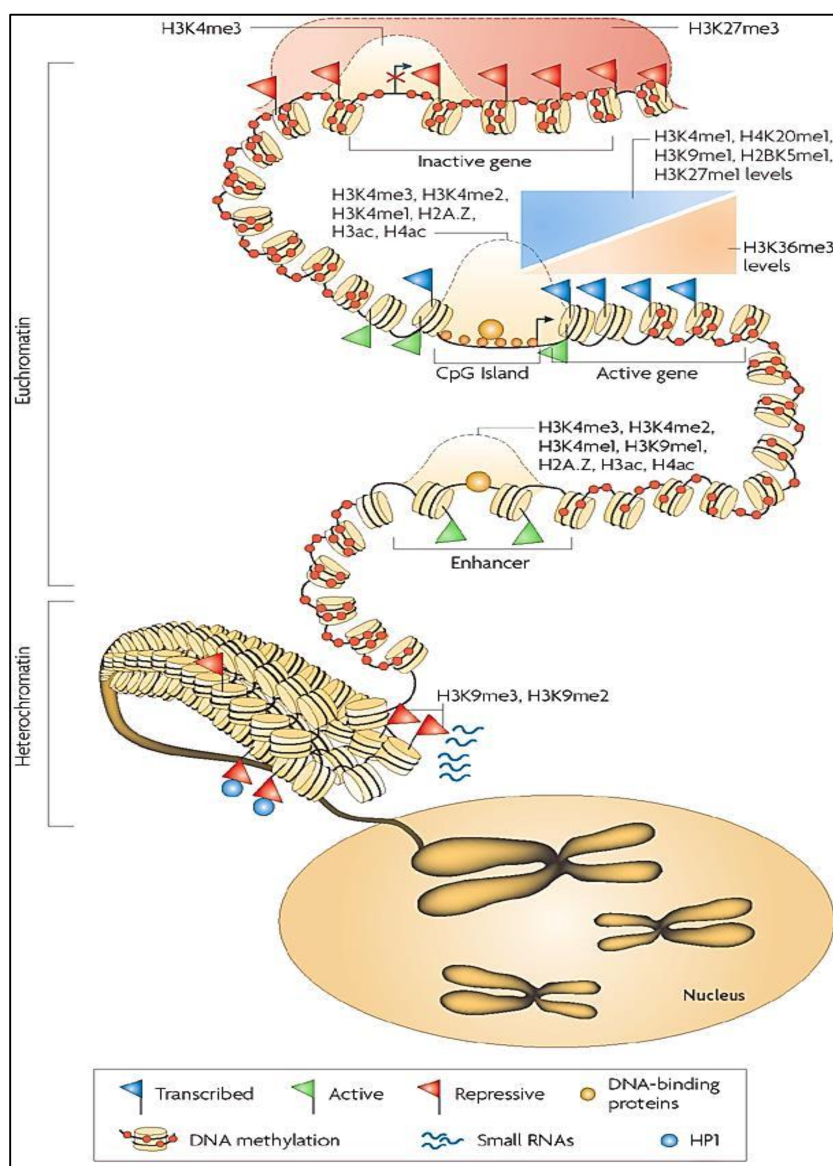



*Figure 1.9: Chromatin structure and modifications. Chromosomes are divided into accessible regions of euchromatin and poorly accessible regions of heterochromatin. Heterochromatic regions are marked with H3K9me3, which serve as a platform for HP1 binding. Small RNAs have been implicated in the maintenance of heterochromatin. DNA methylation is persistent throughout genomes, and is missing only in regions such as CpG islands, promoters and possibly enhancers. The H3K27me3 modification is present in broad domains that encompass inactive genes and usually coincides with H3K4me2/3 to form so called bivalant domains. H3K4me3, H3K4me2, H3K4me1 as well as histone acetylation and histone varient H2A.Z mark the transcription start site regions of active genes. The monomethylations of H3K4, H3K9, H3K27, H4K20 and H2BK5 mark actively transcribed regions, peaking near the 5' end of genes. The trimethylation of H3K36 also marks actively transcribed regions, but peaks near the 3'end of genes. High levels of H3 and H4 acetylation are typically detected at the promoter and 5' regions. Adapted from Schones and Zhao, 2008.*

Chromatin immunoprecipitation experiments have revealed that active genes are methylated at lysine 4 of histone H3 (H3K4), H3K36 and H3K79 *(Figure 1.9)* (Barski et al., 2007; Pokholok et al., 2005; Saunders et al., 2006; Schubeler et al., 2004). These modifications are thus thought to have a role in transcription. H3K4me peaks around the transcription start site and is gradually diminished further 3'. H3K36me and H3K79me display a broader distribution within the gene body, starting just downstream of the H3K4me peak *(Figure 1.8)* (Bell et al., 2007; Wirbelauer et al., 2005). H3K4 methylation has been implicated in transcriptional activation pathways since many chromatin remodeling and co-activator complexes bear a module which specifically recognizes H3K4me2/3 (Wysocka et al., 2006).

More recent data from mammalian systems indicates that in contrast to invertebrates H3K4me2/3 are not exclusively marking actively transcribed regions *(Figure 1.9)* (Bernstein et al., 2006; Guenther et al., 2007; Mikkelsen et al., 2007; Roh et al., 2006; Weber et al., 2007). Interestingly, these loci are CpG-rich sequences and many bear low but detectable levels of Pol II and acetylated histone H3 (Guenther et al., 2007; Roh et al., 2006). Inactive loci display a different set of methylation marks mainly consisting of methylation of H3K9, H4K20, and H3K27. H3K9 and H4K20 di- and tri-methylation play essential roles in heterochromatin maintenance at pericentromeric repeat regions and are further present at repetitive, transposable and retroviral elements in mammalian genomes (Lehnertz et al., 2003; Mikkelsen et al., 2007; Peters et al., 2003).

Only few regulatory regions have so far been identified to be methylated at H3K9 and/or H4K20. These are mostly CpG-poor promoters of large gene families such as the olfactory receptor clusters or zinc finger proteins (Mikkelsen et al., 2007; Vogel et al., 2006), suggesting that recognition is based on the repetitive nature of their genomic organization. H3K27 di- and tri-methylation in turn is excluded from regions carrying H3K9 methylation and predominantly localizes to CpG-rich regions, which strongly implies different functions of these two repressive histone methylation marks.

Epigenetic modifications, especially histone and DNA methylations, have a large impact on the regulation of gene expression and are critical in establishing patterns of gene repression during development (Cedar H, Bergman Y, 2009). Previous genome-wide maps of histone H3 lysine 4 and lysine 27 trimethylation (H3K4me3 and

H3K27me3) showed a very clear correlation between H3K4me3 and expressed genes and H3K27me3 and repressed genes in embryonic stem cells ((Bernstein BE et al., 2006) (Mikkelsen TS et al., 2007) (Pan G et al., 2007) (Zhao XD et al., 2007), T cells (Wei G et al., 2009), and hematopoietic stem cells/progenitor cells (Cui K et al., 2009). DNA methylation (DNAMe) is a widely accepted gene expression silencing mark and was considered as coupled to H3K27me3 through enzymatic interaction (Cedar H, Bergman Y, 2009). Genome wide mapping of DNAMe, however, revealed that most strong CpG island promoters are unmethylated even when they were inactive and low CpG content promoters are predominantly methylated although this methylation does not preclude gene expression (Weber M et al, 2007).

### 1.12. Current status of SCs research in treating retinal degenerative diseases:

Over the past few years a great deal of interest has been generated in using SCs to treat degenerative diseases that afflict different tissues, including retina. The interest is due to the defining properties of SCs, 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 SCs and directed differentiation of the ESCs have fuelled the hope for cell and gene therapy using SCs from heterologous sources. Despite the success of isolation and maintenance of embryonic RPCs 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 RPCs display limited self-renewal capacity. Second, they 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 uses because of the source of these cells are 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 RPCs.

In this context many studies have been performed on isolation and differentiation of SCs from the adult iris and ciliary body of animal models like rats, mice, chick and

pigs. The Iris- and Ciliary Body-derived cells form spheres and undergo neural differentiation is a new and distinct mechanism for reprogramming epithelia like Iris- and Ciliary Body-derived cells to retinal neural cells. Acquisition of retinal neuronal properties by these cells involves various factors which are not well understood. Our study, therefore, draws attention toward the necessity for rigorous characterization of Iris and ciliary body derived SCs and their differentiation into retinal lineage and offers a model for characterizing neuronal potential of heterologous SCs that may shed light on their biology as well as on their therapeutic potential.

# CHAPTER 2: OUTLINE OF THE WORK

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.

In 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*.

## 2.1. Specific objectives of the study:

### 2.1.1. 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*.

### 2.1.2. 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.

### 2.1.3. 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.

### 2.1.4. 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.

### 2.1.5. 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.

# CHAPTER 3: METHODOLOGY

## 3.1. Sample collection and preparation:

### 3.1.1. Isolation of the Ciliary epithelial cells and Iris pigment cells

After the nearest relative of the diseased person has given the consent for

- Enucleation of the eye ball
- Corneal tissue to be used for transplantation
- Rest of the tissues to be used for research purposes

A detailed medical history regarding the cause of death and any other illness suffered by the individual in the past is obtained by an ophthalmologist and if the diseased person is found to be fit then eye ball is procured by $360^o$ limbal peritomy, disinsertion of the extra ocular muscles and ligation of the optic nerve. Blood sample is also taken for HIV and Hepatitis B analysis, if found to be positive then the eye ball is not used for surgical or research purposes. Donor eyeball is then stored in a moist chamber and is brought to the eye bank within 6hrs of death of the diseased individual. Corneoscleral button is made with 4mm of sclera from the limbus also being included in the button.

### 3.1.2. Dissection of iris:

Once the button is made anterior pupillary zone (1.5mm) of iris is removed until the collaret by cutting with vannas scissors and this part is discarded. The posterior ciliary zone of the iris is removed from its attachment to the ciliary body (pars plicata) in a zipper like fashion as a whole for $360^o$ and is transferred to the medium under strict aseptic precautions.

Contents of the removed iris includes: Endothelium, Stroma, Dilator muscle, Basement membrane, two layers of pigment epithelium anterior and posterior.

### 3.1.3.Dissection of ciliary body:

After removing the iris a horizontal cut is made & the whole ciliary body (pars plicata region) about 2.0mm width of tissue is dissected with wescott's scissor from the pars plana. Pars plicata is differentiated from the pars plana by the ciliary processes, which are present in pars plicata on the posterior surface the tissue. The tissue is then transferred under strict aseptic conditions into the medium.

Contents of the removed ciliary body includes: Supraciliary layer, Ciliary muscle, Stroma, Epithelial layer: Pigment epithelium &non-pigment epithelium

### 3.1.4. Dissection of Retina:

After removing the iris and ciliary body from the eye ball, the lens was removed using a spatula. Vitreous humor was emptied out of the eye ball using a 5ml syringe and discarded. On the inside of the eyeball, a thin fleshy film with blood vessels is attached to the back of the eye at just one spot (Blind spot). The retina as a whole is cut at the blind spot using wescott's scissor and pulled carefully without tearing using smooth & blunt forceps. The tissue is then transferred under strict aseptic conditions into the medium.

Contents of the removed retina includes: Inner limiting membrane, Nerve fibre layer, Ganglion cell layer, Inner plexiform layer, Inner nuclear layer, Outer plexiform layer, Outer nuclear layer, External limiting membrane, Photoreceptor layer – rods/cones, Retinal pigment epithelium.

### 3.1.5. Culturing of the ciliary pigment epithelium:

- The dissected ciliary tissue was brought to the laboratory in the transport medium in sterile conditions.
- The ciliary tissue was washed thrice carefully with HBSS (with $Ca^{2+}$ and $Mg^{2+}$) to remove the vitreous remnants.
- The ciliary tissue was then placed carefully in HBSS (with $Ca^{2+}$ and $Mg^{2+}$) and with the help of St. Martin forceps and Pierce Hoskins forceps.

- The non-pigmented epithelium gently peeled off holding from the pars plana region to pars plicata of the Ciliary body.
- The ciliary tissue was then placed in a sterile Petri plate and incubated with 200 units of dispase for 45mins at 37$^{o}$C.
- After incubation the Dispase was removed, the Pigmented epithelial layer was scraped off gently from the underlying stroma with sterile 15mm bard parker blade.
- The Pigmented epithelial layer was incubated with 0.02% trypsin for 15 minutes at 37$^{o}$C.
- To stop the action of trypsin, Trypsin inhibitor- 10%fetal bovine serum was added and triturated for 25-30 times.
- The separated cells were then centrifuged at 1000rpm for 3minutes and the supernatant was discarded.
- The pellet was washed thrice with DMEM/F12 medium at 1000rpm for 3 minutes.
- The cell pellet was then re-suspended in 310µl of the Neurosphere medium.
- The suspended cells were then plated (100µl in each well) in a 12 well plate containing the Neurosphere medium.

### 3.1.6.Culturing of the iris pigment epithelium:

- The dissected iris tissue was brought to the laboratory in the transport medium in sterile conditions.
- The iris tissue was washed thrice carefully with HBSS (with $Ca^{2+}$ and $Mg^{2+}$) to remove the vitreous.
- The iris tissue was then placed in a sterile Petri plate and incubated with 200 units of dispase for 5mins at 37$^{o}$C.
- After incubation the Dispase was removed, the posterior pigmented epithelial layer was scraped off gently from the underlying stroma with sterile 15mm bard parker blade.

- The pigmented epithelial layer was incubated with 0.02% trypsin for 10 minutes at 37°C.
- To stop the action of trypsin, Trypsin inhibitor- 10%fetal bovine serum was added and triturated for 25-30 times.
- The separated cells were then centrifuged at 1000rpm for 3minutes and the supernatant was discarded.
- The pellet was washed thrice with DMEM/F12 medium at 1000rpm for 3 minutes.
- The cell pellet was then re-suspended in 310µl of the Neurosphere medium.
- The suspended cells were then plated (100µl in each well) in a 12 well plate containing the Neurosphere medium.

### 3.1.7.Culturing of the Retina:

- The dissected retinal tissue was brought to the laboratory in the transport medium in sterile conditions.
- The retinal tissue was gently washed thrice carefully with HBSS (with $Ca^{2+}$ and $Mg^{2+}$) to remove the vitreous.
- The retinal tissue was cut into tiny bits using a scalpel and incubated with 0. 25% trypsin for 15 minutes at 37°C.
- To stop the action of trypsin, Trypsin inhibitor- 10%fetal bovine serum was added and triturated for 25-30 times.
- The separated cells were then centrifuged at 1000rpm for 3minutes and the supernatant was discarded.
- The pellet was washed thrice with DMEM/F12 medium at 1000rpm for 3 minutes.
- The cell pellet was then re-suspended in 310µl of the Retinal culture medium.
- The suspended cells were then plated (100µl in each well) in a 12 well plate containing the Retinal culture medium.

### 3.2.Cell count:

The suspended cells were then loaded on Neubar chamber and cell count was done. The cell count was done in the four corner squares of the Neubar chamber and calculated with the following formulae.

No. of cells/Area x Depth factor

### 3.3.Cell Viability Assays:

#### 3.3.1.Cell viability- Trypan blue:

To quantify the viable cells in the cultures, 0.4 % trypan blue dye exclusion test was used. A mixture of 1 volume of dye and 9 volumes of cell suspension was made in Phosphate buffered saline (pH 7.4). This was allowed to stand at room temperature for 10 minutes. Charged on to an improved neubaur chamber, the four corner 1mm sized squares were counted. Cells touching upper and left boundaries were included and those touching lower and right boundaries were excluded. Number of stained and unstained cells was separately noted. Calculations were done to express the number of viable cells as percentage. The viable cells do not take up the stain while the dead cells were stained. Cell count was carried out thrice to confirm results.

**Calculation**

*The cell count (cells per ml) was determined as follows:*

Cell count per ml = average cell count per square x dilution factor x $10^4$

*The cell viability (%) was determined as follows:*

Cell viability (%) = cell count (viable) / total cell count (viable + non-viable) x 100

#### 3.3.2.Cell viability – Propidium iodide:

The viability assay for iris and ciliary pigment epithelial cells for plating was performed by the fluorescence methods to avoid the interference of the natural pigmentation of these cells. To quantify the viable cells in the cultures, 0.5µg/ml Propidium iodide was used. A mixture of 1 volume of Propidium Iodide and 9 volumes of cell suspension was made in Phosphate buffered saline (pH 7.4). This was allowed to stand at room temperature for 10

minutes in dark. Charged on to an improved neubaur chamber, the four corner 1mm sized squares were counted.

Cells touching upper and left boundaries were included and those touching lower and right boundaries were excluded. Total number of cells were counted under the phase contrast microscope and noted. The number dead cells were counted under the fluorescent microscope. Calculations were done to express the number of viable cells as percentage. The viable cells do not take up the stain while the dead cells were stained. Cell count was carried out thrice to confirm results.

**Calculation**

*The cell count (cells per ml) was determined as follows:*

Cell count per ml = average cell count per square x dilution factor x $10^4$

*The cell viability (%) was determined as follows:*

Cell viability (%) = cell count (viable) / total cell count (viable + non-viable) x 100

**3.4.Sphere suspension assay:**

- The neurospheres from the primary cultures are counted and collected into a sterile vial and spun at 1000 rpm for 3mins
- To the pellet 0.02% trypsin solution was added and incubated at 37ºC for 5 minutes.
- Then to inhibit the action of trypsin 10% Fetal bovine serum was added and triturated gently 15 times
- The cell suspension was then washed thrice with DMEM/F12.
- The cell pellet was suspended in 210µl of DMEM/F12 medium
- Then cell count was done and plated onto a 6 well plate containing the poly D-lysine and laminin coated coverslips and the differentiation medium.
- Once the cells are differentiated and full grown, they are used for RNA extraction and immunocytochemistry.

### 3.5.Coating Glass Cover slips with Poly D-Lysine:

- Autoclaved coverslips are used (22mm glass coverslips will fit exactly onto a precleaned glass microscope slide and will fit in a six-well plate, though they are smaller than the wells).
- Each coverslip was placed in a different well of a six-well plate.
- The coverslips were rinsed thoroughly with autoclaved double distilled water to remove any debris and to uniformly wet the surface of the coverslip.
- Enough amount of poly-D-lysine was added to cover the surface of the coverslip.
- The coverslips in poly-D-lysine was incubated for at least 30 minutes and rinsed thoroughly with sterile distilled water.
- Aspirate and let coverslips dry completely (overnight). Store coated coverslips at 4°C until needed.

### 3.6.Coating of glass cover slips with laminin:

- Laminin is always stored in -20$^{o}$C. Laminin coating of coverslips is done only on the day of plating. For thawing laminin it is kept at 2-8$^{o}$C to avoid the formation of gel.
- Diluted (5ug/ml) in sterile HBSS, or in sterile water, and culture surface was coated with a minimal volume.
- The coverslips along with laminin was incubated at 37$^{o}$C for 2 hours.
- The coverslips are then washed thrice with Phosphate buffered saline (PBS) and the cells are plated.

### 3.7. RNA isolation and Reverse Transcriptase polymerase chain reaction:

- Total RNA was isolated from the neurospheres at the end of 7th day of incubation to determine the pattern of expression of different stem cell markers specific for retinal progenitor cells by Reverse Transcriptase polymerase chain reaction RT-PCR.

- Cells were collected and the total RNA was extracted by the RNeasy mini kit method, according to the manufacturer's recommended protocol and the total RNA was stored at -80ºC before use.

**3.7.1.RNA isolation Protocol:**

- The cells are harvested from the cultures and collected in a vial.
- The harvested cells are centrifuged at 10000rpm for 5-10 minutes and the supernatant is discarded using a pipette.
- To the pellet 350µl of the RLT buffer (given in the kit) was added.
- The tissue lysate was centrifuged for 3minutes at a maximum speed in a microfuge.
- The supernatant was transferred to a new microfuge.
- 350µl of 70% ethanol was added to clear the lysate and mixed immediately by pipetting.
- 700µl of the sample was applied on to the RNeasy mini column placed in 2ml collection tube including the precipitate that has formed.
- Centrifuged for 15 seconds at 10,000rpm. The flow through was discarded and the same collection tube was used.
- To the column 700µl of RW1 buffer (given in the kit) was added and centrifuged for 15 sec at 10,000rpm for 15 seconds.
- The spin column was transferred to a new collection tube. Then 500µl of RPE buffer (given in the kit) was added and centrifuged at 10,000rpm for 15 seconds.
- The flow through was discarded and another 500µl of the RPE buffer was added, centrifuged at 10,000rpm for 2minutes.
- The column was placed in a new vial and 30-40µl of RNase free water was added directly on to the membrane and centrifuged at 10,000rpm for1minute.
- 5µl of the total RNA extracted was run in 2% agarose gel to see the quality of RNA extracted and the rest was stored at -80ºC before use. RT-PCR was

performed using sensiscript reverse transcripatases, which is a recombinant heterodimeric enzyme.

### 3.7.2.cDNA conversion protocol: (Sensiscript RT Kit)

| | | |
|---|---|---|
| 10 x buffers | : | 2.0µl |
| DNTPs | : | 2.0µl |
| Oligo dT | : | 2.0µl |
| RNase out | : | 1.0µl |
| Sensiscript RT | : | 1.0µl |
| RNAse free water | : | Variable |
| Template RNA | : | 100ng to 1µg → 60 minutes at $37^0$C. |

### 3.7.3.PCR Amplification:

PCR amplification of the first – strand cDNAs were performed using specific primer pairs, along with Housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as internal control.

***Reaction cocktail:***

| | | |
|---|---|---|
| 10 X buffer | : | 2.5 µl |
| dNTPs | : | 2.5 µl |
| Forward Primer | : | 1 µl |
| Reverse Primer | : | 1 µl |
| Taq polymerase | : | 0.3 µl |
| MilliQ water | : | Variable |
| cDNA | : | 1.0 µg |

***Profile:***

| | | | |
|---|---|---|---|
| Initial denaturation | : | 94ºC – 5minutes | 35 Cycles |
| Denaturation | : | 94ºC – 45seconds | |
| Annealing | : | As per tabulation – 45 second | |
| Extension | : | 72ºC – 45seconds | |
| Final Extension | : | 72ºC – 2 minutes | |
| Hold | : | 4ºC - ∞ ∞ | |

### 3.7.4. List of Primer sequences: *(Table 3.1)*

| Sl. No. | Gene symbol | Primer Sequence |
|---|---|---|
| **Retinal/neural SCs genes** | | |
| 1 | GAPDH | 5' GCCAAGGTCATCCATGACAAC 3'<br>5' GTCCACCACCCTGTTGCTGTA 3' |
| 2 | ABCG2 | 5' TCAGGTAGGCAATTGTGAGG 3'<br>5' AGTTCCATGGCACTGGCCATA 3' |
| 3 | NES | 5' CAGCTGGCGCACCTCAAGATG 3'<br>5' AGGGAAGTTGGGCTCAGGACTGG 3' |
| 4 | PAX 6 | 5' AGATGAGGCTCAAATGCGAC 3'<br>5' GTTGGTAGACACTGGTGCTG 3' |
| 5 | MSI1 | 5' CGCCTGGTCCATGAAAGTGAC 3'<br>5' GAGACTGACGCGCCCCAGCC 3' |
| 6 | TUJ | 5' AGATGTACGAAGACGACGAGG 3'<br>5' GTATCCCCGAAAATATAAACA 3' |
| 7 | GFAP | 5' GTGACTCATCCTCTTGAAGAT 3'<br>5' ACAGATCCCACCAGTCTGCTC 3' |
| 8 | SOX2 | 5' GCCGAGTGGAAACTTTTGT 3'<br>5' GTTCATGTGCGCGTAACTG 3' |
| 9 | NOTCH 1 | 5' GACATCACGGATCATATGGA 3'<br>5' CTCGCATTGACCATTCAAAC 3' |
| **Retinal specific genes** | | |
| 10 | Brn3b | 5' GGAGAAGAAGCGCAAGC 3'<br>5' TCTGGAGAGGCCAAGAGTC 3' |
| 11 | THY 1 | 5' TGCCTAGTGGACCACAGCCTT 3'<br>5' TCACAGGGACATGAAATCCGT 3' |

| | | |
|---|---|---|
| 12 | CHX 10 | 5' AGCTAGAGGAGCTGGAGAAG 3'<br>5' CATGATGCCATCCTTGGCTG 3' |
| 13 | RHO | 5' TACGTGCCCTTCTCCAATGCG 3'<br>5' GCCATGACCCAGGTGAAGGCA 3' |
| 14 | RCVRN | 5' AGCTGCAGCTGAACACCAAG 3'<br>5' TCGTCTGGAAGGAGCTTCAC 3' |
| 15 | OPN1SW | 5' TAGCAGGTCTGGTTACAGGAT 3'<br>5' GAGACGCCAATACCAATGGTC 3' |
| 16 | NRL | 5' GTGCCTCCTTCACCCACC 3'<br>5' CAGACATCGAGACCAGCG 3' |
| 17 | STX1 | 5' TGACAAGATCGCAGAGAACG 3'<br>5' TTGATGATCTCACTGTGCCG 3' |
| 18 | CALB2 | 5' ATCCTGCCAACCGAAGAGAAC 3'<br>5' GCAGGAAGTTTTCCTGGACAG 3' |
| **Pluripotency genes** | | |
| 19 | GAPDH | 5' AGAAGGCTGGGGCTCATTTG 3'<br>5' AGGGGCCATCCACAGTCTTC 3' |
| 20 | c-MYC | 5` TCAAGAGGTGCCACGTCTCC 3`<br>5' TCTTGGCAGCAGGATAGTCCTT 3` |
| 21 | KLF4 | 5` CGAACCCACACAGGTGAGAA 3`<br>5` TACGGTAGTGCCTGGTCAGTTC 3` |
| **Pigmentation genes** | | |
| 22 | RPE65 | 5` CGCCGCTCACAGCTCAT 3`<br>5` ATCGAAGGAGACTGCCGGT 3` |
| 23 | TYR | 5` CGGCCAACGATCCCATT 3`<br>5` TGCCTTCGCAGCCATTG 3` |
| 24 | PALMD | 5` CACAGTGCAGCATACAATGG 3`<br>5` GGGATTGGCATATACAGGCT 3` |

| **Retinal Histogenesis** | | |
|---|---|---|
| 25 | NEUROD | 5` GTTATGAGACTATCACTGCTCAGGACC3`<br>5` AGAAGTTGCCATTGATGCTGAGC 3` |
| 26 | Rx | 5` TGTTTAAGCACGCTCAGGAC 3`<br>5` CAGTCCTCCTCTTTCCCTGA 3` |
| 27 | CRX | 5` GACAGCAGCAGAAACAGCAG 3`<br>5` TCTAGGCCGCTGAAATAGGA 3' |
| 28 | NRL | 5' GTGCCTCCTTCACCCACC 3'<br>5' CAGACATCGAGACCAGCG 3' |
| 29 | MITF | 5` AGGAACTTGAAATGCAGGCT 3`<br>5` TGATGCTGAAGGAGGTCTTG 3` |
| 30 | IRBP (Rbp3) | 5` CTTTCCATAGCCCAGGACAT 3`<br>5` CGGCAGAGGCATAGTTATCA 3` |
| 31 | S-ARRESTIN | 5` TTGCCCTGTTCAGTGATGTT 3`<br>5` AGTAATCGCACGGAGCTCTT 3` |
| **Cell cycle genes** | | |
| 32 | CYCLIN D1 | 5′ CCGTCCATGCGGAAGATC 3′<br>5′ ATGGCCAGCGGGAAGAC 3′ |
| 33 | CYCLIN D3 | 5' CGTGGTCGGTGTAGATGC 3'<br>5' TGGATGCTGGAGGTATGTG 3' |
| 34 | CYCLIN B1 | 5' CAGTCAGACCAAAATACCTACTGGGT 3'<br>5' ACACCAACCAGCTGCAGCATCTTCTT 3' |

*Table 3.1: List of primers used in the study.*

### 3.7.5.Gel Electrophoresis:

PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% Ethidium bromide with molecular marker 100bps ladder to confirm the size of the resultant product.

## 3.8. Immunofluorescence Assay:

### 3.8.1. Immunofluorescence assay for the detection of 5-bromo-2'-deoxy-uridine (BrdU) incorporated into the cellular DNA:

After the 7 days of incubation the neurospheres were plated on the poly-D-lysine coated slides and incubated with the BrdU labeling medium for 48 hours. And the BrdU labeling was done by BrdU labeling and Detection kit from Roche applied sciences, Germany. The labeling and detection was done according to the kit protocol.

***Preparation of the kit working solution:***

- BrdU labeling medium 1:1000 dilution of the stock with sterile cell culture medium. Prepared shortly before use.
- Anti-BrdU working solution 1:10 dilution of the stock with incubation buffer.
- Anti-mouse-Ig-fluorescein stock solution Anti-mouse-Ig-fluorescein is dissolved in 1ml of double distilled water.
- Anti-mouse-Ig-fluorescein working solution 1:10 dilution of the stock with PBS. Prepared shortly before use.
- Washing Buffer Washing buffer concentrate 10x is 1:10 diluted with double distilled water.
- Ethanol fixative 50mM Glycine solution in 70ml of absolute Ethanol to get 100ml of fixative pH 2.0.

***Protocol for detection:***

- BrdU labeling was done on the primary neurosphere formed from CPE and IPE.
- Neurosphere was transferred to poly D-lysine coated coverslips in a 6 well plate and incubated along with 10µM BrdU labeling medium and allowed them to attach to the coverslips for 48 hours.

- The medium was aspirated and then the cover slip was washed thrice with washing buffer.
- All samples were fixed in cold methanol for 20 min at 25°C. The preparation was washed thrice with washing buffer.
- The cells are covered with the anti-BrdU working solution and incubated for 30 minutes at 37°C.
- The preparation was again washed thrice with the washing buffer.
- The cells are covered with the Anti-mouse-Ig-fluorescein working solution and incubated for 30 minutes at 37°C.
- The preparation was washed thrice with the washing buffer.
- The labeled preparations are mounted in glycerol and examined in a fluorescence microscope at a wavelength of 515-565nm.
- The counting can be done under microscope using 40X objective to assess the BrdU labeling indices.
- The labeling index was expressed as the number of positively labeled nuclei/the total number of nuclei x 100%.

### 3.8.2. Immunofluorescence assay for the detection of specific protein expression:

***Preparation of cells for Immunofluorescence:***

- Coverslips were coated with poly-D-lysine and laminin as mentioned above.
- The cells were grown on the glass coverslips for at least 24hrs.
- The cells grown on coverslip were rinsed briefly in PBS (with $Ca^{2+}$ and $Mg^{2+}$).

***Fixation:***

- The samples were fixed with either ice-cold methanol or acetone for 1-10 min or in 4% paraformaldehyde for 20 min at room temperature.
- The samples were then washed thrice with ice cold PBS.

(Note: Fixation is performed to immobilize antigens while retaining cellular and subcellular structure. It also allows for access of antibodies to all cells and subcellular components. (better at preserving cell structure).)

***Permeablisation:***

- The samples were incubated for 20 min with PBS containing Triton X-100 (0.1% for Membrane proteins, 0.5% for cytoplasmic proteins and 1.0% for nuclear proteins – PBST).
- The cells were washed thrice with PBS each 5 min.

(Note: Permeablisation was required for intracellular epitopes when the antibody required access to the inside of the cell to detect the protein. It is also required for detection of transmembrane proteins if the epitope is in the cytoplasmic region.)

***Blocking and Incubation:***

- Cells were incubated with Blocking solution - 1% BSA or 10% serum (from the species that the secondary antibody was raised in) in PBST for 30 min to block unspecific binding of the antibodies.
- Cells were incubated in the Primary antibody (Table 3.2) diluted with the blocking solution in a humidified chamber for 4hr at room temperature or overnight at 4°C.
- The solution was decanted and the cells were washed thrice in PBS 5 min each wash.
- Cells were incubated with secondary antibody in blocking solution for 2hr at room temperature in dark.
- The solution was decanted and the cells were washed thrice in PBS 5 min each wash in dark.

***Counter staining:***

- Cells were incubated with DAPI/PI diluted in blocking solution for 1 min.

- The solution was decanted and the cells were washed thrice in PBS 5 min each wash in dark.

***Mounting:***

- Coverslips were mounted with a drop of Antifade mounting medium.
- Coverslips were sealed with nail polish to prevent drying and movement under microscope. The slides were stored in dark at -20 or 4°C.

**3.8.3.List of Antibodies:** *(Table 3.2)*

| Antibody Name | Cell type | Localisation | Dilution |
|---|---|---|---|
| ABCG2 | Putative stem cell marker | Membrane protein | 1:30 |
| Beta actin | Internal Control | Cytoplasmic mRNP granules | 1:500 |
| Beta III tubulin | Neural cells | Cytoplasmic protein | 1:500 |
| Cytokeratin | Epithelial cells | Cytoplasmic protein | 1:300 |
| GFAP | Glial progenitor cells | Cytoplasmic protein | 1:400 |
| Musashi-1 | Progenitor cells | Cytoplasmic protein | 1:500 |
| Nestin | Neural progenitor cells | Cytoplasmic protein | 1:200 |
| Neuralfilament 160 | Neural cells | Filament | 1:40 |
| Pax6 | Progenitor cells | Nuclear protein | 1:300 |
| Recoverin | Rod cells | Cytoplasmic protein | 1:200 |
| S-opsin | Cone cells | Membrane protein | 1:1000 |
| Rhodopsin | Rod cells | Membrane protein | 1:500 |
| Vimantin | Muller cells | Cytoskeletal protein | 1:400 |

*Table 3.2: List of antibodies used in the present study*

## 3.9.SyBR green Real time Polymerase chain Reaction:

RNA extraction for the SyBR green real time PCR was performed as mentioned in the RNA isolation protocol above.

### 3.9.1.First strand cDNA synthesis:

| | | |
|---|---|---|
| 2x RT Reaction mix | : | 10.0µl |
| RT Enzyme mix | : | 2.0µl |
| RNA | : | Up to 1.0µg |
| DEPC treated $H_2O$ | : | Made up to 20µl |

***Amplification Profile:***

| | | |
|---|---|---|
| 25$^{o}$C | : | 10mins |
| 50$^{o}$C | : | 30mins |
| 85$^{o}$C | : | 5mins |
| Chill on ice, add 1.0µl (2U) of E. coli RNAse H | | |
| 37$^{o}$C | : | 20mins |
| Store at -20$^{o}$C until use | | |

### 3.9.2.Second strand synthesis:

| | | |
|---|---|---|
| SyBR Green PCR Supermix | : | 25.0 µl |
| Forward Primer (10µM) | : | 1.0µl |
| Reverse Primer (10µM) | : | 1.0µl |
| ROX dye (optional) | : | 1.0µl/0.1µl |
| First strand cDNA template | : | 5.0–10.0µl |
| RNasefree  water | : | Made up to 50µl |

***Amplification Profile:***

| | | |
|---|---|---|
| UDG Incubation | : | 50$^{o}$C – 2mins hold |
| Initial Denaturation | : | 95$^{o}$C – 10mins hold |

| | | | |
|---|---|---|---|
| Denaturation | : | 95ºC – 30sec | 40 cycles |
| Annealing | : | *60ºC – 60sec | |

*temperature varies according to the target primer sequences/sets.

| | | |
|---|---|---|
| Melt curve | : | 95ºC – 15sec |
| | | 60ºC – 1min |
| | | 95ºC – 15sec |

**3.10. RT$^2$ q-PCR:**

- RT$^2$ Profiler PCR Arrays are known to be reliable tools for analyzing the expression of a focused panel of genes.
- Each 96-well plate, PCR array includes SYBR® Green-optimized primer assays for a panel of relevant, pathway- or disease-focused genes.
- RT$^2$ Profiler PCR Arrays contain primer designs that can be amplified simultaneously under uniform cycling conditions.
- This provides the RT$^2$ Profiler PCR Array with the specificity and the high amplification efficiencies required for accurate real-time SYBR® Green results.
- RT$^2$ Profiler PCR Arrays are sensitive enough for use with RNA prepared from regular samples (0.1–5 µg RNA), FFPE samples, and small samples (1–100ng RNA).
- The mRNA levels of 84 genes each associated with Stem cell signaling were examined in CPE-NS with (test) and without SAPNS (control) and extracellular matrix and cell adhesion molecules were examined simultaneously in CPE-DC with (test) and without SAPNs (control) using human RT$^2$ profiler arrays according to the manufacturer's instructions.
- Total RNA (250ng) was reverse transcribed using the First Strand Synthesis Kit and cDNA was subjected to Real-time PCR using SYBR green/ROX Master Mix on a 7500 Real-Time PCR System (Applied Biosystems, USA).

- Array data were analyzed using $RT^2$ Profiler PCR Array Data Analysis software; SA Biosciences.
- For each condition, three biological replicates were analyzed.

**3.11. Glutamate receptor activity:**

- Cells were plated on coverslips, placed in open perfusion chamber, and perfused on the stage of an inverse, fixed-stage microscope with an Oxygenated solution.
- Experiments were performed at room temperature.
- Cells were incubated for 45 minutes in Fluo-3AM (10 µM) plus nonionic detergent (10 µM - Pluronic F127).
- After loading, cells were rinsed (1 mL/min) for 30 minutes by perfusion with the bathing solution.
- Test solutions were applied by bath perfusion as mentioned in results.
- Cells were viewed through a 20x objective (1.0 numerical aperture [NA]; Ziess) and fluorescence changes were monitored with a cooled charge-coupled device (CCD) camera.
- Images were acquired every 5 to 10 seconds, using 2 x 2 binning with 0.5-1 second acquisition times. For measuring the fluorescence changes with time, images were processed using Ziess AxioVision software V4.8.
- Fluorescence intensity before (Fb') and after addition of the test solutions (Ft') were determined. Background fluorescence (an area without any cells) was subtracted from the values of Ft' and Fb' for each cell to get the Normalized fluorescence intensity before (Fb) and Normalized fluorescence intensity on addition of test solution (Ft).
- The intensity values for the calcium flux were determined as follows $\Delta F/F = (Ft - Fb)/Fb$ for every time point. The values of $\Delta F/F$ were obtained for every cell and plotted.

## 3.12. Synaptic vesicle - Endocytosis and Exocytosis:

***Preparation of Dye:***

- FM-143 dye was prepared by diluting in deionized distilled water to make stock solution (1mg/ml). This solution can be stored for several months 2-8ºC.

***Preparation of synapse for labelling:***

- The cells were cultured for 2-3 weeks before labelling. The cultures used for the imaging should be clear and without any debris.
- In the culture preparation recurrent activity was blocked for the all the following steps with specific antagonist CNQX.

***Staining Procedure:***

- The perfusion chamber/apparatus was prepared by purging air from the tubing with appropriate saline solution. The flow rate was set to 2-3ml/min.
- Vacuum line was connected to remove buffer from imaging chamber during perfusion.
- The cells were loaded into the unheated open imaging chamber of the perfusion apparatus. The excess saline solution was gently aspirated using a pasteur pipette leaving some saline in the chamber to avoid drying of the cells.

***Dye loading:***

- A working concentration of the FM-143 dye was added to the saline solution for the particular preparation.
- (Addition of the dye will label exposed membranes immediately)

- Synaptic vesicle exocytosis was stimulated using nerve stimulation by a depolarizing solution. (High potassium – typically 50-100mM). (This process typically labels all terminals from many neurons)
- The time required for stimulation varies depending on the preparation used and typically 1-5mins.
- The nerve terminals are allowed to recover in the presence of the dye to allow for complete endocytosis of all released vesicles. This recovery procedure may require upto 10-15mins.
- 1mM ADVASEP-7 was applied to the washing solution, for reducing the time duration (hours to minutes) and reduce background fluorescence.

***Washing:***

- The extracellular FM 143 dye was washed away by exchanging the solution multiple times with fresh saline solution. This will remove excess dye in exposed membranes.
- Washing was continued for at least 15 min before imaging to minimize background fluorescence and to allow all endocytosed membrane to reform into release-competent vesicles.

***Imaging:***

- The sample was placed under a fluorescence microscope and optics were set to fluorescein (FM -143).
- Total fluorescence levels were imaged to quantify the relative number of synaptic vesicles endocytosed during the stimulation and recovery periods.

***Dye Unloading:***

- To image vesicle release, this was started by collecting several control images. The synaptic vesicle exocytosis was stimulated while imaging. As vesicles containing dye exocytose, dye releases into extracellular space and quickly

washes away. (Any loss in fluorescence measured during stimulation is indicative of the rate and amount of synaptic vesicle exocytosis.

- The nerve terminal was thoroughly stimulated (either prolonged nerve stimulation or repeated high potassium applications) to unload completely all releasable FM 143 dye.
- Dye remaining after this step was be used as the terminal background level (This background level is attributed to non-vesicular fluorescence as well as non-releasing vesicles).

***Analysis:***

- The amount of fluorescence was quantified in each terminal for each time point.
- Using image analysis software (Image J was used, open source freeware; http://rsb.info.nih.gov/ij/), the nerve terminal was outlined and fluorescence intensity was recorded inside the region.
- The fluorescence was measured in the image background or non-synaptic area of the image.
- The background is attributed to non-terminal FM dye, auto fluorescence or image detector settings. This value was subtracted from the terminal fluorescence for each time point. The resulting background-subtracted fluorescence reflects the amount of vesicle release.
- To measure any loss of fluorescence as a result of imaging, the fluorescence in the control images taken before stimulating was used. Any measurable loss is likely due to photobleaching of the terminal.
- If loss is occurring before stimulation, it is likely occurring during stimulation as well. Therefore, the decline in fluorescence during destaining needs to be corrected to account for photobleaching.

## 3.13. Affymetrix gene expression analysis:

### 3.13.1. Preparation of RNA for Gene expression analysis:

The best quality and yield of RNA is obtained by Trizol method followed by Qiagen RNeasy column purification.

***Harvest of RNA:***

- The fresh tissue/frozen tissue/Cell lines (Pellet) were transferred into 1ml of TRIzol reagent per 50-100mg of tissue/cell pellet.
- The samples were homogenized until they are uniformly homogeneous.
- The homogenized samples are incubated at room temperature for 5mins.
- 0.2ml of chloroform per 1ml of TRIzol Reagent was added. The tubes were shaken vigorously by hand for 15mins and incubated at room temperature for 3mins.
- The samples were centrifuged at 12,000xg for 15mins at 4$^{o}$C.
- The aqueous phase was transferred to a fresh tube.
- 0.5ml of Isopropyl alcohol was added per 1ml of TRIzol reagent used initially.
- The samples were incubated at Room temperature for 10mins and centrifuged at 12,000xg for 10mins at 4$^{o}$C.
- The pellets were washed with 1ml of 75% ethanol per 1ml TRIzol used initially at 12,000xg for 10mins at 4$^{o}$C.
- The ethanol was air dried and the RNA was dissolved in RNase-free water completely at a concentration not less than 1µg/µl (estimated from the pellet size).
- RNA yield was measured by OD using NanoDrop.

***Qiagen column clean-up:***

- The elution buffer was preheated at 60-65$^{o}$C.
- Aliquot of 100µg RNA was taken in a 1.5ml tube.

- The volume was adjusted to 100µl with RNase-free water.
- 350µl of RLT buffer (pre added 3.5µl of β-ME to it) was added to the sample and mixed thoroughly.
- 250µl of (96-100%) ethanol was added to the sample tubes, mixed well by pipetting.
- The 700µl of the sample was applied to a RNeasy mini spin column sitting on a 2ml collection tube. Centrifuge for 30sec at 10,000rpm.
- Reapply the sample from the flow-through onto the column. Centrifuged again for 30sec at 10,000rpm.
- The column was transferred into a new 2ml collection tube.
- 500µl of RPE buffer was added and let it stand for 1min. Centrifuge for 30sec at 10,000rpm and discard the flow-through.
- Another 500µl of RPE buffer was added to the column and let stand for 1min. Centrifuge for 30sec at 10,000rpm and discard the flow-through.
- Centrifuge at a maximum speed for another 3min.
- The column was transferred into a new 1.5ml collection tube, 30µl of pre-heated RNase-free water was added directly onto the membrane. Let it stand for 1min at Room temperature.
- The tubes were centrifuged at maximum speed for 1min. The elution was repeated again using 10µl of RNase-free water.
- RNA yield was measured by OD using NanoDrop.

### 3.13.2. Qualitative and Quantitative analysis of RNA:

The standard method for qualitative and quantitative analysis of RNA samples prior to analysis on microarray systems is through 2100 Bioanalyzer system which enables rapid, accurate and reproducible analysis of the quality and composition of RNA.

The RNA analysis was carried out using Agilent RNA 6000 Nano Kit, the sample preparation and analysis was carried out as per manufacturer's instruction. (Kit insert: part no. G2938-90034, 08/2006).

### 3.13.3. Gene chip Whole Transcript (WT) Target labeling Assay, Hybridization and scanning:

The Affymetrix Gene chip Whole transcript Target labeling assay, Hybridization and scanning is performed to generate amplified and biotinylated sense strand DNA targets from the entire expressed genome without bias. The entire protocol is performed as per the manual supplied by the Manufacturer. (Part no.701880 Rev. 5).

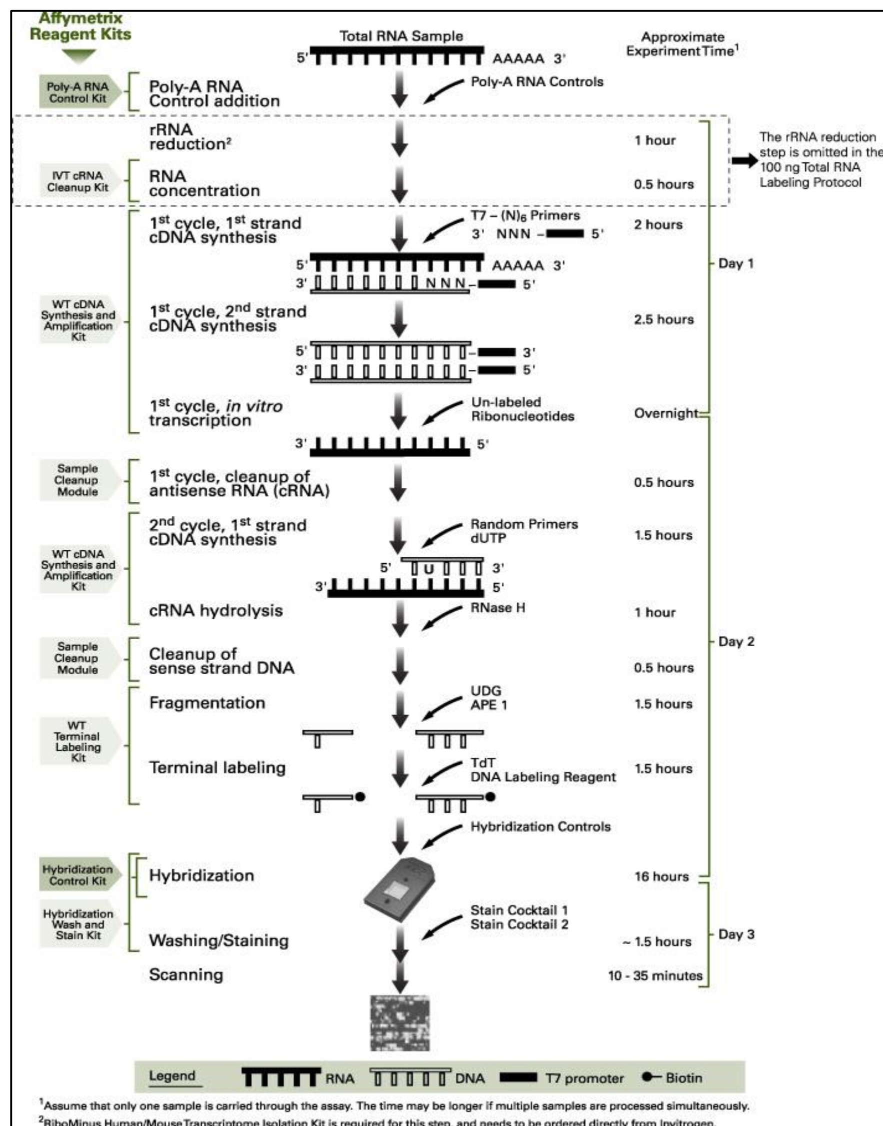



*Figure 3.1: Overview of Gene expression microarray (Adapted from manufacturers booklet Part no.701880 Rev. 5)*

### 3.13.4. Statistical Analysis of the Affymetrix gene expression data:

The intensity values of different probe sets (genes) generated by Affymetrix GCOS were imported into Gene Spring GX version 11.5 software (Agilent Technologies, Inc., Santa Clara, CA) for data analysis. Raw data summarization and normalization was done using Gene Spring GX v 11.5 from Agilent Technologies, USA. Summarization was done using GCRMA algorithm and Quantile normalization was done. Further baseline transformation of samples in each batch was done to the respective control samples (Iris / Ciliary samples were considered as control for all other conditions for each batch). Considering inherent batch to batch and biological variation, probes which were minimum 2 fold up or down regulated in each condition were qualified as differentially regulated genes. P-value was derived based on Student T-test and Benjamin Hocheberg FDR test for each of the differentially regulated gene across the biological replicates. Differentially regulated genes with a p-value of less than or equal to 0.05 were considered for further functional analysis. Functional analysis of differentially regulated genes based on Gene Ontology categories was done using Gene Spring GX v 11.0. Categories that were statistically significant with a p-value of less than 0.05 were considered as significantly differentially regulated. The working gene list was submitted to hierarchical clustering to identify samples with similar patterns of gene expression. The smooth correlation for distance measure algorithm (Gene Spring) was used.

## 3.14. DNA Methylation Microarray (MeDIP-on-Chip):

### 3.14.1. DNA Extraction: (QIAamp DNA Mini Kit)

- The cells were harvested from the cultures and collected in a vial.
- The harvested cells were centrifuged at 10000rpm for 5-10 minutes and the supernatant is discarded using a pipette.
- 200µl of AL buffer and 20µl of proteinase K was added to the pellet. Incubated at 60oC for 10mins.
- 200µl of ethanol was added to the samples and mixed well for 15sec by vortexing.

- The entire sample was transferred to column sitting on a 2ml collection tube and centrifuged at 8000rpm for 1min.
- The column was transferred to a new collection tube and 750µl of AW1 buffer was added. Centrifuged at 8000rpm for 1min.
- The column was transferred to a new collection tube and 500µl of AW2 buffer was added. Centrifuged at 14000rpm for 3min.
- The flow through was discarded and empty column was spin at 14000rpm for 1min.
- 100µl of AE buffer was added to the column sitting on a new 2ml collection tube and incubated at room temperature for 5mins. Centrifuge at 8000rpm for 1min.
- The collected DNA sample was stored at -20$^{o}$C.
- The isolated DNA was measured using standard NanoDrop spectrophotometer.

### 3.14.2. Methylated DNA Immunoprecipitation (MeDIP), Sample labelling, Hybridization and Scanning**:

Immunoprecipitation of the Methylated DNA using 5-Methylcytosine antibody, Sample labelling, Hybridization and Scanning was performed as per Agilent Microarray Analysis of Methylated DNA Immunoprecipitation Protocol Part No. G4170-90012 Version 1.1, May 2010.

### 3.14.3. Statistical Analysis of the MeDIP-on-Chip data:

MeDIP-on--Chip data were analyzed using Agilent's DNA Analytics 1.3 software. Raw data was obtained by Agilent Feature extraction software. Median blank subtraction and Intra-array lowess normalization was performed. Peaks were detected using Whitehead Per-array Neighborhood Model v1.0. Maximum distance for two probes to be considered as neighbors is 50bp, a probe is considered "bound" if P(Xbar)<0.05 and central probe has P(X)<0.05 and at least one neighboring probe has P(X)<0.05. To achieve quantitative analysis of epigenetic modifications, total signal intensities of epigenetic marks were calculated as follows: 1) All negative intensity values were considered as zero. 2) All

intensity values including promoter and gene body of each gene were summarized. 3) Genes with total intensities above 0.4 were defined as marked genes. Mapping of bound probes was performed using human genome HG19 Feb. 2009.

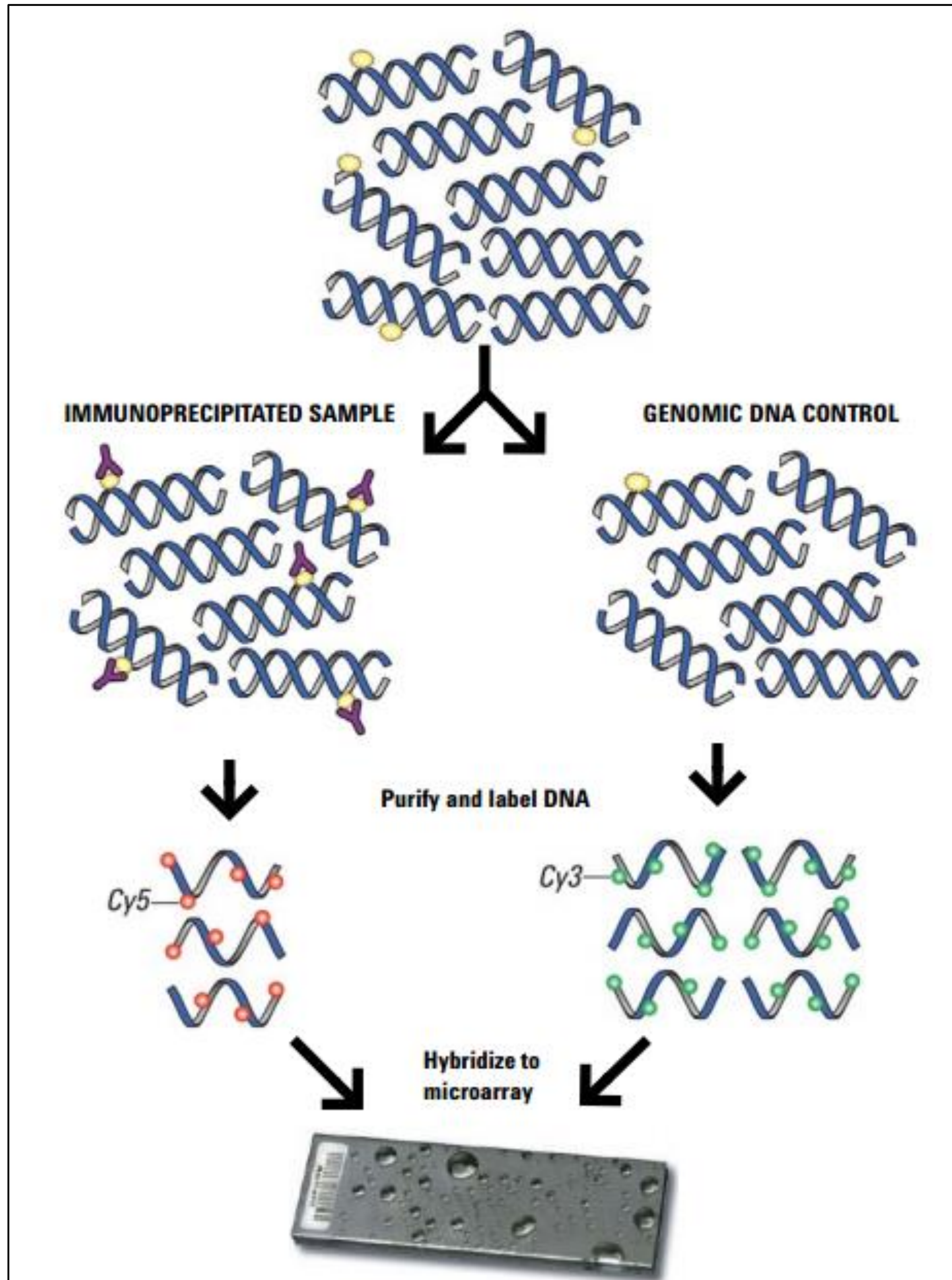



*Figure 3.2: Overview of MeDIP-on-Chip Microarray (Adapted from manufacturer's booklet Part No. G4170-90012 Version 1.1, May 2010.)*

## 3.15. Chromatin Methylation Microarray (ChIP-on-Chip):

### 3.15.1. Prepare the cells and cross-link proteins to DNA:

5 x $10^7$ to 1 x $10^8$ cells were used for each immunoprecipitation.

***CPE derived neurospheres:***

- Fresh 11% Formaldehyde solution was added directly to 1/10 of cell culture media volume in the flasks.
- Flasks were swirl briefly and let them sit at room temperature for 20 minutes.
- Add 1/20 of the cell culture volume of 2.5 M glycine to flasks to quench the formaldehyde.
- Spin down the cells at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor.
- Resuspend the pellets in 50 mL 1X PBS, spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor. Discard supernatant. Repeat once.
- Resuspend in 10 mL of 1x PBS per 108 cells. Transfer 1 x $10^8$ cells to 15 mL conical tubes and spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor. Discard the supernatant.
- If the cells were not used immediately, they were flash freeze in liquid nitrogen and stored the pellets at –80°C.

***CPE neurosphere derived differentiated cells:***

- Fresh 11% Formaldehyde solution was added directly to 1/10 of cell culture media volume in the flasks. Formaldehyde solution was added directly to culture media or to PBS.
- The plates or flasks were swirled briefly and left at room temperature for 10 minutes.
- 1/20 volume of 2.5 M glycine was added to plates or flasks to quench the formaldehyde.

- The cells were rinsed with 5 mL 1X PBS. Another 5 mL of 1X PBS was added to the flask and the cells were harvested using a silicone scraper.
- The cells were poured into the required number of 50 mL conical tubes and spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor. The supernatant was discarded.
- The pellet was resuspended in 10 mL 1X PBS per $10^8$ cells. Transfer 5 x $10^7$ to 1 x $10^8$ cells to 15 mL conical tubes and spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor. The supernatant was discarded.
- If the cells were not used immediately, they were flash freeze in liquid nitrogen and stored the pellets at –80°C.

### 3.15.2. Immunoprecipitation of Chromatin (ChIP), Sample labelling, Hybridization and Scanning**:

Immunoprecipitation of the Chromatin bound Methylated DNA using H3K4me3, H3K27me3 and RNA Pol II antibodies, Sample labelling, Hybridization and Scanning was performed as per Agilent Mammalian ChIP-on-Chip Protocol Part No. G4481-90010 Version10.2, March 2011.

### 3.15.3. Statistical Analysis of the ChIP-on-Chip data:

ChIP-chip data were analyzed using Agilent's DNA Analytics 1.3 software. Raw data was obtained by Agilent Feature extraction software. Median blank subtraction and Intra-array lowess normalization was performed. Peaks were detected using Whitehead Per-array Neighborhood Model v1.0. Maximum distance for two probes to be considered as neighbors is 1000bp, a probe is considered "bound" if $P(Xbar)<0.05$ and central probe has $P(X)<0.05$ and at least one neighboring probe has $P(X)<0.05$. To achieve quantitative analysis of epigenetic modifications, total signal intensities of epigenetic marks were calculated as follows: 1) All negative intensity values were considered as zero. 2) All intensity values including promoter and gene body of each gene were summarized. 3)

Genes with total intensities above 0.4 were defined as marked genes. Mapping of bound probes was performed using human genome HG19 Feb. 2009.

**Note:** ***Part of the work was performed at Genotypic technologies Pvt. Ltd. Bangalore, Karnataka, India.*

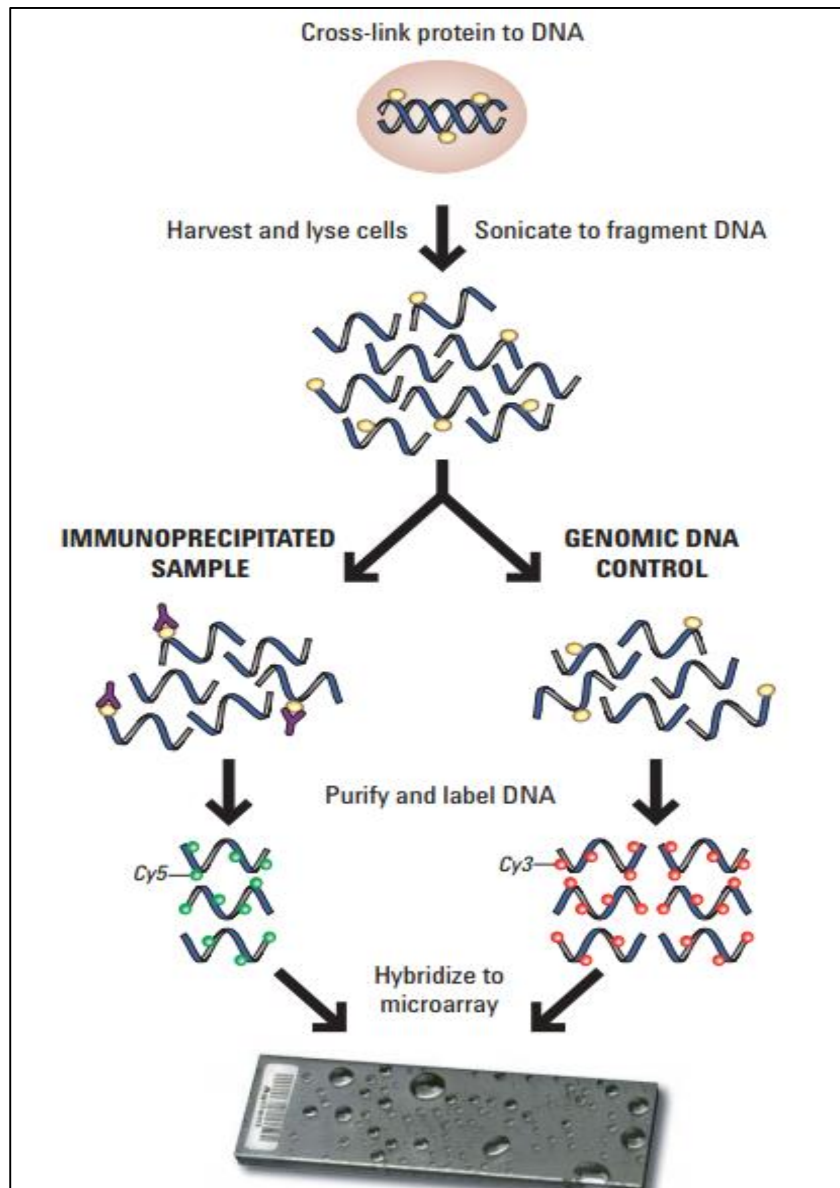



*Figure 3.3: Overview of ChIP-on-Chip Microarray (Adapted from manufacturer's booklet Part No. G4481-90010 Version10.2, March 2011.)*

## 3.16. Transmission Electron Microscopy:

- The cells collected were fixed in primary fixative and left for fixation for 4 to 8hrs at 8ºC.
- The cells were then washed thrice with the Sodium cacodylate buffer for 10mins.
- Cells were then transferred to a secondary fixative and left for 2hrs at 8ºC.
- The excess fixative was washed with the Sodium cacodylate buffer thrice for 10mins.
- The cells were then treated with graded series of acetone 30%, 50%, 70%, 80% and 90% 10mins each and with 100% acetone twice for 10mins, each time followed by propylene oxide treatment twice for 10mins.
- The cells were then infilterated with the resin mixture consisting of Epon 812 resin, DDSA (Dodecenyl Succinic Anhydride) and NMA (Nadic® Methyl Anhydride) starting with 25%, 50%, 75% and finally with 100% resin mixture for 2hrs each.
- They were then embedded using the same resin mixture with added catalyst (DMP 30) in “Easymoulds” at 60ºC for 48hrs.
- The resin blocks were removed from the mould, trimmed and sectioned using Leica Ultracut R Ultramicrotome with a diamond or glass knives.
- Initially semi thin sections were cut which were stained with toluidine blue and screened using the light microscope to check for the area of interest in those sections.
- **Then ultrathin sections were cut, collected on copper grids, stained with saturated solutions of Uranyl acetate followed by lead citrate
- Screened in JEOL JEM 100SX transmission electron microscope at an accelerating voltage of 80kV.

**Note:** ***Part of the work was performed at Cancer Institute, Chennai, Tamilnadu.*

## 3.17. Culturing of CPE cells in Self Assembling peptide nanofibre scaffolds/Puramatrix:

### 3.17.1. Preparation of Peptide Hydrogel/SAPNs:

- The viscosity of the self-assembling peptide nanofibre scaffold (SAPNs) stock solution (1% w/v) was decreased by vortexing for 30 minutes every time the stock is used. If air bubbles are present, the aliquot was centrifuged.
- The appropriate volume of SAPNs was prepared in a microtube by diluting the stock with sterile 20% sucrose to generate a 2X concentration of the scaffold in 10% sucrose. A total volume of 100µl per 24-well cell culture insert (i.e., 50µl of 2X scaffold in 10% sucrose and 50µl of cells at 2X in 10% sucrose).
- SAPNs, prepare a stock of 0.30% Scaffold in 10% sucrose (for 1ml, combine 500µl of 20% sucrose, 300µl Scaffold, and 200 µl of sterile $H_2O$).
- Mix by gentle pipetting.

| Cell culture Plate | Growth Area ($cm^2$) | Volume Per Well (µl) |
|---|---|---|
| 6-well | 9.60 | 1200 |
| 24-well | 2.00 | 250 |
| 96-well | 0.32 | 50 |

### 3.17.2. 3D Cell Encapsulation in Cell Culture Plates:

- SAPNs/cell/sucrose mixture was added to the center of the well carefully, without introducing bubbles.
- The gelation of the SAPNs was initiated by gently running culture media down the side of the well on top of the hydrogel.
- This was repeated until cells have been plated in all wells.
- The media was changed very gently two times over the next one hour to further equilibrate the pH of the hydrogel.

### 3.17.3. Immunostaining for cells cultured in Puramatrix:

- For immunocytochemistry, CPE-NS and CPE-DC cells along with the SAPNs were fixed with 4% paraformaldehyde for 30min.
- The cells were then incubated for 24 h in blocking buffer.
- Subsequently the cells were incubated for 16-18hrs at 4ºC with the primary antibody dissolved in blocking buffer.
- After washing the cells 4-6 times for 3hrs, the secondary antibody (FITC/TRITC conjugated), dissolved in blocking buffer was added, and incubated for 8hrs at 4ºC in the dark.
- The samples were washed 4–6 times for 3hrs followed by cell-nuclei labeling with DAPI, 100ng/mL in PBS for 2hrs at room temperature.
- Subsequently, matrices were mounted with prolonged gold Antifade.
- For analyzing the immunofluorescence labeling, pictures were taken with a Zeiss Axiobserver.

### 3.17.4. Cell Proliferation (BrdU labeling) Assay for cells cultured in Puramatrix:

- Cell proliferation assay for the cultured neurospheres encapsulated in various concentrations of SAPNs was assessed by measuring BrdU incorporation during DNA synthesis in proliferating cultured cells.
- The BrdU labelling was performed according to manufacturer's instruction with slight modifications.
- Briefly the cells were incubated with BrdU (10 microM) for 24 hr, followed by an incubation for 48 hr (Day 7).
- The samples were fixed with 4% paraformaldehyde solution for 30 min at room temperature, washed with 1x PBS, and were incubated for 24 h in blocking buffer, followed by anti-BrdU antibody solution in incubation buffer (1:10 dilutions) for 4hrs at 37°C.
- After incubation, samples were washed three times with washing buffer for 3hrs.

- The samples were then incubated for 2hr at 37°C in dark with a secondary antibody conjugated with FITC, washed three times with washing buffer and then incubated for 1hr with DAPI.
- The samples were placed on a microscope slide for imaging and the pictures were captured with a Zeiss Axiobserver.

# CHAPTER 4: ISOLATION AND CHARACTERIZATION OF STEM/PROGENITOR CELLS ISOLATED FROM THE ADULT IRIS AND CILIARY BODY OF HUMAN CADAVERIC EYE

## 4.1.Introduction:

The Iris and Ciliary body of the human eye are of the neuro-ectodermal derivative like retina which could be a good source for SCs isolation (Mohamed Abdouh & Gilbert Bernier, 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* (Hanako Ikeda et al., 2005) (Deepak A. Lamba et al., 2006) (Jason S. Meyera et al., 2009). Recent *in vitro* studies have demonstrated the presence of the multipotent SCs in the CPE using neurosphere culture from adult mice, rats, rabbits, porcine and humans (Ahmad I et al., 2000) (Tropepe V et al., 2000) (Yuji Inoue et al., 2005) (MacNeil A et al., 2007) (Fischer, A.J. et al., 2003) (Brenda L. K. Coles et al., 2004) (Moe MC et al., 2009). Likewise SCs were also demonstrated from the IPE cells of chicken (Maki Asami et al., 2007), adult rodents and primates (TadamichiAkagi et al., 2005), non-human mammals (Masatoshi Haruta et al., 2001) and also from humans (Frøen RC et al., 2011). Although their functions and roles *in vivo* remain unknown, the SCs proliferate *in vitro* and differentiate into cells expressing markers of certain mature retinal neurons, such as bipolar cells, photoreceptor cells, raising the possibility that these cells can be used as a potential cell source in transplantation therapy for retinal diseases (T.A. Reh & E.M. Levine, 1998) (MacLaren R.E. et al., 2006). However, the potential of isolated SCs from IPE and CPE of the human cadaveric eyes remain unexplored.

Much of the knowledge available about the neural SCs comes from studies carried out *in vitro* from the adult CNS of rodents (Reynolds and Weiss, 1992) and humans (Kukekov et al., 1999; Palmer et al., 2001). But most of the previous works are performed with SCs obtained from subventricular zone and other animals, leaving questions about SCs derived from other regions of humans. In the previous experiments performed on SCs isolated from rodents (Reynolds and Weiss, 1992), the

cells were allowed to proliferate in the presence of mitogens. After incubation for a couple of days these cells produced aggregates (of cells) characterized as "neurospheres". The cells present within these neurospheres express Nestin, an intermediate filament protein characteristically present in progenitor cells of diverse tissues (Reynolds and Weiss, 1992; Seaberg et al., 2004; Wiese et al., 2004). These neurospheres could be passaged and differentiated into neurons and glial cells when the mitogens were withdrawn from the cultures. Side-lining the argument of *in vitro* artifact, these cells could generate neurons upon transplantation. Because of the easy experimental access and defined cell culture conditions, manipulation of adult SCs *in vitro* allow the analysis of both intrinsic and extrinsic mechanisms that regulate the various steps involved in neurogenesis.

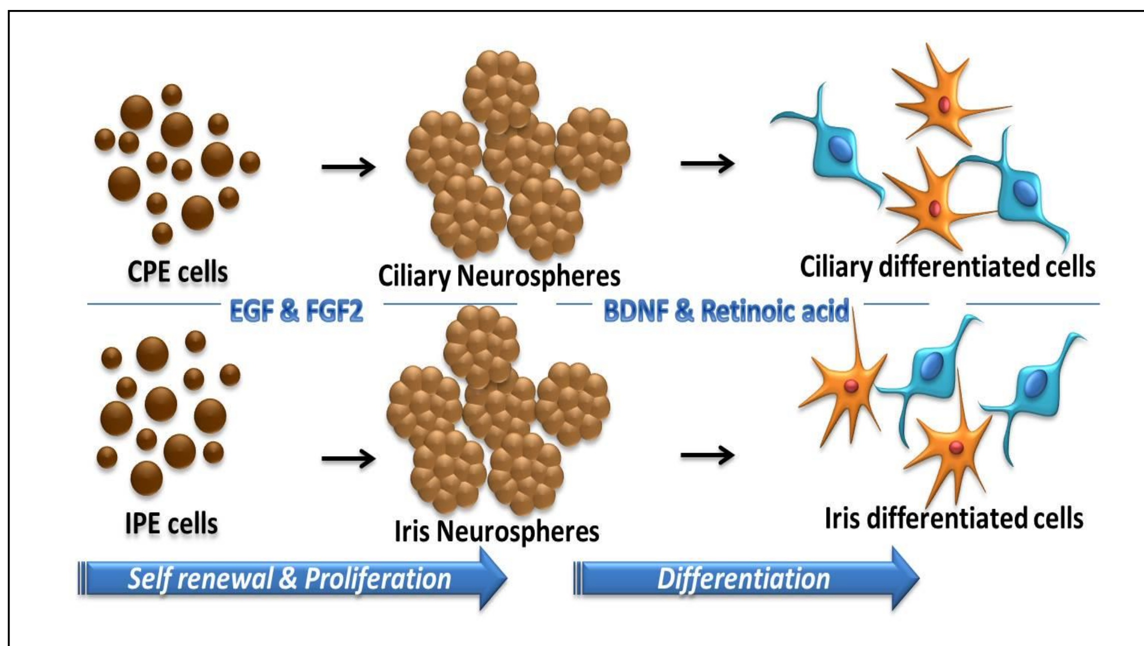



*Figure 4.1: A Schematic representation of methodology for the culturing of SCs in vitro. The desired region from which the neural precursor cells are to be cultured is enzymatically digested and plated in mitogen containing serum free medium. The precursor cells proliferate and populate the culture dish.*

The neurospheres generated in the cultures harbours several hundreds to thousands of cells that are highly proliferative. In the absence of any attachment factors the cells remain in the sphere form and the cellular interaction within the sphere is heterogenous (Reynolds and Rietze, 2005). The inner most cells of a sphere do not come in contact

with the culture medium and the fate of these cells is likely to be dependent on the factors secreted by the adjacent cells.

Studies have shown that cells within neurospheres have certain characteristics of SCs like self-renewal in the presence of appropriate mitogens, clonal expansion and ability of producing major cell types: neurons, astrocytes and oligodendrocytes (Tropepe V et. al., 2000) (Yuji Inoue et. al., 2005) (MacNeil A et. al., 2007) (Fischer, A.J. 2003). Several proteins have been characterized that are restricted in their expression pattern to SCs. Molecular markers are the vital elements in the identifying and characterizing SCs along with the cell differentiation and fate commitment, as defined sets of markers are needed to assign cells to specific types and their hierarchical positions.

Due to the limitations in the availability of these cells, functional characterization of the SCs have been restricted to *ex vivo* method, by bringing the cells to a tissue culture environment and induced to differentiation with specific growth factors *(Figure 4.1).* Upon plating the Pigment epithelial cells in the presence of mitogens the cells proliferate and the SCs outnumber other cell types. By controlling the plating density, each neurosphere could be made to arise from a single cell. After trypsinization and single cell dissociation, the cells are again plated with mitogens *(Figure 4.2).* Any subsequent formation of secondary neurospheres is indicative of self-renewal of the cells.

Demonstration of self-renewal is a strong indicator of the presence of stem cells in the culture. The neurospheres that arise from a single cell are then differentiated. Special media conductive for differentiation can switch early progenitor cells present in neurosphere cultures to spontaneously undergo differentiation along the cell lineage as a function of time in culture (Brenda L. K. Coles, et al., 2004). The generation of neurons and glial cells from these suggests the presence of multipotent stem cells *(Figure 4.2).* The available studies are not sufficient to explain the mechanisms by which multipotent SCs can differentiate into lineage-specific progenitors or further into retinal neural cell types.

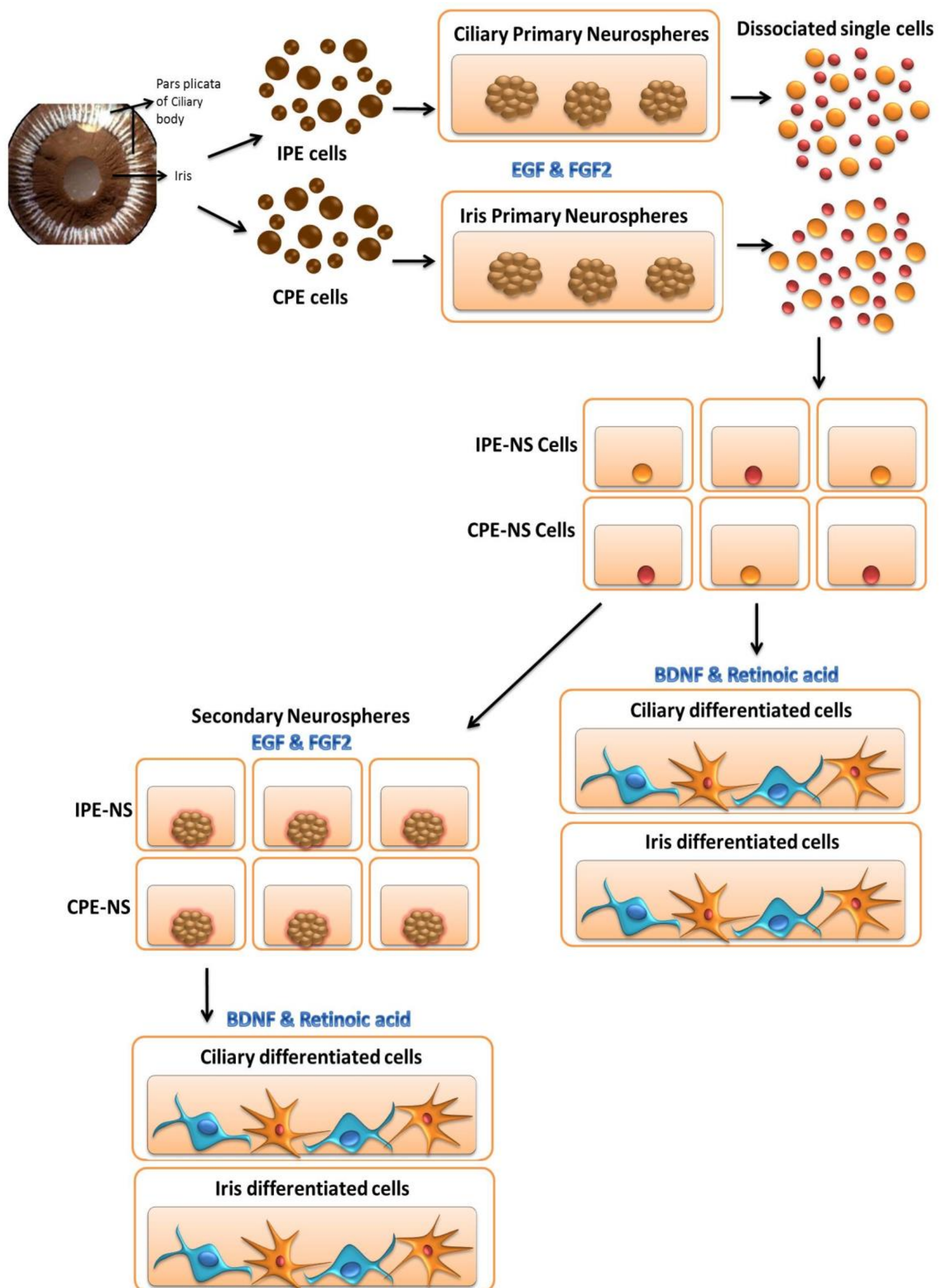



*Figure 4.2: Schematic representation of the assay undertaken to study the self-renewal and multipotentiality of adult IPE and CPE cells.*

In the current study, we explored the changes involved in stem/progenitor cells from IPE and CPE cells to give rise to cells of heterologous lineage of retinal neural cell types. Here, we report the neuronal potential of cells isolated from the IPE and CPE cells of adult eye. We have observed that IPE and CPE cells, when removed from the eye and cultured in the presence of mitogens, begin to express neural progenitor markers. In differentiation conditions these cells express retinal neuronal specific markers suggesting their differentiation along the retinal lineage. Our study, therefore, draws attention toward the necessity for rigorous characterization of IPE and CPE differentiation into retinal lineage and offers a model for characterizing neuronal potential of heterologous stem cells/progenitors that may shed light on their biology as well as on their therapeutic potential.

## 4.2.Materials and methods:

### 4.2.1.Isolation of IPE and CPE cells from human cadaveric eye:

The dissociation and cultures of IPE and CPE were performed as described in methodology.

### 4.2.2. Sphere-formation, expansion and differentiation of IPE and CPE cells:

We employed the sphere formation assay to assess the rate of proliferation at which single dissociated cells could form sphere colonies in the presence of mitogens. For this the isolated single pigmented cells from both the IPE and CPE were plated in 96 well plates at a density of one cell per well in retinal culture medium and confirmed under the microscope. The culture plates were inspected for the presences and absence of spheres after 7 days in culture and the number of spheres generated was counted. Using either sphere suspension or adherent culture conditions the expansion capacity of IPE and CPE cells was assessed.

***Sphere suspension assay:*** After every 7 days of culture in retinal culture medium the spheres formed were counted, collected and digested in 0.02% trypsin-EDTA (Invitrogen) for 15 min at 37$^{o}$C. The cells were then subjected to mechanical trituration after addition of trypsin inhibitor and centrifuged at 1000rpm for 10 min and washed

twice to generate single cells. These cells were re-suspended and plated at a density of $2x10^4$cells/ml in retinal culture medium.

***Adherent/monolayer culture for retinal differentiation potential:*** The dissociated cells were plated at a density of $1x10^6$cells/ml in retinal differentiation medium for adherent/monolayer culture and the culture medium was changed every 2-3 days. The cells were treated with 0.02 % trypsin-EDTA for 10 min at 37°C after reaching 80-90% confluence and collected for further analysis.

### 4.2.3. Clonal analysis of the Neurospheres:

The isolated cells from the ciliary and iris pigment epithelium were counted and seeded into 96 well plates at a dilution of 1 cell per well. At the end of the 7th day the Neurospheres formed in the 96 well plates were counted (Change of medium given every 2nd day). 20 neurospheres were extracted, individually and dissociated by 0.02% trypsin and the resulting single cell suspension from each neurosphere was inoculated into individual wells of a six-well plate at a plating density of one cell per $mm^2$ for analysing the formation of secondary neurospheres.

### 4.2.4. Characterization of stem/progenitor cells:

***Quantitative Characteristics Neurospheres:*** The characteristics and the number of neurospheres were assessed at the end of 7 days in culture. All spheres in the well were counted and the results were expressed as number of neurospheres/50,000 cells.

***Analysing the Proliferative potential of the neurospheres:*** At the end of 6 days *in vitro*, neurospheres were labelled with BrdU (10µM) for 48h. The neurospheres were placed on poly-D-lysine coated glass slides and allowed to settle for 4 hours prior to fixation with 4% Para formaldehyde (PFA) for 20 minutes at room temperature (RT) for BrdU immunocytochemistry. The BrdU labeling was performed according to the kit protocol (Roche diagnostics).

### 4.2.5. Immunofluorescence analysis on the IPE and CPE neurospheres and differentiated cultures:

As described in methodology.

**4.2.6.RNA isolation and RT-PCR:**

As described in methodology.

**4.3. Results:**

**4.3.1.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 *(Figure 4.3 A&B)*.

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 *(Figure 4.3 C&D)*. 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 *(Figure 4.3 E)*. 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 *(Figure 4.4 A-B)*. 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 *(Figure 4.4 C)*. 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.

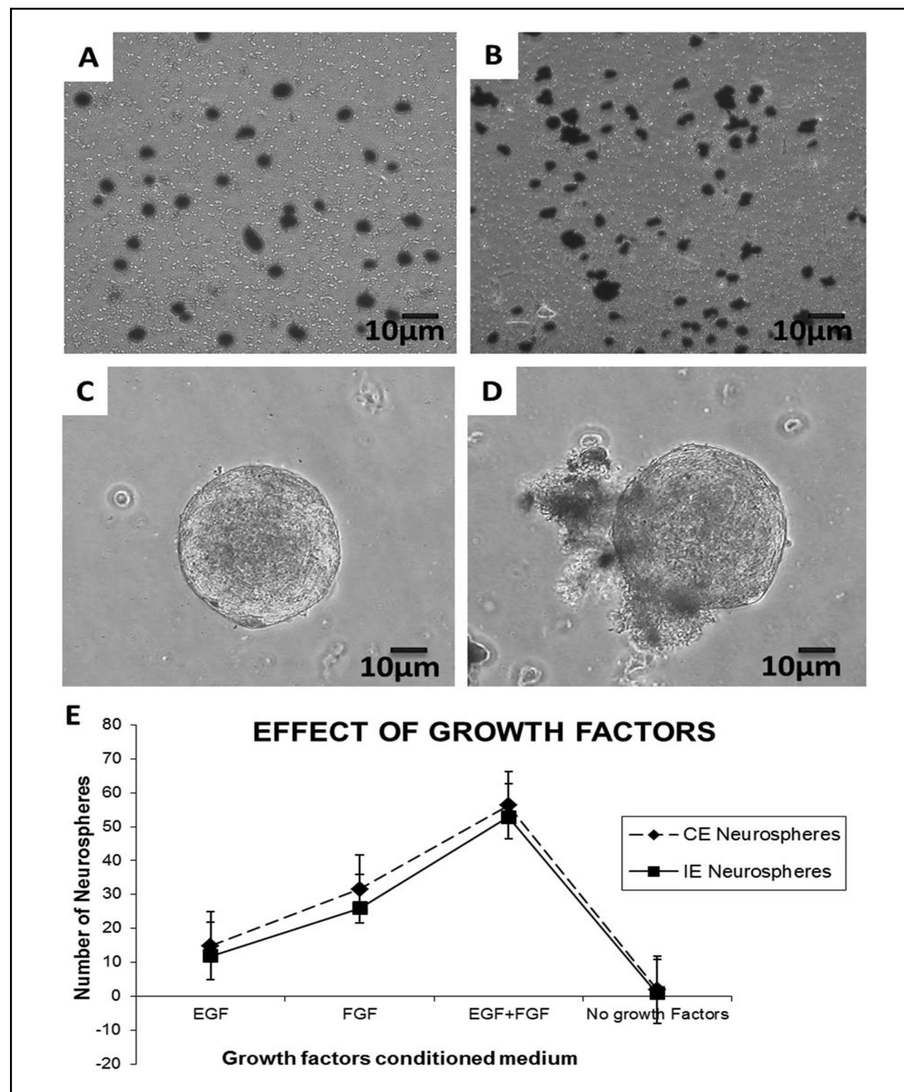



*Figure 4.3: Single dissociated cells isolated from the IPE and CPE of human cadaveric eye when cultured in serum-free medium supplemented with mitogens generated neurospheres. Representative images of dissociated cells of the iris (A) and ciliary body (B) on the day of plating, Representative images of neurospheres generated from dissociated cells of the IPE (C) and CPE (D) after 7 days in tissue culture. Scale bars = 10µm. IPE and CPE stem cells/progenitors display differential ability to generate neurospheres in the presence of EGF, FGF2, and EGF+FGF2 (E). (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

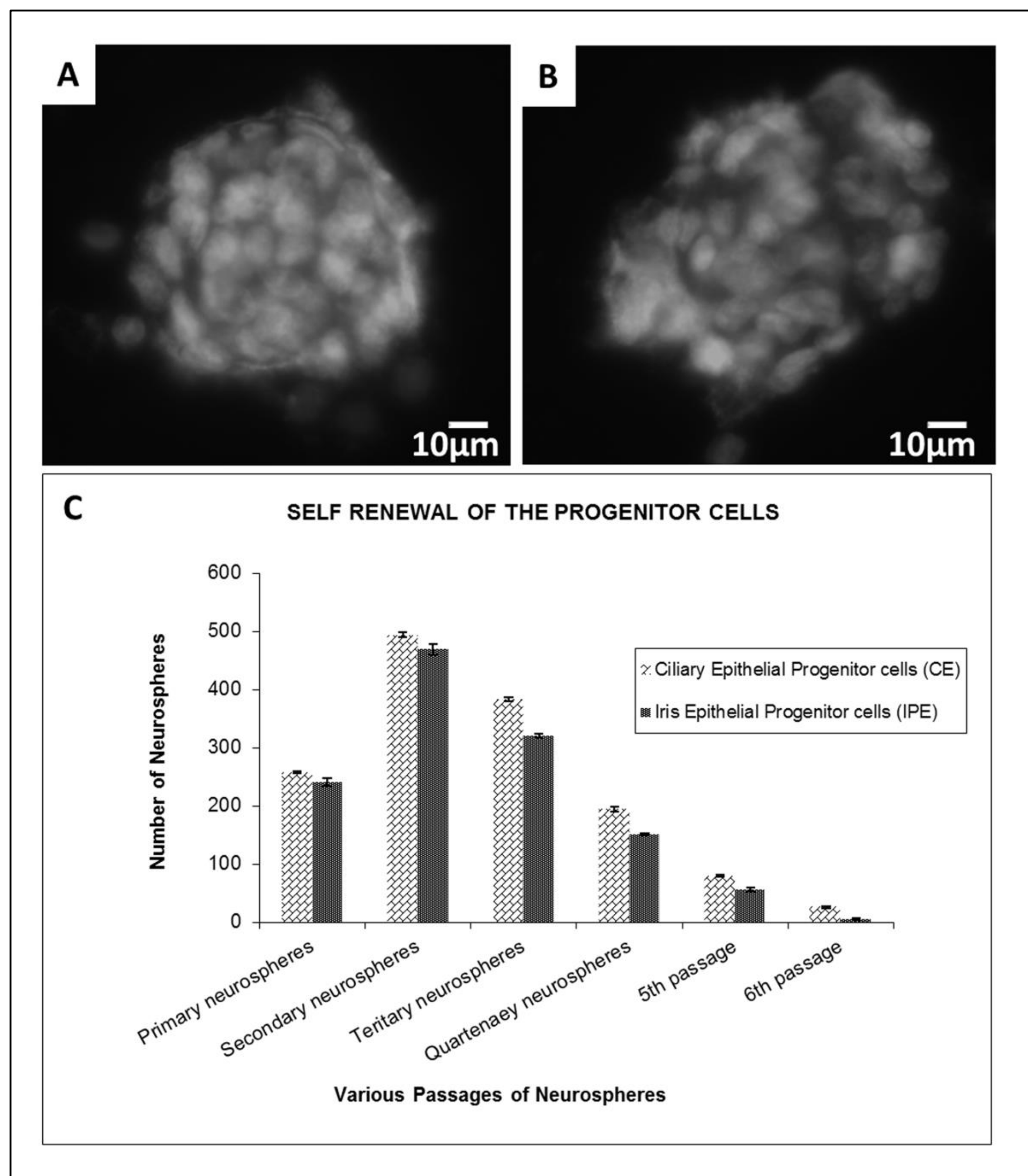



*Figure 4.4: Comparison and expansion of Neurospheres (Self renewal potential) derived from IPE and CPE cells in the presence of mitogens and the proliferative potential. Proliferative potential as indicated by BrdU labeling of the IPE neurosphere (A) and CPE neurosphere (B). Plot of the average total neurosphere number as a function of passage number (N=3). (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

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 (Fig 4.5 A&B). The morphological characteristics of the neurospheres were analysed and the quantitative characteristics were tabulated *(Table 4.1)*.

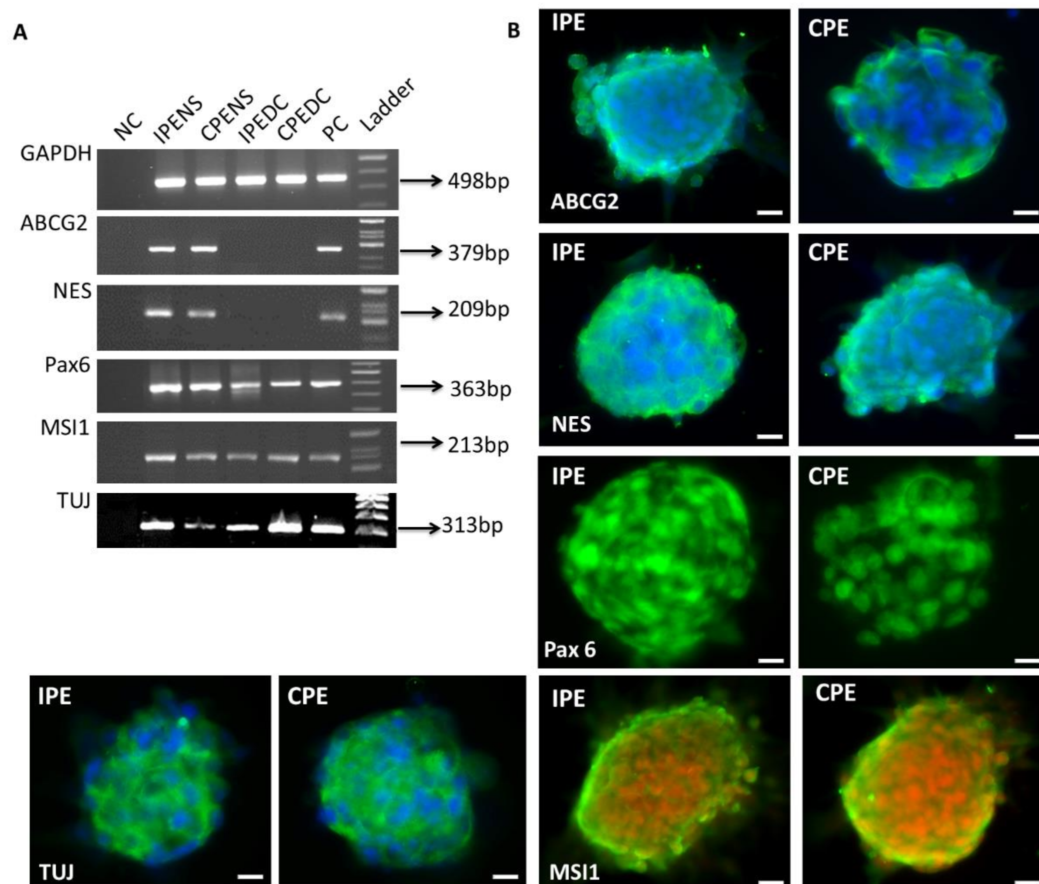



*Figure 4.5: Stem cell potential of neurospheres from IPE and CPE tissues. (A) RT-PCR analysis of the progenitor markers in both the neurosphere and differentiated cells derived from the IPE and CPE cells. (B) Immunofluorescence images of neurospheres stained for the presence of ABCG2, NES, PAX6, β III tubulin and MSI1 expression. Neurospheres were generated from dissociated cells of the IPE and CPE and grown for 7 days in serum free media containing FGF and EGF. Nuclei were labelled with DAPI (for ABCG2, NES PAX6 and β III tubulin) and PI (for MSI1). Scale bars 10µm. (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

| **Characteristics** | **Ciliary Pigment Epithelial cells (CPE)** | **Iris Pigment Epithelial cells (IPE)** |
|---|---|---|
| Cell diameter | 24±0.4 | 26±0.6 |
| NS diameter | 163±19.09 | 269±14.46 |
| Cells isolated per eye | $2.8 \times 10^6$ | $1.8 \times 10^6$ |
| NS-forming cells (%)* | 2.5 | 1.3 |
| Cell number per NS | 511 | 685 |

*Table 4.1: Quantitative Characteristics of Progenitor Cells (Human cadaveric eyes: n=7): Diameters are expressed as mean ± SD.* From clonal analysis experiments of ciliary and iris cells.*

**4.3.2.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 *(Table 4.2)*.

The IPE and CPE derived neurospheres showed negative expression when stained for the epithelial marker like pan-cytokeratin *(Figure 4.6)* indicating the distinct pattern of differentiation. The IPE and CPE derived neurospheres expressed the neural precursor marker Nestin and β III tubulin *(Figure 4.5 B)*.

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 *(Figure 4.7 A&B)*. These results suggest that the isolated and expanded IPE and CPE cells can be induced to differentiate into a retinal neuronal direction.

| Marker | % of cells positive IE NS cells | % of cells positive CE NS cells |
|---|---|---|
| **ABCG2** | 17.4 | 16.3 |
| **PAX6** | 34.7 | 38.7 |
| **NES** | 26.9 | 28.9 |
| **MAS1** | 23.1 | 24.5 |
| **TUJ** | 21.2 | 22.2 |
| **GFAP** | 19.5 | 21.5 |

*Table 4.2: Quantitative Characteristics of Progenitor markers in the neurospheres (Human cadaveric eyes: n=7)*

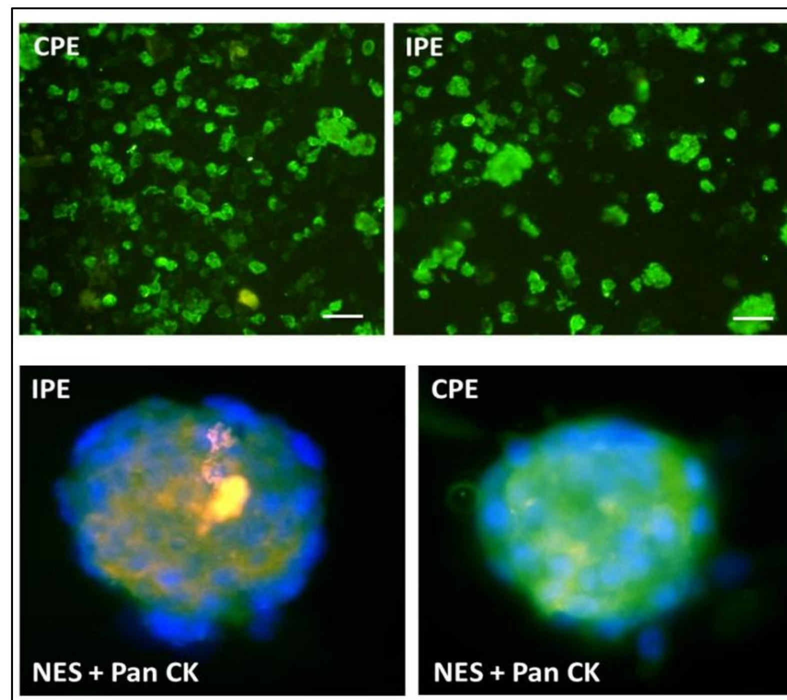



*Figure 4.6: Expression of pan cytokeratin in first day cells of IPE and CPE. Scale bars 10µm.Expression of Pan Cytokeratin and Nestin in the IPE and CPE derived neurospheres. Neurospheres were generated from dissociated cells of the IPE and CPE and grown for 7 days in serum free media containing FGF and EGF. Nuclei were labelled with DAPI, Pan Cytokeratin labelled with TRITC (Red) and Nestin labelled with FITC (Green). (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

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 unpigmentedmelanosomes 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 *(Figure 4.8)* 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.

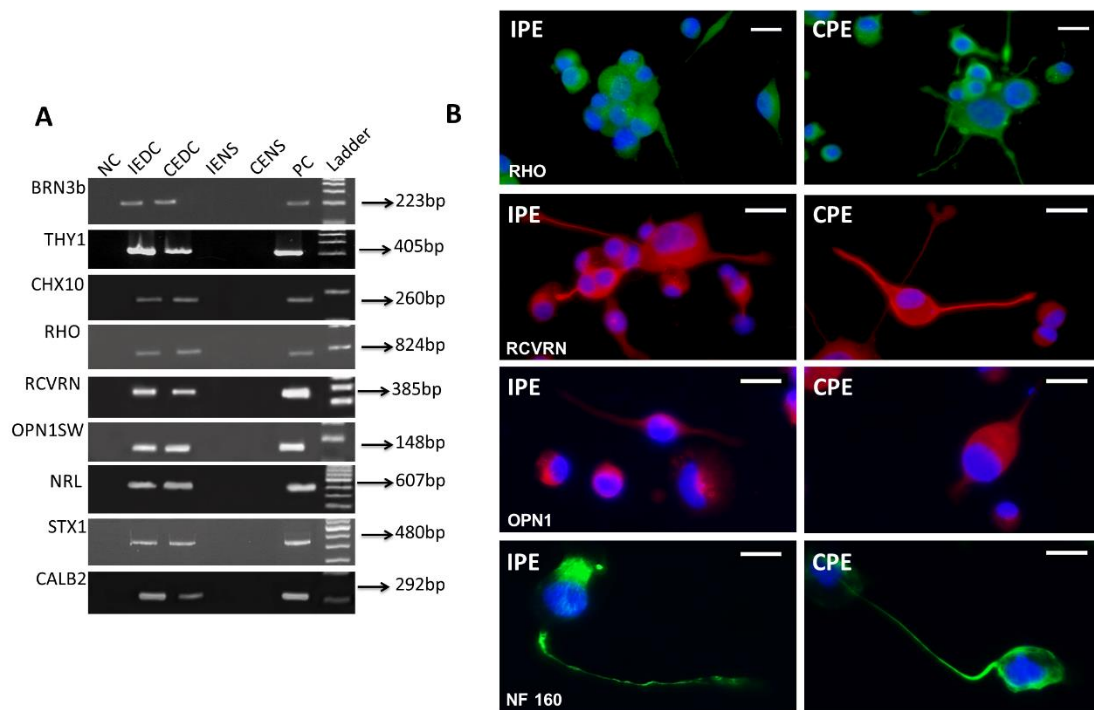



*Figure 4.7: Differentiation potential of IPE and CPE derived neurospheres. Primary neurospheres from IPE and CPE derived neurospheres were dissociated and cultured on laminin-coated tissue culture plates under differentiation conditions for 21 days. The dissociated cells expand to form a monolayer which was analysed by RT-PCR for the retinal cell markers like Brn3b, Thy1, Chx10, Rhodopsin, Recoverin, s-Opsin, Nrl and Syntaxin1, Calretinin and immuno- stained for the presence of Rhodopsin, s-Opsin, Recoverin, and Neural filament (NF) 160. Nuclei were labelled with DAPI. Light microscope images show neuronal morphology of differentiated cells from each region. Scale bar = 100μm. (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

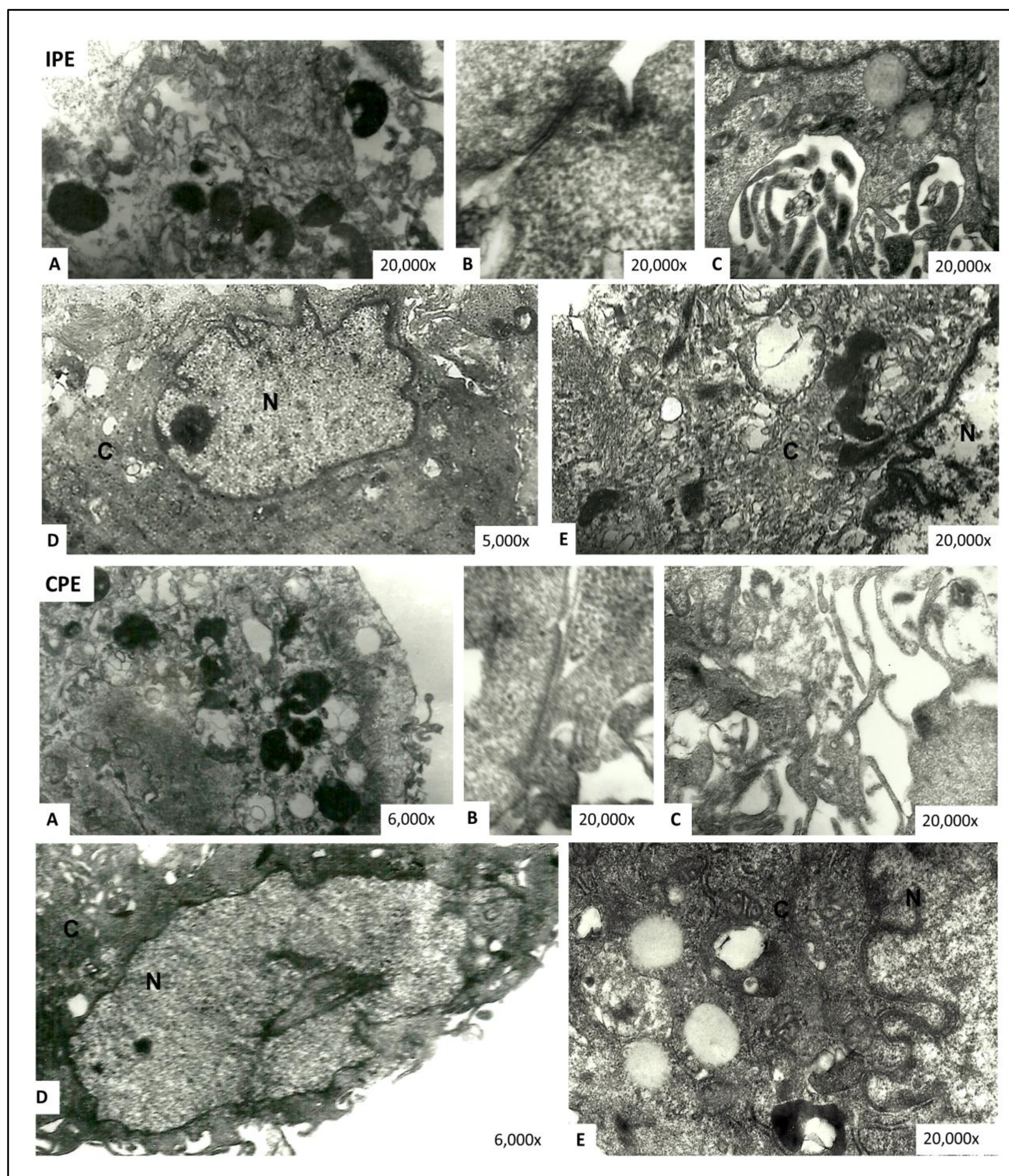



*Figure 4.8: Transmission electron micrographs of adult human iris pigment epithelial cells (IPE)/Ciliary pigment epithelial cells (CPE) derived Neurospheres and differentiated cells. (A) Neurosphere cell showing pigments, (B) Cells within the neurospheres showing adherent-like junction, (C) Differentiated cells showing Intermediate filaments (D) Differentiated cells without any pigments (E) Differentiated cells without any pigments higher magnification. N; nucleolus and C; cytoplasm. (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

## 4.4. Conclusion:

In conclusion, our results shown that the CPE and IPE regions of the human cadaveric eye could be cultured and yield cells that are capable of significant expansion *in vitro* indicating that the CPE and IPE of human cadaveric eye contains stem/progenitor cells. A progenitor enriched population was obtained by neurosphere assay indicating the presence of neural stem/progenitor cell characteristics. These sphere colonies could be differentiated into retinal cell phonotype *in vitro*.

# CHAPTER 5: GENE EXPRESSION PROFILES OF STEM/PROGENITOR CELLS DERIVED FROM HUMAN IRIS AND CILIARY PIGMENT EPITHELIUM AND THE RETINAL NEURONAL POPULATION

## 5.1.Introduction:

Microarray experiments are used to quantify and compare gene expression on a large scale. Microarray-based gene profiling has become an important technique to measure changes in gene expression on a genome-wide scale. DNA microarrays comprise different types of probes: oligonucleotides, complementary DNA (cDNA), or genomic regions. In case of a cDNA microarray, thousands of PCR-amplified cDNAs can be spotted and immobilized onto a glass slide (Schena et al., 1995). The RNA of interest (target) is reverse-transcribed into cDNA, while incorporating either fluorescent molecules or a tag that is later bound to a fluorescent molecule. These targets are hybridized to the microarray, and the fluorescence at each spot is detected using a laser scanner. To compare gene expression levels from two RNA samples, they are co-hybridized to a single microarray and detected with different fluorescent dyes. Since every sequence has distinct hybridization characteristics, this method does not provide an absolute measurement for the presence of a given RNA molecule. Instead, it compares the co-hybridized sample pools and determines the relative abundance for each RNA species (Duggan et al., 1999; Butte, 2002).

A cDNA microarray can be composed of a few, well-known clones for the analysis of isolated biological systems. It can also contain thousands of clones to cover as much of the genome as possible. Such large arrays are used for screening studies, when little is known about the molecular biology of the examined system. They allow the simultaneous identification of thousands of expression patterns, effectively generating a snapshot of transcriptome differences between the two compared samples. The transcriptome is the entirety of all RNA molecules (transcripts) in a cell at a particular time point. The targets of interest are usually derived from cells or tissues, and comparisons are frequently drawn between disease versus control samples, treated versus untreated samples, or different biological states. Whereas most experimental

setups are static, time course experiments permit observation of gene expression changes over time (Yang and Speed, 2002).

Very little is known about the molecular processes that govern the proliferation and migration of SCs derived from IPE & CPE and their differentiation into specific cell types. A more thorough understanding of the molecular biology of these cells and their niche is of crucial importance. To shed more light on these molecular mechanisms and to discover the genes involved in these processes, IPE and CPE derived SCs should be cultured, expanded, and differentiated *in vitro*, providing a model system for their behavior in situ.

The gene expression status of IPE and CPE derived SCs and of differentiated cells derived from these were to be compared by means of cDNA microarray analysis. In the present chapter this approach should identify many genes involved in the biological processes linked to the maintenance and differentiation of these cells. Special attention was given to genes that take part in the regulation of cellular fate by participating in signal transduction, and also in genes that control the stemness and differentiation into retinal cell phenotype. Therefore, this chapter aimed at identifying candidate genes and proteins involved in the functioning of adult IPE and CPE derived SCs.

## 5.2.Materials and methods:

### 5.2.1.RNA isolation and RT PCR

Following trypsinization of the neurospheres and the differentiated cells, total RNA was extracted using RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions.

### 5.2.2.Microarray analysis:

Neurospheres were generated as described in methodology, and their stem cell properties were determined by RT-PCR or Immunostaining before collecting for the microarray analysis. RNA from the neurospheres and the differentiated cells was isolated using RNeasy mini Kit and the RNA was quantified using NanoDrop and the quality of RNA was analysed using Agilent RNA Nano chip in Bioanalyzer as mentioned in methodology. Samples having RNA integrity number (RIN) value above

8 was used for further processing. All the microarray steps were performed as mentioned in methodology.

**5.2.3.Statistical Analysis of the Microarray Data:**

As described in methodology.

**5.2.4.Verification of the Microarray data with real time PCR:**

Quantitative PCR was carried out by adding 5 µl of template cDNA to a final 25 µl reaction of 100 picomoles of each forward and reverse primer, and 2.5 µl of Syber Green master mix (Qiagen). Real-time PCR was performed using the 7500 instrument (Applied Biosystems) as described in methodology.

**5.3.Results:**

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

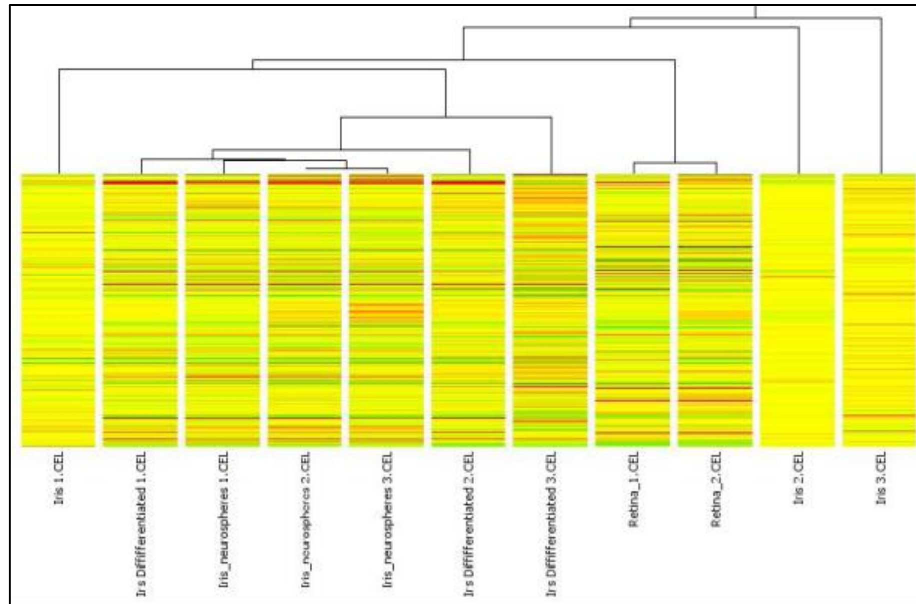
To understand the underlying mechanism of distinct proliferation and differentiation potential of stem/progenitor cells, we carried out gene expression profiling of these cells in proliferating and differentiating conditions using microarray analysis. Analysis of Average Difference (AD) values for the 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 *(Figure 5.1A and B).*

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 as described in Materials and Methods. The cluster analysis *(Figure: 5.2)* 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.*(Table 5.1).*

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).

**(A) Hierarchical Condition Tree – IPE-NS and IPE-DC**




**Hierarchical Condition Tree – CPE-NS and CPE-DC**

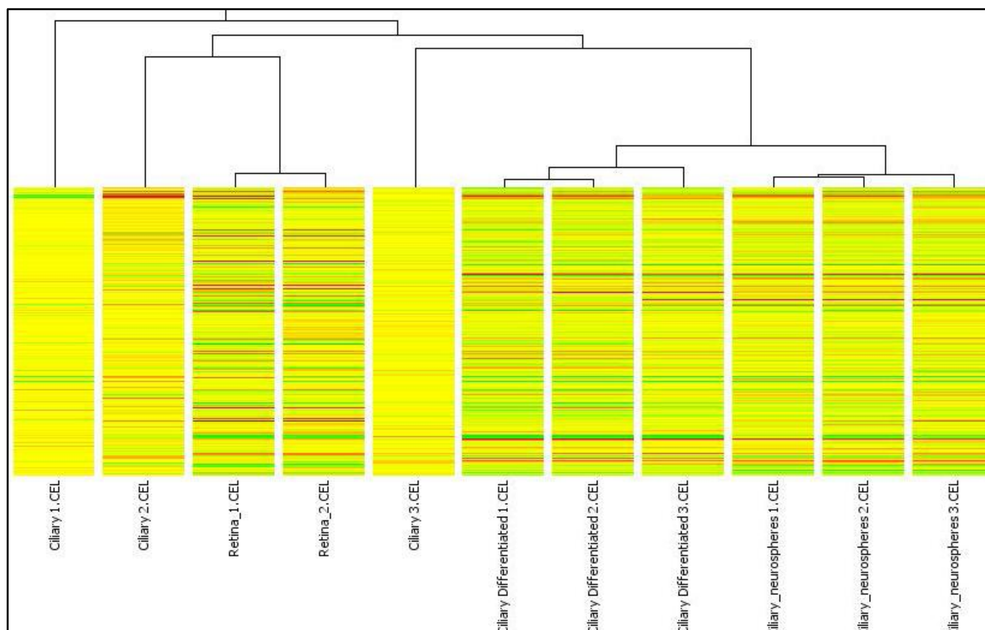
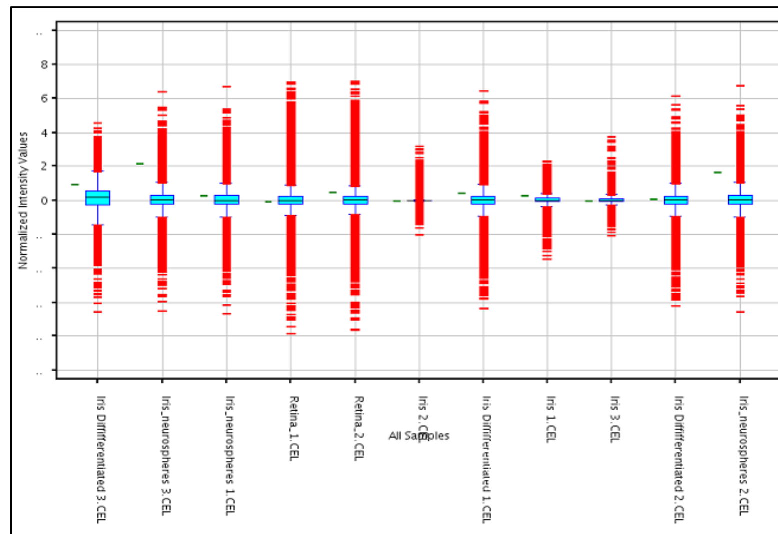



*Figure 5.1: (A) Hierarchical Condition Tree of the relative expression level of the IPE and CPE derived neurospheres and differentiated cells where IPE and CPE cell are represented as controls.*

**(B) Box Whisker Plot – IPE-NS and IPE-DC**




**Box Whisker Plot – CPE-NS and CPE-DC**

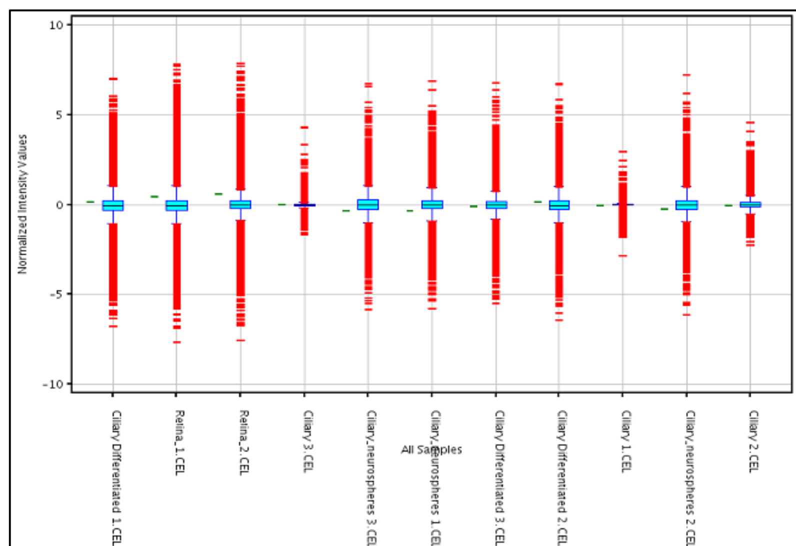
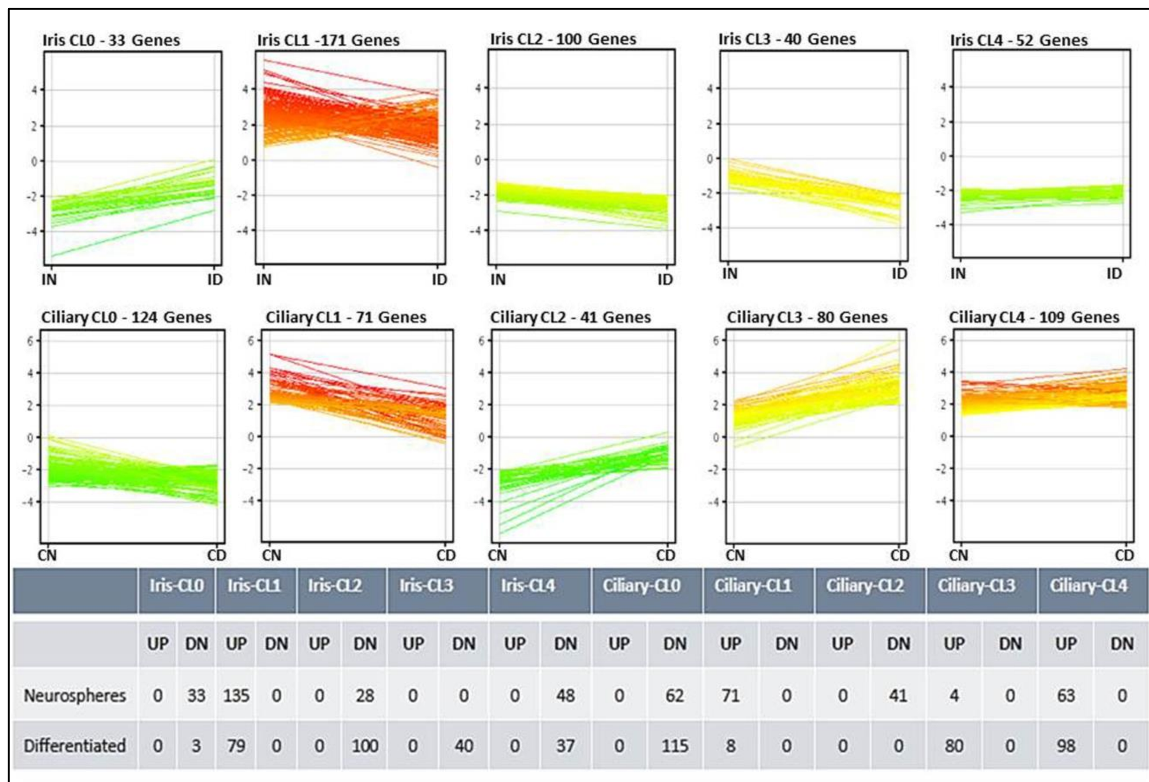



*Figure 5.1: (B) Box and Whisker plot of the relative expression level of the IPE and CPE derived neurospheres and differentiated cells where IPE and CPE cell are represented as controls. (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

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).




| | Iris-CL0 | | Iris-CL1 | | Iris-CL2 | | Iris-CL3 | | Iris-CL4 | | Ciliary-CL0 | | Ciliary-CL1 | | Ciliary-CL2 | | Ciliary-CL3 | | Ciliary-CL4 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UP | DN | UP | DN | UP | DN | UP | DN | UP | DN | UP | DN | UP | DN | UP | DN | UP | DN | UP | DN |
| Neurospheres | 0 | 33 | 135 | 0 | 0 | 28 | 0 | 0 | 0 | 48 | 0 | 62 | 71 | 0 | 0 | 41 | 4 | 0 | 63 | 0 |
| Differentiated | 0 | 3 | 79 | 0 | 0 | 100 | 0 | 40 | 0 | 37 | 0 | 115 | 8 | 0 | 0 | 0 | 80 | 0 | 98 | 0 |

*Figure 5.2: The dynamic behavior of the genes that are consistently upregulated or downregulated in both the IPE NS-DC and the CPE NS-DC are clustered K-mean analysis and shown separately according to the genes with similar expression changes between Proliferating conditions (Neurospheres) and differentiating conditions (Differentiated cells). For each series, 5 clusters were found. Clusters IrisCL0- IrisCL4 contains upregulated & downregulated genes of IPE derived neurospheres and differentiated cells; clusters CiliaryCL0–CL4 contain upregulated &downregulated genes of CPE derived neurospheres and differentiated cells. The x-axis depicts the NS and DC points. The y-axis shows relative fold changes; that is, expression changes. These relative numbers are estimated logarithmic fold changes and therefore not identical to the estimated fold changes. (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

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.

| Gene Symbol | Gene Description | Cluster | CPENS p-value | CPEDC p-value | CPENS (FC) | CPEDC (FC) | IPENS p-value | IPEDC p-value | IPENS (FC) | IPEDC (FC) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cell Adhesion proteins** | | | | | | | | | | |
| **CDH13** | cadherin 13, H-cadherin | CL4 | 0.00 | 0.03 | 2.33 | 2.11 | 0.02 | 0.02 | 1.20 | 0.28 |
| **CNTNAP3** | contactin associated protein-like 3 | CL2 | 0.00 | 0.01 | -2.22 | -1.37 | 0.00 | 0.05 | -1.43 | -1.28 |
| **PKP2** | plakophilin 2 | CL2 | 0.01 | 0.01 | -1.13 | -1.60 | 0.05 | 0.05 | -1.70 | -3.14 |
| **ICAM1** | intercellular adhesion molecule 1 (CD54), | CL4 | 0.00 | 0.04 | 2.15 | 2.02 | 0.01 | 0.02 | 1.11 | 1.00 |
| **CNTN1** | contactin 1 | CL0 | 0.01 | 0.01 | -1.93 | -2.92 | 0.00 | 0.05 | -3.35 | -4.30 |
| **CNTN4** | contactin 4 | CL0 | 0.04 | 0.02 | -1.66 | -2.85 | 0.02 | 0.10 | -1.36 | -1.74 |
| **NRCAM** | neuronal cell adhesion molecule | CL1 | 0.00 | 0.05 | 2.37 | 1.23 | 0.05 | 0.03 | 0.42 | -0.24 |
| **Extracellular matrix components and cell–matrix interaction** | | | | | | | | | | |
| **ADAMTS12** | ADAM metallopeptidase with thrombospondin type 1 motif, 12 | CL3 | 0.05 | 0.01 | 0.89 | 2.66 | 0.01 | 0.03 | 1.56 | 1.86 |
| **COL1A2** | collagen, type I, alpha 2 | CL4 | 0.00 | 0.00 | 1.34 | 2.25 | 0.01 | 0.04 | 0.80 | 0.33 |
| **COL3A1** | collagen, type III, alpha 1 | CL3 | 0.01 | 0.02 | 0.86 | 3.64 | 0.04 | 0.04 | -0.56 | -0.43 |
| **C1QC** | complement component 1, q subcomponent, C chain | CL0 | 0.05 | 0.00 | -1.64 | -3.09 | 0.02 | 0.05 | -1.04 | -0.94 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **SPON1** | spondin 1, extracellular matrx protein | CL0, CL3 | 0.00 | 0.00 | -2.13 | -4.19 | 0.03 | 0.01 | -1.46 | -2.85 |
| **CHL1** | cell adhesion molecule with homology to L1CAM | CL2 | 0.00 | 0.06 | -1.31 | -2.56 | 0.01 | 0.04 | -2.29 | -2.77 |
| **CXCL5** | chemokine (C-X-C motif) ligand 5 | CL1 | 0.02 | 0.03 | 2.74 | 0.93 | 0.02 | 0.03 | 1.87 | 0.80 |
| **CXCL6** | chemokine (C-X-C motif) ligand 6 | CL1 | 0.01 | 0.05 | 3.26 | 2.29 | 0.02 | 0.05 | 1.39 | 2.00 |
| **IL8** | interleukin 8 | CL1 | 0.00 | 0.05 | 4.28 | 2.03 | 0.03 | 0.05 | 3.12 | 2.76 |
| **CCL20** | chemokine (C-C motif) ligand 20 | CL1 | 0.01 | 0.50 | 2.88 | -0.09 | 0.03 | 0.05 | 2.42 | 1.40 |
| **IL1A** | interleukin 1, alpha | CL1 | 0.00 | 0.01 | 2.50 | 1.37 | 0.05 | 0.01 | 1.89 | 1.16 |
| **IL1B** | interleukin 1, beta | CL1 | 0.02 | 0.05 | 2.21 | 1.96 | 0.05 | 0.02 | 1.93 | 2.75 |
| **IL33** | interleukin 33 | CL1 | 0.01 | 0.05 | 2.30 | 0.20 | 0.05 | 0.01 | 0.57 | -1.36 |
| **IL6** | interleukin 6 | CL1 | 0.00 | 0.05 | 5.14 | 3.01 | 0.05 | 0.04 | 1.99 | -0.67 |
| **MMP1** | matrix metallopeptidase 1 | CL1 | 0.00 | 0.03 | 4.15 | 1.93 | 0.01 | 0.03 | 5.14 | 1.58 |
| **MMP16** | matrix metallopeptidase 16 | CL4 | 0.04 | 0.01 | 1.77 | 2.70 | 0.00 | 0.04 | 1.38 | 0.89 |
| **MMP2** | matrix metallopeptidase 2 | CL4 | 0.02 | 0.00 | 2.45 | 4.06 | 0.03 | 0.04 | 1.46 | 0.96 |
| **MMP3** | matrix metallopeptidase 3 | CL1 | 0.01 | 0.04 | 4.30 | 1.47 | 0.01 | 0.04 | 5.03 | 1.22 |
| **TREM1** | triggering receptor expressed on myeloid cells 1 | CL1 | 0.00 | 0.04 | 5.16 | 1.39 | 0.01 | 0.02 | 3.62 | 0.74 |
| **TNC** | Tenascin C | CL1 | 0.01 | 0.03 | 3.44 | 2.84 | 0.01 | 0.03 | 3.20 | 1.56 |

| **Cytoskeleton** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **ACTN1** | actinin, alpha 1 | CL4 | 0.00 | 0.00 | 1.98 | 2.67 | 0.01 | 0.05 | 1.32 | 0.48 |
| **ARHGAP11A** | Rho GTPase activating protein 11A | CL3, CL1 | 0.03 | 0.01 | 1.54 | 2.94 | 0.01 | 0.02 | 2.84 | 1.61 |
| **ARHGAP20** | Rho GTPase activating protein 20 | CL2 | 0.03 | 0.23 | -0.58 | 0.71 | 0.03 | 0.04 | -1.84 | -2.48 |
| **ARHGAP28** | Rho GTPase activating protein 28 | CL4 | 0.01 | 0.01 | -1.32 | -1.26 | 0.00 | 0.02 | -2.28 | -2.24 |
| **DYNC1I1** | dynein, cytoplasmic 1, intermediate chain 1 | CL2 | 0.01 | 0.01 | -1.99 | -2.01 | 0.02 | 0.05 | -2.20 | -2.72 |
| **GSN** | gelsolin | CL2 | 0.01 | 0.00 | -1.30 | -1.51 | 0.01 | 0.04 | -2.12 | -2.50 |
| **S100A11** | S100 calcium binding protein A11 | CL4 | 0.00 | 0.00 | 1.87 | 2.24 | 0.03 | 0.04 | 1.01 | 0.49 |
| **S100A16** | S100 calcium binding protein A16 | CL1 | 0.00 | 0.01 | 2.69 | 2.41 | 0.00 | 0.03 | 2.69 | 1.65 |
| **TAGLN** | transgelin | CL3 | 0.05 | 0.00 | 0.93 | 2.63 | 0.02 | 0.05 | -1.28 | -1.22 |
| **TUBA1C** | tubulin, alpha 1c | CL4 | 0.00 | 0.01 | 2.65 | 2.35 | 0.00 | 0.03 | 2.15 | 1.26 |
| Cell proliferation | | | | | | | | | | |
| **CDC20** | cell division cycle 20 homolog | CL4,CL1 | 0.01 | 0.01 | 2.26 | 3.03 | 0.03 | 0.03 | 2.69 | 0.93 |
| **CCNB1** | cyclin B1 | CL4,CL1 | 0.01 | 0.00 | 2.33 | 3.47 | 0.01 | 0.04 | 3.05 | 1.42 |
| **CCNB2** | cyclin B2 | CL4,CL1 | 0.01 | 0.01 | 2.26 | 3.41 | 0.02 | 0.03 | 2.83 | 1.57 |
| **KIF23** | kinesin family member 23 | CL1 | 0.01 | 0.01 | 2.49 | 4.11 | 0.01 | 0.03 | 2.61 | 1.54 |
| **PBK** | PDZ binding kinase | CL4,CL1 | 0.02 | 0.01 | 2.53 | 3.78 | 0.02 | 0.03 | 3.27 | 2.02 |

| **Signal transduction** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **CAMK1G** | calcium/calmodulin-dependent protein kinase IG | CL4 | - | - | - | - | 0.00 | 0.05 | -2.33 | -2.06 |
| **IGFBP3** | insulin-like growth factor binding protein 3 | CL1 | 0.00 | 0.00 | 4.58 | 4.02 | 0.01 | 0.03 | 3.89 | 1.86 |
| **NR1D1** | nuclear receptor subfamily 1, group D, member 1 | CL2 | 0.00 | 0.00 | -2.01 | -1.67 | 0.01 | 0.03 | -2.01 | -2.46 |
| **THRA** | thyroid hormone receptor, alpha | CL2 | 0.00 | 0.00 | -2.11 | -1.53 | 0.00 | 0.01 | -2.23 | -1.66 |
| **NR4A2** | nuclear receptor subfamily 4, group A, member 2 | CL2 | 0.01 | 0.05 | 0.23 | -0.21 | 0.01 | 0.01 | -1.47 | -2.23 |
| **ENPP1** | phosphodiesterase 1 | CL3 | 0.02 | 0.04 | -0.78 | -0.60 | 0.02 | 0.05 | -0.96 | -2.54 |
| **ENPP2** | phosphodiesterase 2 | CL0 | 0.00 | 0.01 | -2.13 | -2.00 | 0.02 | 0.02 | -0.78 | -1.62 |
| **RGS4** | regulator of G-protein signaling 4 | CL3 | 0.02 | 0.00 | 1.28 | 3.52 | 0.01 | 0.06 | -1.35 | -0.62 |
| **TRIP13** | thyroid hormone receptor interactor 13 | CL4 | 0.01 | 0.01 | 2.04 | 2.73 | 0.02 | 0.05 | 2.02 | 0.74 |
| **Chromatin-associated components and nuclear factors** | | | | | | | | | | |
| **KPNA2** | karyopherin alpha 2 | CL4,CL1 | 0.01 | 0.00 | 2.14 | 3.13 | 0.00 | 0.03 | 2.17 | 1.20 |
| **SNRPN** | small nuclear ribonucleoprotein polypeptide N | CL2,CL0 | 0.02 | 0.02 | -0.94 | -0.97 | 0.00 | 0.03 | -1.87 | -1.39 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Metabolism** | | | | | | | | | | |
| **CA9** | carbonic anhydrase IX | CL1 | 0.00 | 0.05 | 2.17 | 0.71 | - | - | - | - |
| **PTGIS** | prostaglandin I2 (prostacyclin) synthase | CL3 | 0.02 | 0.01 | 0.86 | 3.60 | 0.04 | 0.05 | -0.65 | -0.34 |
| **Ion channels–transporters, lipoproteins, and steroids** | | | | | | | | | | |
| **CLUL1** | clusterin-like 1 (retinal) | CL4 | 0.00 | 0.01 | -1.83 | -2.29 | 0.00 | 0.05 | -2.19 | -2.07 |
| **SORL1** | sortilin-related receptor, L(DLR class) A repeats-containing | CL0,CL3 | 0.00 | 0.00 | -1.98 | -2.36 | 0.01 | 0.02 | -0.91 | -2.25 |
| **Cell type-specific changes** | | | | | | | | | | |
| **PLP1** | proteolipid protein 1 | CL2 | 0.04 | 0.05 | 1.15 | -0.37 | 0.01 | 0.05 | -1.96 | -2.68 |
| **PRKCA** | protein kinase C, alpha | CL3 | 0.00 | 0.01 | 1.30 | 2.70 | 0.05 | 0.05 | 0.39 | 0.59 |
| **NCALD** | neurocalcin delta | CL0 | 0.01 | 0.00 | -1.77 | -3.82 | 0.05 | 0.03 | -1.08 | -1.44 |

*Table 5.1: Cluster Analysis differentially expressed genes of the IPE and CPE derived neurospheres and differentiated cells. FC- Fold Change.*

### 5.3.2.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. The Genes involved in these pathways are listed according to their fold change *(Table 5.2)*

| Gene symbol | IPE-NS (FC) | P-value | CPE-NS (FC) | P-value |
|---|---|---|---|---|
| **NOTCH SIGNALING** | | | | |
| ADAM17 | 1.3 | 0.00 | 1.8 | 0.00 |
| CNTN1 | -3.3 | 0.00 | -1.9 | 0.01 |
| DNER | 2.4 | 0.01 | 1.1 | 0.00 |
| JAG1 | 1.2 | 0.00 | 1.6 | 0.02 |
| WDR12 | 1.2 | 0.00 | 1.0 | 0.03 |
| **WNT SIGNLAING** | | | | |
| CALCOCO1 | -1.8 | 0.01 | -1.4 | 0.01 |
| CCND1 | 1.4 | 0.02 | 2.7 | 0.00 |
| CXXC4 | -1.3 | 0.01 | -2.3 | 0.00 |
| FZD7 | -1.5 | 0.01 | -1.7 | 0.00 |
| LDLR | 1.1 | 0.00 | 2.6 | 0.00 |
| MAPK10 | -1.4 | 0.00 | -1.3 | 0.00 |
| MYC | 1.5 | 0.00 | 2.1 | 0.00 |
| PLAU | 4.8 | 0.00 | 5.2 | 0.00 |
| SFRP5 | -1.1 | 0.01 | -3 | 0.01 |
| SOSTDC1 | -2.6 | 0.03 | -4.4 | 0.01 |
| WIF1 | -4.9 | 0.00 | -1.5 | 0.00 |
| WNT2B | -1.5 | 0.04 | -1.1 | 0.00 |
| WNT5A | 1.2 | 0.05 | 2.9 | 0.00 |
| **TGF β SIGNALING** | | | | |
| BMP4 | -1.3 | 0.01 | -1.2 | 0.01 |
| CREBBP | -1.5 | 0.00 | -1.1 | 0.00 |
| ENG | 1.1 | 0.04 | 1.6 | 0.00 |
| EP300 | -1.4 | 0.00 | -1.1 | 0.00 |
| FKBP1A | 1.5 | 0.01 | 1 | 0.00 |
| FST | 3.2 | 0.04 | 1.3 | 0.01 |
| INHBA | 3.7 | 0.01 | 3.9 | 0.00 |
| LIF | 4.1 | 0.00 | 4.7 | 0.00 |
| SERPINE1 | 5.7 | 0.00 | 6.4 | 0.00 |
| SLC15A4 | 1.2 | 0.00 | 1.3 | 0.01 |

| Gene symbol | IPE-DC (FC) | P-value | CPE-DC (FC) | P-value |
|---|---|---|---|---|
| TGFB1 | 1.2 | 0.01 | 1.6 | 0.00 |
| TGFB2 | -1.1 | 0.00 | -1.7 | 0.02 |
| TGFBI | 2.3 | 0.01 | 2.9 | 0.00 |
| TGFBR3 | -2 | 0.01 | -2.6 | 0.00 |
| **Gene symbol** | **IPE-DC (FC)** | **P-value** | **CPE-DC (FC)** | **P-value** |
| **NOTCH SIGNALING** | | | | |
| HEY2 | -2.30 | 0.00 | -1.60 | 0.01 |
| **WNT SIGNALING** | | | | |
| KREMEN1 | -1.6 | 0.04 | -1.7 | 0.03 |
| MAPK10 | -1.8 | 0.05 | -1.4 | 0.01 |
| MYC | 1.0 | 0.02 | 2.4 | 0.01 |
| PRKCZ | -1.0 | 0.04 | -1.1 | 0.02 |
| WIF1 | -4.9 | 0.02 | -2.4 | 0.00 |

*Table 5.2: List of molecular pathway genes involved in the stem cell differentiation.*

**FC – Fold change against IPE cells/CPE cells for NS cells and IPE-NS/CPE-NS for DC cells.*

### 5.3.3. Verification of Microarray analysis:

To verify the results of the microarray analysis with an independent method, qRT-PCRs were performed for some of the genes with relevant expression changes and biological importance. By this means, the differential regulation of gene expression detected by the microarrays was confirmed *(Table 5.3)*.

## 5.4.Conclusion:

In conclusion, 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 signalling 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 into retinal cell lineage.

| Sl. No. | Gene Name | CPE NS | | CPE DC | | IPE NS | | IPE DC | |
|---|---|---|---|---|---|---|---|---|---|
| | | Microarray | Q PCR | Microarray | Q PCR | Microarray | Q PCR | Microarray | Q PCR |
| **Pluripotency genes** | | | | | | | | | |
| 1 | c-Myc | 2.6 | 4.3 | -0.4 | -2.3 | 1.5 | 4.0 | -0.4 | -1.4 |
| 2 | Klf4 | 1.3 | 2.0 | -0.2 | -1.3 | 2.2 | 3.2 | -0.2 | -1.3 |
| 3 | Oct 4 | 1.6 | 1.8 | -0.3 | -1.6 | 1.4 | 1.9 | -0.4 | -1.6 |
| 4 | Sox 2 | 1.5 | 1.9 | -0.6 | -1.3 | 2.6 | 2.3 | -0.5 | -1.5 |
| 5 | Pax 6 | * | 2.3 | * | 1.9 | * | 2.5 | * | 1.9 |
| 6 | Nanog | 0.9 | 1.0 | -0.1 | -1.2 | 0.7 | 0.9 | -0.1 | -1.0 |
| **Pigmentation genes** | | | | | | | | | |
| 7 | Rpe65 | -2.6 | -3.7 | -2.5 | -3.0 | -0.4 | -0.9 | -2.9 | -4.3 |
| 8 | Tyr | -3.0 | -4.8 | -3.8 | -5.2 | -3.4 | -4.9 | -2.5 | -5.0 |
| 9 | Palmd | -0.8 | -1.0 | -1.3 | -1.6 | -0.8 | -1.2 | -0.9 | -1.0 |
| **Retinal Histogenesis** | | | | | | | | | |
| 10 | NeuroD | 0.3 | 2.5 | 0.09 | 1.4 | 0.1 | 2.6 | 0.1 | 1.2 |
| 11 | Rx | * | 4.3 | * | 4.6 | * | 4.2 | * | 3.8 |
| 12 | Crx | * | 4.5 | * | 4.3 | * | 3.9 | * | 3.5 |
| 13 | Mitf | -1.4 | -2.6 | -2.0 | -2.5 | -2.9 | -2.3 | -2.4 | -2.8 |
| 14 | Irbp (Rbp3) | 1.2 | 2.7 | 2.4 | 3.0 | 1.0 | 1.6 | 3.0 | 3.6 |

| 15 | S-arrestin | 0.8 | 1.5 | 1.6 | 1.8 | 0.5 | 1.0 | 1.4 | 1.5 |
|---|---|---|---|---|---|---|---|---|---|
| **Cell cycle genes** | | | | | | | | | |
| 16 | Cyclin D1 | 2.7 | 2.4 | 2.7 | 2.5 | 1.4 | 2.0 | 0.3 | 0.7 |
| 17 | Cyclin D3 | * | 2.2 | * | 0.2 | * | 2.0 | * | 0.3 |
| 18 | Cyclin B1 | 2.3 | 3.1 | 3.4 | 3.6 | 3.0 | 3.6 | 1.4 | 1.9 |
| **Retina Specific genes** | | | | | | | | | |
| 19 | Brn3b | * | 0.9 | * | 3.1 | * | 0.5 | * | 2.8 |
| 20 | Chx10 | * | 1.0 | * | 2.7 | * | 1.4 | * | 3.0 |
| 21 | RCVRN | * | 0.1 | * | 3.5 | * | 0.2 | * | 4.1 |
| 22 | OPN1SW | * | 0.1 | * | 4.1 | * | 0.4 | * | 2.0 |
| 23 | Nrl | * | 1.8 | * | 1.8 | * | 1.5 | * | 1.5 |
| 24 | CALB2 | * | 0.2 | * | 1.5 | * | 0.1 | * | 1.9 |

*Table 5.3: Verification of the Microarray results for the IPE and CPE derived neurospheres and differentiated cells.*

*Note: * indicates p value not significant so data not considered; – indicates downregulated genes; + indicates upregulation.*

***Note:** CPE NS – Ciliary pigment epithelial derived Neurospheres, CPE DC – Ciliary pigment epithelial derived differentiated cells, IPE NS – Iris pigment epithelial derived Neurospheres, IPE DC – Iris pigment epithelial derived differentiated cell.*

# CHAPTER 6: FUNCTIONAL NEURONAL PROPERTY OF THE DIFFERENTIATED CELLS OBTAINED FROM THE IRIS AND CILIARY DERIVED STEM/PROGENITOR CELLS

## 6.1.Introduction:

Adult iris and ciliary body derived SCs hold remarkable potential for the study of potential treatment of a variety of retinal degenerative diseases. Many studies have shown that under the appropriate conditions a variety of SCs are capable, of adopting new morphologies that are very similar to neuronal cells and these cells often express neuronal markers (Black and Woodbury 2001; Brazelton et al. 2000; Kim et al. 2002; Levy et al. 2003; Woodbury et al. 2000). However, before these cells may be used for the treatment purposes, it is essential to fully understand the differentiation of these cells and how they respond to their milieu. Along with the marker studies in recent years, the functionality of differentiated cells has become increasingly important (Tsai et al. 2002; Wagers et al. 2002), as previous reports of trans- differentiation/de-differentiation have often failed to demonstrate the functional characteristics of the newly acquired phenotype. Therefore, for demonstrating that the cells primary fate has been redirected along a different lineage, a variety of tests should be performed among which the application of calcium imaging for the *in vitro* cellular functional studies is being used.

Calcium is a ubiquitous intracellular signal messenger that regulates many diverse cellular processes (Berridge et al., 2000). It also regulates the vital processes like transcriptions, proliferation, differentiation and cell death (Lu and Means, 1993; Orrenius et al., 2003). Other than the major cellular processes it is also involved in the exocytosis and muscle contraction. In the nervous system, because of the complex morphology of neurons, the calcium ions extend their high degree of versatility. The intracellular calcium concentration varies 10 to 100 times during rest and activity (Berridge et al., 2000). *Figure 6.1* summarizes a schematic representation of some of the most important sources of neuronal calcium signalling. There are multiple mechanisms underlying the calcium influx in a cell from the extracellular space,

including glutamate receptors, nicotinic acetylcholine receptors (nAChR), and transient receptor potential type C (TRPC) channels (Fucile, 2004; Higley and Sabatini, 2008; Ramsey et al., 2006) and voltage gated calcium channels. The most important aspects of calcium signalling in neurons is mediated by the glutamate receptors, which are of two types: Ionotropic glutamate receptors and metabotropic glutamate receptors.

### 6.1.1.Ionotropic Glutamate Receptors

Ionotropic receptors are those receptors which, when a ligand binds, change their conformation to allow ions to pass through a channel. Previously, it was thought that glutamate only acted upon ionotropic receptors. Like all ligand-gated ion channels, they are composed of a ligand-binding domain and an associated ion channel. There are three main types of ionotropic glutamate receptors, which were named from their specific agonists.

***NMDA Receptor:*** N-methyl-D-aspartate (NMDA) receptors are highly permeable to Ca2+ and are permeable to Na+ and K+ to a lesser extent. These receptors are both ligand gated and voltage gated (Alford et al., 1989). The Ca2+ ion permeability of this receptor is enhanced by increased phosphorylation of the receptors and vice versa (Skeberdis et al., 2006; Sobczyk and Svoboda, 2007). The NMDA receptor is recognized as part of a large complex of cell surface proteins, receptors and intracellular mediators at the post-synaptic density, which interact to regulate excitatory neurotransmission and synaptic plasticity.

***AMPA Receptor:*** The two other types of ionotropic glutamate receptors are often referred to as non-NMDA receptors due to their physiological similarities. One of these, the AMPA receptor, is preferentially activated by α–amino–3–hydroxy–5-methylisoxazole-4-propionate (AMPA). It is permeable to Na+ and K+ and to some extent Ca2+ (Bowie and Mayer, 1995) (Liu and Zukin, 2007) exhibit fast gating kinetics (Geiger et al., 1995). It undergoes desensitization, which effectively limits ion flow as a result of glutamate binding to the receptor for only a few milliseconds (Rosenmund and Mansour, 2002). They are found in many forms of GABAergic neurons (Jonas et al., 1994).

***Kainate Receptor:*** Kainate receptors are permeable to NA+ and K+ and to some extent Ca+ like the other glutamate receptors (Bowie and Mayer, 1995). As the name implies, the preferred agonist of this receptor type is kainic acid. Kainate receptors also are highly susceptible to desensitization (Rosenmund and Mansour, 2002).

### 6.1.2. Metabotropic Glutamate Receptors

Previously, it was reported that glutamate stimulated inositol triphosphate (IP3) formation in cultured striatal neurons, hippocampal slices, cultured astrocytes and cultured cerebellar granule cells through non-AMPA, -kainate, or -NMDA pathways (Sladeczek, 1985) (Nicoletti et al, 1986a) (Pearce et al, 1986) (Nicoletti et al, 1986b) which led to invention of "metabotropic" glutamate receptors (mGluRs). Since then, eight such receptor subtypes have been identified. These have been subdivided into 3 groups based on sequence homology, second messenger coupling, and pharmacology: group I (mGluR1 and 5 subtypes), group II (mGluR2 and 3 subtypes), and group III (mGluR4 and 6-8 subtypes) (Kew and Kemp, 2005).

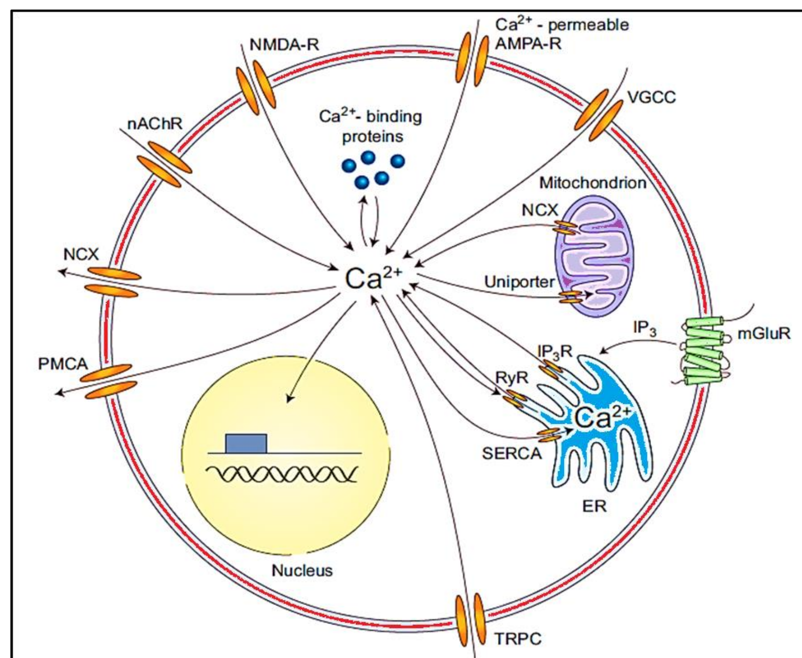



***Figure 6.1:*** *Neuronal Calcium Signalling: A schematic representation showing the receptors that mediate calcium influx and efflux. The mitochondria are also important for neuronal calcium homeostasis. Adapted from Christine Grienberger and Arthur Konnerth. Imaging Calcium in Neurons. Neuron 73, March 8, 2012.*

| Currently Accepted Name [a] | Glutamate site | NMDA Glycine site | Other | AMPA | Kainate |
|---|---|---|---|---|---|
| Alternate Name | — | — | Quisqualate | — | |
| Structural Information | NR1 (920 aa human)<br>NR2A (1464 aa human)<br>NR2B (1484 aa human)<br>NR2C (1233 aa human)<br>NR2D (1329 aa rat)<br>NR3A (1115 aa rat) NR3B (1003 aa mouse) | Not known | Not known | iGluR1 (889 aa human)<br>iGluR2 (883 aa human)<br>iGluR3 (894 aa human)<br>iGluR4 (881 aa rat) | iGluR5 (978 aa human)<br>iGluR6 (877 aa rat)<br>iGluR7 (919 aa human)<br>KA1 (956 aa human)<br>KA2 (962 aa human) |
| Subtype Selective Agonists | N-Methyl-D-aspartic acid **(M3262)**,<br>Quinolinic acid **(P63204)** | Glycine **(G7126)**,<br>D-Serine **(S4250)**,<br>R(+)-HA-966 (partial) **(H130)** | Cyclothiazide **(C9847)**, [d]<br>LY503430 [d] | AMPA **(A0326)**,<br>S(–)-5-Fluorowillardiine **(F2417)**,<br>CX-614, [d]<br>ATPA (iGluR5) **(A263)** | Kainic acid **(K0250)**,<br>Domoic acid **(D6152)**,<br>4-Methylglutamate **(G137)** |
| Subtype Selective Antagonists | D(–)-AP-5 **(A169)**,<br>D(–)-AP-7 **(A167)**,<br>CGS19755 **(C105)**,<br>CGP37849,<br>LY382884 **(GluR5)**,<br>CPP, (±)-, D- **(C104, C189)**,<br>D-CPPene,<br>EAA-090 | 7-Chlorokynurenic acid **(C0306)**,<br>5,7-Dichlorokynurenic acid **(D138)**,<br>MNQX,<br>L-689,560,<br>L-701,324 **(L0258)**,<br>GV 150526 | Ro 25-6981 (NR2B) **(R7150)**,<br>Ro 8-4304 (NR2B) **(R8900)**,<br>CP 101,606 (NR2B),<br>Ifenprodil (NR2B) **(I2892)**,<br>SPD-502 | NBQX **(N183)**,<br>GYKI 52466 **(G119)**, [c]<br>GYKI 53655, [c]<br>CNQX **(C239)**,<br>DNQX **(D0540)**,<br>YM90K,<br>LY294486,<br>Ro 48-8587 | CNQX **(C239)**,<br>DNQX **(D0540)**,<br>NS 102 **(N179)**, [b]<br>LY293558 (iGluR5),<br>NS3763 (iGluR5) |

**Abbreviations**
**AMPA:** α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
**AP-5:** 2-Amino-5-phosphonopentanoic acid
**AP-7:** 2-Amino-7-phosphonoheptanoic acid
**ATPA:** (RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid
**D-CCPene:** D-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonene
**CGP37849:** D,L-(E)-2-Amino-4-methylphosphono-3-pentanoic acid
**CGS19755:** 4-Phosphonomethyl-2-piperidinecarboxylic acid (Selfotel)
**CNQX:** 6-Cyano-7-nitroquinoxaline-2,3-dione
**CNS 1102:** N-(1-Naphthyl)-N'-(3-ethylphenyl)-N'-methyl-guaniaine HCl
**CP 101,606:** (1S,2S)-1-(4-Hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol
**CPP:** 3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid
**CX-614:** 2H,3H,6aH-Pyrolidino[2",1"-3',2']1,3-oxazino[6',5',-5,4]benzo[e]1,4-dioxan-10-one
**DNQX:** 6,7-Dinitroquinoxaline-2,3-dione
**EAA-090:** [2-(8,9-Dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)-ethyl]phosphonic acid
**GV 150526:** 3-[2-(Phenylamino)carbonyl]ethenyl-4,6-dichloroindole-2-carboxylic acid
**GYKI 52466:** 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine
**GYKI 53655:** 1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine
**HA-966:** 1-Hydroxy-3-aminopyrrolid-2-one
**L-689,560:** (±)-4-(trans)-2-Carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline
**L-701,324:** 7-Chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(H)-quinolinone
**LY293558:** (3S,-4aR,6R,8aR)-6-[2-(1Htetrazol-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid
**LY382884:** 3S,4aR,6S,8aR-6-((4-Carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid
**LY503430:** (R)-4'-[1-Fluoro-1-methyl-2-(propane-2-sulfonylamino)-ethyl]-biphenyl-4-carboxylic acid methylamide
**MNQX:** 5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione
**NBQX:** 2,3-Dihydro-6-nitro-7-sulphamoyl-benzo(f)quinoxaline
**NMDA:** N-Methyl-D-aspartic acid
**NS 102:** 5-Nitro-6,7,8,9-tetrahydrobenzo[G]indole-2,3-dione-3-oxime
**NS3763:** 5-Carboxyl-2,4-dibenzamido-benzoic acid
**Ro 25-6981:** R-(R*,S*)-α-(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol
**Ro 8-4304:** 4-{3-[4-(4-Fluorophenyl)-3,6-dihydro-2H-pyridin-1-yl]-2-hydroxy-propoxy}-benzamide
**Ro 48-8587:** 9-(1H-Imidazol-1-yl)-8-nitro-[1,2,4]triazolo[1,5-c]quinazoline-2,5(3H,6H)-dione
**SPD-502:** 8-Methyl-5(4-(N,N-dimethylsulfamoyl)phenyl)6,7,8,9,-tetrahydro-1H-pyrrolo[3,2-h]-isoquinoline-2,3-dione-3-O-(4-hydroxybutyrate-2-yl)oxime
**YM90K:** 6-(1H-Imidazol-1-yl)-7-nitro-2,3(1H,4H)-quinoxalinedione

*Table 1. Glutamate receptor agonists and antagonists. Adapted from Sigma-RBI eHandbook.*

### 6.1.3.Synaptic vesicle recycling- Exocytosis and Endocytosis:

Neurotransmission is the principal activity of neurons which involves the fusion of vesicles containing neurotransmitters with the plasma membrane of the nerve terminal. Calcium influx triggers exocytosis of synaptic vesicles with neurotransmitters in the presynaptic terminals (Neher and Sakaba, 2008). Likewise, a calcium influx (increased calcium level) in dendritic spines is essential for the induction of activity dependent synaptic plasticity during postsynaptic activity (Zucker, 1999). Following the process of exocytosis, the synaptic vesicles are endocytosed and recycled in order to sustain further synaptic transmission. The process of endocytosis and exocytosis can be explained as the 'kiss-and-run', clathrin-dependent and bulk endocytosis.

Briefly, the Kiss-and-run endocytosis involves the transient fusion of synaptic vesicles with the plasma membrane, these vessels do not completely lose their identity and they can be rapidly reused after being refilled with a neurotransmitter (Valtorta et al., 2001) (Ceccarelli et al., 1973) (Aravanis et al., 2003). Clathrin-dependent endocytosis is mainly observed during mild neuronal activity and during clathrin-dependent exocytosis, vesicles fully collapse before clathrin-mediated invagination and fission occur (Granseth et al., 2006 (Heuser and Reese, 1973; Heuser, 1989). By contrast, bulk endocytosis is provoked by strong stimulation, which involves large fragments of membrane and the generation of endosome-like structures from which vesicles bud off (Heuser and Reese, 1973; Koenig and Ikeda, 1989; Koenig and Ikeda, 1996; Takei et al., 1996; Teng and Wilkinson, 2000; Richards et al., 2000; Holt et al., 2003; Wu and Wu, 2007).

In the present chapter, we have used the fluorescent dye methods to measure the ionotrophic glutamate receptor activity using various Ca2+ channel blockers and also the bulk exocytosis and endocytosis activity in the differentiated progeny of the IPE-NS and CPE-NS.

## 6.2. Materials and Methods:

### 6.2.1.Glutamate receptor activity:

As described in the methodology.

### 6.2.2.Synaptic vesicle - Endocytosis and Exocytosis:

As described in the methodology.

## 6.3.Results:

### 6.3.1.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 *(Figure 6.2)*. 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 (Figure 6.2). 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 *(Figure 6.2)*. The fluorescence, within the marked region of interest, was plotted as a function of time *(Figure 6.2)*. 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.

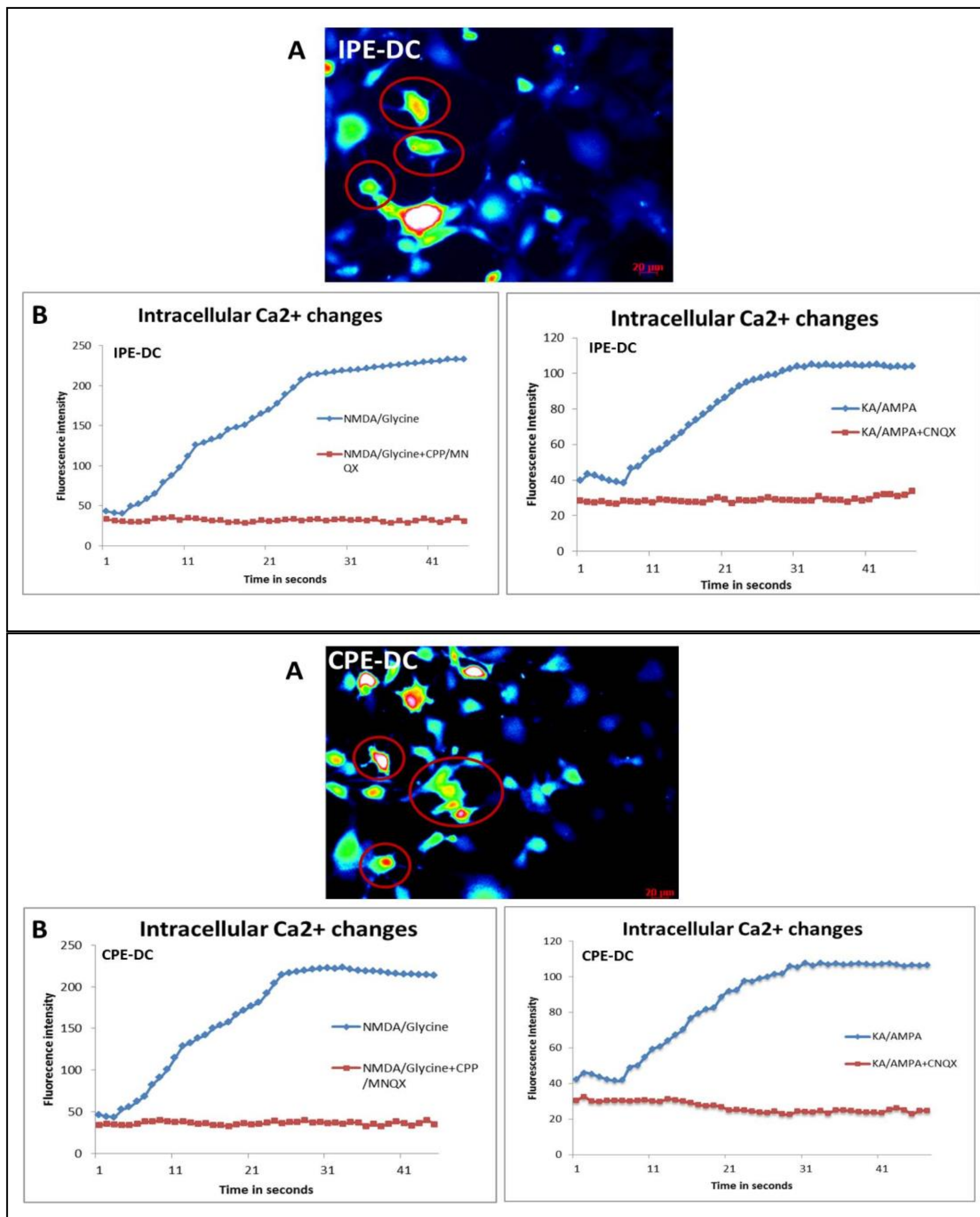



***Figure 6.2 A:*** *IPE-DC and CPE-DC showing neuronal morphology (marked in red) used for the functional studies – calcium imaging.*

***Figure 6.2 B:*** *Activation of glutamate receptors elevates cytosolic Ca2+ in cultured IPE-DC and CPE-DC in vitro measured with Fluo-3AM. Average response of (n=3) fluo-3-loaded IPE-DC and CPE-DC demonstrates increase in intracellular Ca2+ in response to NMDA+glycine, and the changes in the intracellular calcium levels upon addition of the inhibitors CPP/MNQX. Also average response of (n=3) fluo-3-loaded*

*IPE and CPE neurosphere derived differentiated cells demonstrates increase in intracellular Ca2+ in response to KA+AMPA and the changes in the intracellular calcium levels upon addition of the inhibitor CNQX. (Published in Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K.Stem Cell Rev. 2012 Dec;8(4):1163-77.)*

### 6.3.2.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 thesecellsto incorporate the fluorescent styryl dye FM1-43 into synaptic vesicles and to unload this dye after stimulation with 100mM K+ *(Protocol for dye loading and unloading is shown in figure A and B)* 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 *(Figure 6.3)*, 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.

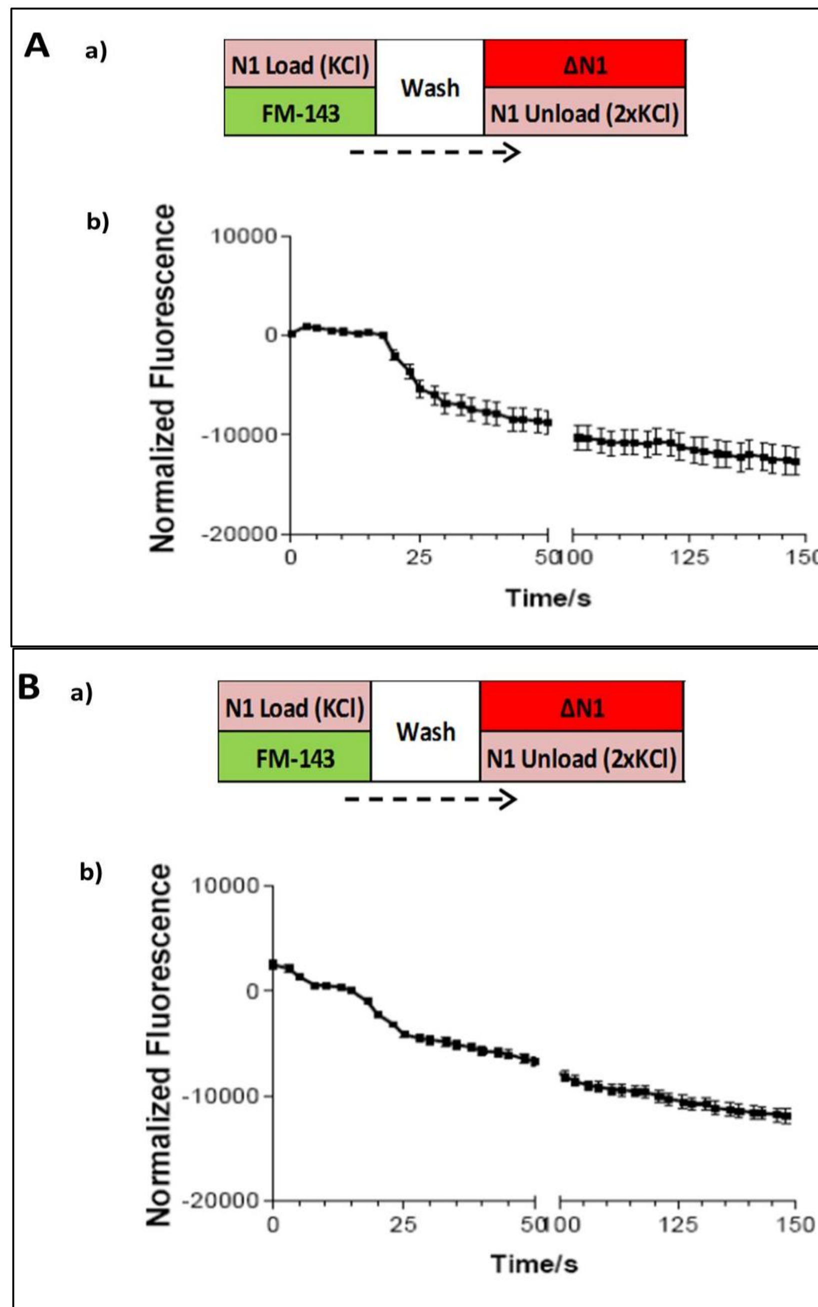



***Figure 6.3:*** *FM-143 assay with synaptic vesicles of the IPE-DC and CPE-DC cells exhibiting strong unloading. [A] (a) The step wise protocol loading and unloading assay in IPE-DC cells were loaded with FM-143 dye and stimulated with 50mM KCl and unloaded with 100mM KCl at ΔN1. b) Graph showing the average fluorescence drop during ΔN1. [B] (a) The step wise protocol loading and unloading assay in CPE-DC cells were loaded with FM-143 dye and stimulated with 50mM KCl and unloaded with 100mM KCl at ΔN1. b) Graph showing the average fluorescence drop during ΔN1.*

### 6.4. Conclusion:

In conclusion, our results show that functional neurons can be generated from CPE and IPE cells. However, numbers and function of the cells generated can vary enormously. Our *in vitro* live cell imaging studies on the differentiated cells derived from the ciliary and iris neurospheres with Fluo-3AM show calcium flux indicating the presence of glutamate receptors which are functionally similar to that of retinal cell phenotypes and FM-143 show the presence of synaptic vesicle recycling, proving that the differentiating conditions promote the expression of various physiological properties commonly associated with retinal neurons.

# CHAPTER 7: EPIGENETIC MODIFICATIONS DEFINING THE DIFFERENTIATION POTENTIAL OF STEM/PROGENITOR CELLS DERIVED FROM ADULT HUMAN CILIARY PIGMENT EPITHELIAL CELLS

## 7.1.Introduction:

Retinal degenerative diseases such as retinitis pigmentosa, retinal dystrophy and age related macular degenerations are the progressive disorders resulting in gradual loss of the photoreceptors leading to blindness (Reh, T. A., & Levine, E. M. 1998) (MacLaren, R. E., et al., 2006) (Lamba D. A, et al., 2009) (Bartsch, U, et al., 2008). (Verhoeff FH 1930) (Klein R, et al., 1997) (Weleber RG and Gregory-Evans K 2001). Current therapeutic strategies involve gene therapy which involves replacement of diseased gene thereby preventing or delaying further cell death. However, the strategy do not replace the cells that are already lost during the disease. An alternative strategy is to replace the degenerated photoreceptors by cells such as retinal progenitors that have potential to generate and restore the cells lost (MacLaren, R. E., et al., 2006) (Klassen HJ, et al., 2004). Due to the lack of stem/progenitor cells in adult retina, the degenerated/damaged cells are lost gradually and do not regenerate. Hence, there is a need for an alternative source of stem cells (SCs) that could differentiate into retinal cells.

The majority of SCs researches are implemented on embryonic stem cells (ESCs) that are known to be effective sources that are capable of forming neural retinal progenitors (Ikeda H, et al., 2005) (Lamba DA, et al., 2006) (Osakada F, et al., 2008) (Osakada F, et al., 2009) (Osakada F, et al., 2009) (Idelson M, et al., 2009) (Meyer JS, et al., 2009). However, limitations such as ethical issues, possibility of immune rejection and teratoma formation and pose a significant barrier for their clinical use. Therefore, 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* (Klein R, et al., 1997) (Weleber RG and Gregory-Evans K 2001) (Reik, W. 2007).

In our previous studies, we have generated functional retinal cells through *in vitro* differentiation from the SCs derived from ciliary pigment epithelium (Srilatha Jasty, et al., 2012). However, the exact molecular mechanisms remain unclear, such as the extent to which epigenetic mechanisms underlie the differentiation process. The epigenetic restrictions such as chromatin or DNA modifications are involved during differentiation of one cell type to alternative lineages, which can modulate DNA accessibility. The cell type specific gene expression patterns, activating differentiation-associated genes and silence stem cell-specific or lineage-unrelated genes are stabilized by these epigenetic mechanisms (Reik, W. 2007) (Kashyap V, et al., 2009) (Khavari DA and Sen GL, Rinn JL 2010) (Meissner A, et al., 2008) (Mohn F, et al., 2008).

Epigenetic modifications, especially DNA and histone methylations, have a large impact on the regulation of gene expression and are critical in establishing patterns of gene repression during development (Cedar H and Bergman Y 2009). DNA methylation is generally involved in stable gene silencing and recent studies also showed a correlation between the “active mark” histone H3 lysine 4 (H3K4me3) and “repressive mark” lysine 27 trimethylation (H3K27me3) in embryonic stem cells (Bernstein BE, et al., 2006) (Mikkelsen TS, et al., 2007) (Pan G, et al., 2007) (Zhao XD, et al., 2007), hematopoietic stem cells/progenitor cells (Cui K, et al., 2009). In this study, we mapped genome-scale DNA methylation and histone methylation in ciliary epithelium derived SCs and retinally differentiated cells and found cell type specific methylation pattern. Furthermore, we correlated promoter DNA methylation with mRNA gene expressions during *in vitro* retinal differentiation from ciliary epithelium derived SCs. The better understanding of these processes may enable us to manipulate ciliary epithelium derived SCs fate and enhance the pool of therapeutically useful retinal cells.

## 7.2.Materials and Methods:

### 7.2.1.Isolation of CPE cells from cadaveric eyes:

Isolation of pigmented ciliary epithelial cells (CPE) and it was carried out as previously described in Methodology.

### 7.2.2.Neurosphere cultures (CPE-NS):

SCs cultures from CPE were carried out as described in Methodology.

### 7.2.3.Retinal Differentiation cultures (CPE-DC):

Retinal differentiation was carried out as described in Methodology.

### 7.2.4.Methylated DNA immunoprecipitation (MeDIP):

MeDIP was performed according to the Agilent Microarray Analysis of Methylated DNA Immunoprecipitation protocol as described in Methodology.

### 7.2.5.Chromatin immunoprecipitation (ChIP):

ChIP was performed according to the Agilent ChIP-chip protocol as described in Methodology.

### 7.2.6.Human promoter microarray profiling:

As described in Methodology.

### 7.2.7.ChIP-chip and MeDIP-chip data analysis:

MeDIP-Chip and ChIP-chip data were analysed using Agilent's Genomic workbench 6.5 software as described in Methodology. MeDIP-chip study accession number is GSE64546 and ChIP-chip study accession number is GSE65268.

### 7.2.8.Gene expression analysis:

As described in Methodology.

### 7.2.9.Real-time quantitative PCR:

Real-time quantitative PCR (qPCR) was performed as described in Methodology.

## 7.3.Results:

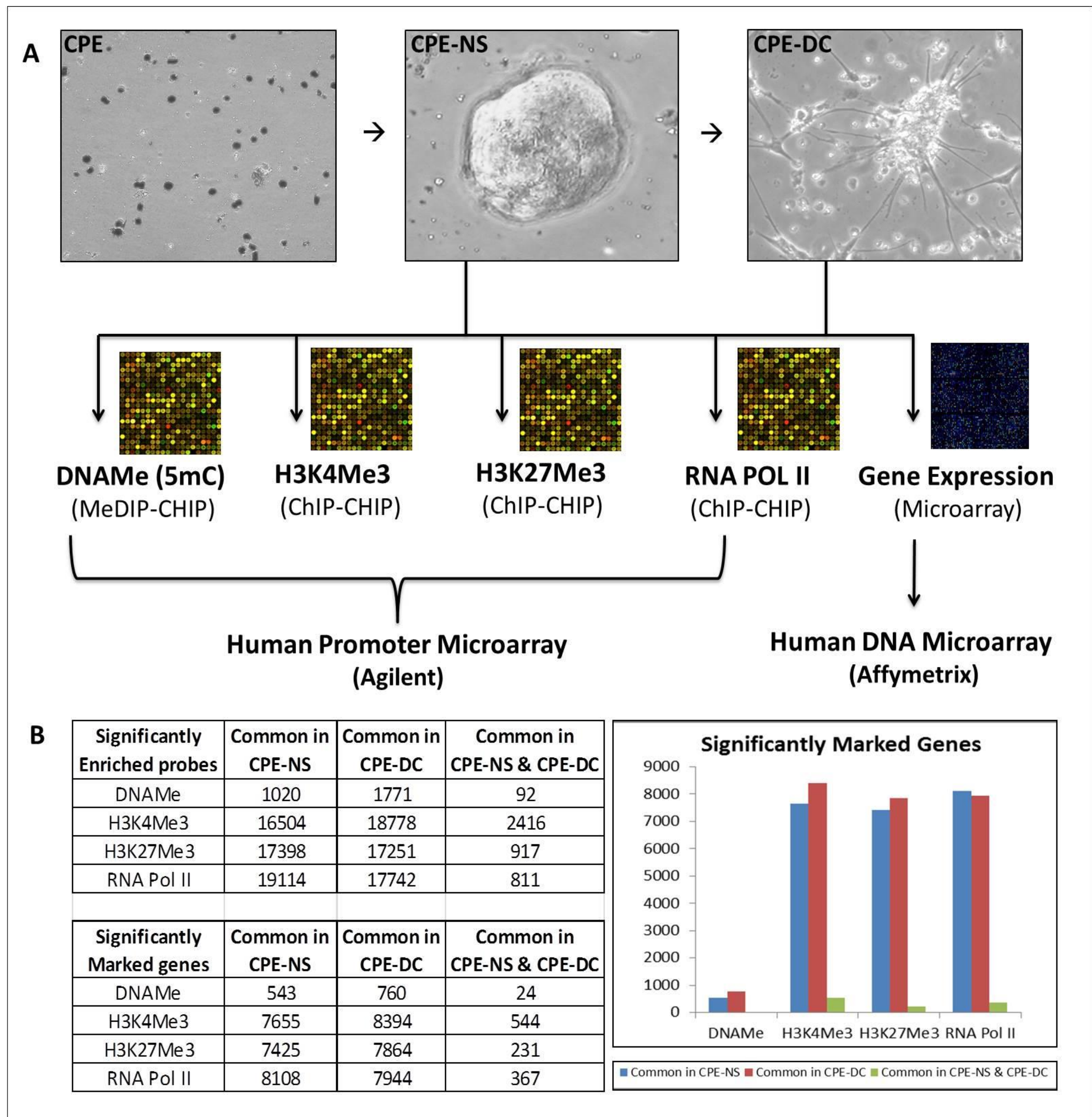
### 7.3.1.Correlation of DNA methylation, histone methylation and gene expression profiling in ciliary epithelium derived stem/progenitor cells and retinally differentiated cells:

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 (Figure 1A). 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 (Figure 1B).

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 (Figure 2A). We compared all the three methylation modifications with gene activities in CPE-NS and CPE-DC (Figure 2B), 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 (Figure 2B), as demonstrated by CPE-NS and CPE-DC cells.




| Significantly Enriched probes | Common in CPE-NS | Common in CPE-DC | Common in CPE-NS & CPE-DC |
|---|---|---|---|
| DNAMe | 1020 | 1771 | 92 |
| H3K4Me3 | 16504 | 18778 | 2416 |
| H3K27Me3 | 17398 | 17251 | 917 |
| RNA Pol II | 19114 | 17742 | 811 |

| Significantly Marked genes | Common in CPE-NS | Common in CPE-DC | Common in CPE-NS & CPE-DC |
|---|---|---|---|
| DNAMe | 543 | 760 | 24 |
| H3K4Me3 | 7655 | 8394 | 544 |
| H3K27Me3 | 7425 | 7864 | 231 |
| RNA Pol II | 8108 | 7944 | 367 |

*Figure 7.1: Overview of Genome-wide profiling of DNA methylation, Histone methylation and gene expression of CPE-NS and CPE-DC cells.*

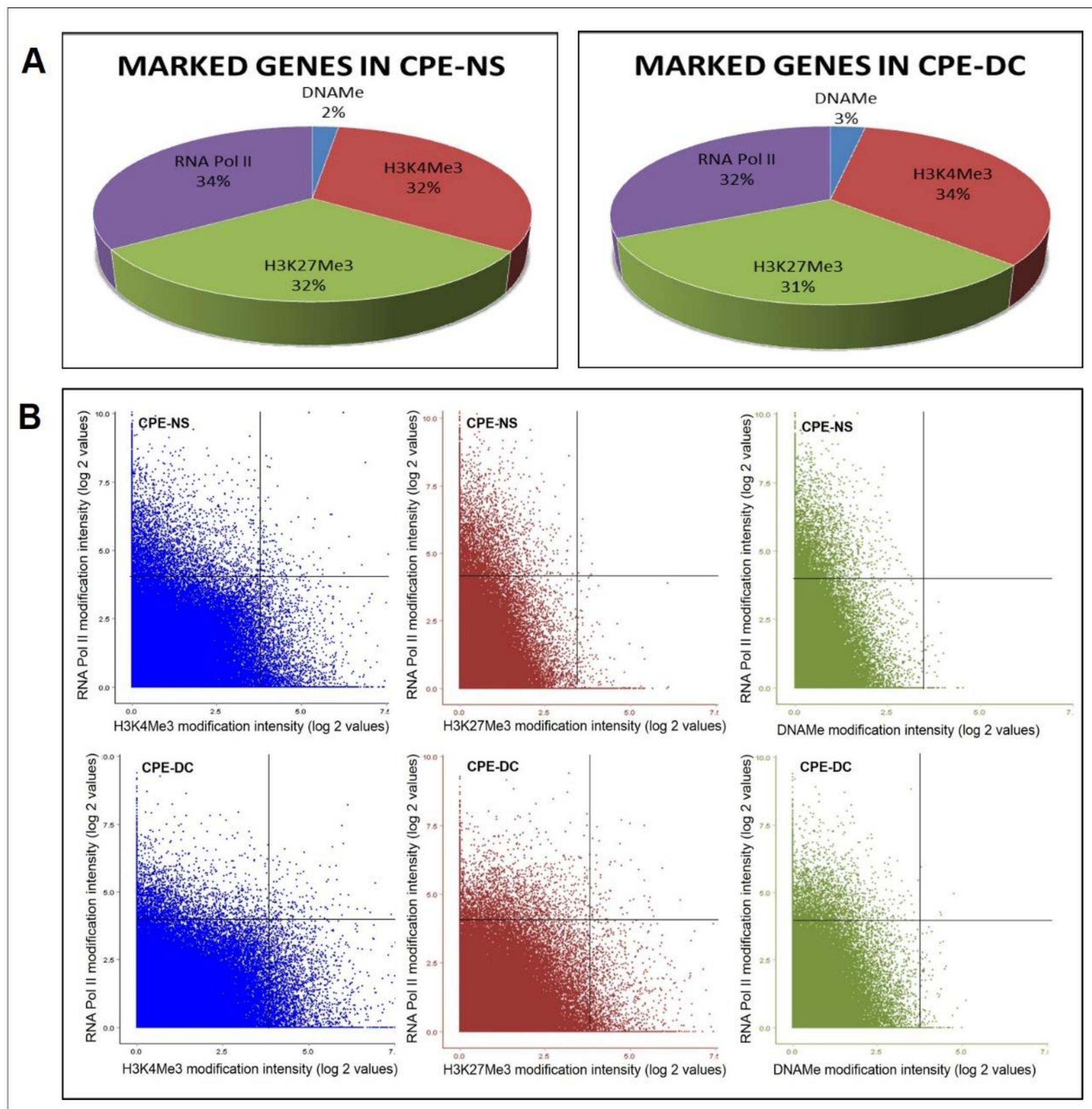
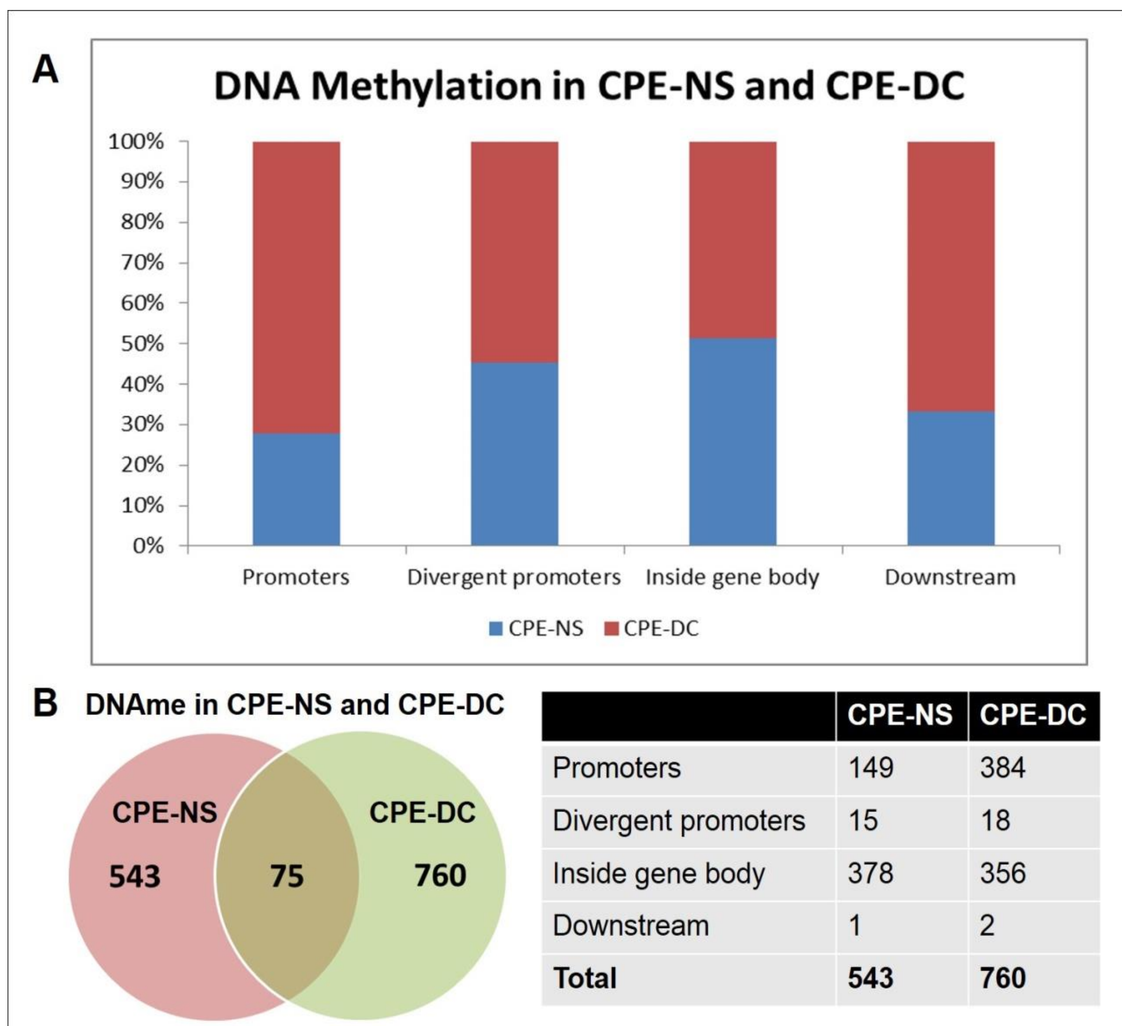



*Figure 7.2: Correlation between H3K4me3, H3K27me3 and DNAMe modifications and gene activities. [A] Genes were ranked according to their expression levels in CPE-NS and CPE-DC cells. The percentages correspond to the marked genes in each for each kind of modification. [B] Scatter plots between RNA Pol II levels and epigenetic modification levels in CPE-Ns and CPE-DC cells.*

### 7.3.2. DNA Methylation changes during Retinal neuronal differentiation:

To analyse 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 (Figure 3A). CPE-NS and CPE-DC cells show commonly marked genes of n=75 (Figure 3B), 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 (Figure 2B).




| | CPE-NS | CPE-DC |
|---|---|---|
| Promoters | 149 | 384 |
| Divergent promoters | 15 | 18 |
| Inside gene body | 378 | 356 |
| Downstream | 1 | 2 |
| **Total** | **543** | **760** |

*Figure 7.3: DNA Methylation Marked genes. [A] Graphical representation of the percentage of DNA methylation marked gene in CPE-NS along with their type of location. [B] Venn diagram showing the number of DNAMe marked genes in CPE-NS and CPE-DC and the commonly marked genes.*

### 7.3.3.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.

### 7.3.4. 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 *(Appendix III)*. 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 *(Appendix IV)*.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 *(Appendix V and VI)* were previously cited.

**7.3.5. 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).

**7.4. Conclusion:**

In conclusion, 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. 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.

# CHAPTER 8: INFLUENCE OF SELF-ASSEMBLING PEPTIDE NANOFIBRE SCAFFOLD ON RETINAL DIFFERENTIATION POTENTIAL OF STEM/PROGENITOR CELLS DERIVED FROM CILIARY PIGMENT EPITHELIAL CELLS

## 8.1. Introduction:

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) (MacLaren RE et al., 2006). However, the adult mammalian retina does not regenerate spontaneously and retinal 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 SCs to the subretinal space (Klassen HJ et al., 2004) (MacLaren RE et al., 2006). Most of the studies have shown that injecting SC suspensions directly into the retinal milieu causes immense cell damage. (Klassen HJ et al., 2004) (MacLaren RE et al., 2006) So there is a critical need for an alternative strategy for building biological substitutes, such as a three-dimensional (3D) culture of retinal cells to repair or replace the damaged cells. Since retinal neurons are not capable of proliferating and neurons in culture are short-lived, there remain significant challenges for retinal tissue engineering and recent advances in SCs biology.

Our earlier study showed that SCs can be isolated from adult ciliary body and iris (Srilatha Jasty et al., 2012), and have the potential to differentiate into retinal neurons and glial phenotypes upon induction by growth factors. Therefore, maintaining and directing the fate of SCs into retinal neuronal cells (Tomita M et al., 2005) and generating functional 3D constructs may serve as good transplantation replacement for tissues or organs. When cells are grown under the appropriate conditions on a 3D scaffold, they become capable of developing mature genetic expression patterns and morphology (Freed LE et al., 1994) (Temenoff JS et al., 2000). Additionally, biodegradable polymers provide temporary scaffolding that is absorbed by the host

upon transplantation. (Tomita M et al., 2005) (Redenti S et al., 2008) Polymer scaffolds such as poly (caprolactone) (PCL), poly- (lactic-co-glycolic acid) (PLGA), poly (lactic acid) (PLA), poly- (glycolic acid) (PGA), poly (glycerol-sebacate), (Colthurst MJ et al., 2000) (Cook A et al., 1997) (Langer R, 2000) (Stephen Redenti et. al. 2009) and hydrogel-based scaffolds are non-cytotoxic to the eye and hence they are widely used in the field of stem cell research. These hydrogel scaffolds provide in vitro model systems that study different aspects of stem cell self-renewal, and differentiation (Andrea Liedmann et al., 2012).

In the present study we use synthetic SAPNs, (RADA16-I- 16-residue peptide composed of alternating hydrophilic arginine, hydrophobic alanine, and hydrophilic aspartic acid (RADARADARADARADA)) which is commercially available and has been used in several studies to investigate the proliferation and neuronal differentiation with SCs of different origins (Andrea Liedmann et al., 2012) (Stefanie Ortinau et al., 2010) (Thonhoff JR et al., 2008). This scaffold has been shown to support cell attachment and proliferation, formation of neurite outgrowth and active synapses, cell entrapment in previous studies (Blow N. 2009) (Taraballi F et al., 2010) (Gelain F et al., 2006) (Allen P et al., 2011) (S Zhang et al., 1995) (TC Holmes et al., 2000) (Semino CE et al., 2004) (Liedmann A et al., 2012). We have investigated the effect of SAPNs on in vitro proliferation and maintenance of SCs derived from the CPE and differentiation of these cells into retinal lineage upon encapsulation. CPE cells encapsulated in SAPNs proliferated in vitro as neurospheres and differentiated towards a heterogeneous population of retinal neurons and glial cells, as demonstrated without SAPNs. The stemness of the proliferated neurospheres and the retinal neuronal properties were demonstrated by changes in mRNA and protein levels.

## 8.2. Materials and Methods:

### 8.2.1. Isolation of Ciliary pigment epithelial cells from cadaveric eyes:

Isolation and culture of SCs from CPE were carried out as described in methodology.

### 8.2.2. Sphere formation assay:

For sphere formation assay, the CPE cells were harvested as mentioned above, embedded in 1.0mg/ml concentration of SAPNs (Puramatrix) at 1x $10^5$cells/mL, and cultured in sphere forming medium [DMEM-F12, supplemented with 10ng/mL of fibroblast growth factor-2 (FGF-2); 20ng/mL of epidermal growth factor (EGF); 1X N2 supplement; L-glutamine, 20mM; 1X Antibiotic and antimycotic solution] for 7 days at 37$^{o}$C and 5% CO2. The SAPNs preparation was performed as per the manufacturer's instructions as described in methodology. The media was changed every alternate day.

### 8.2.3. Differentiation Assay:

Preparation of cells: The neurospheres/SAPNs mixture was washed with 500µl HBSS and transferred to a 15ml conical tube containing culture medium. The neurosphere/SAPNs mixture was disrupted by mechanically triturating several times and subsequently the solution was centrifuged at 3000xg for 5 min. The cell solution was treated with 0.25% Trypsin EDTA solution for 20mins and the cell pellet was resuspended several times in culture medium by pipetting up and down. The cell suspension was centrifuged at 3000xg for 5mins and washed with HBSS to remove debris. The cell suspension was passed through a cell strainer to remove aggregates and the cells in cell culture medium was collected and plated for differentiation assay. (TC Holmes, et. al. 2000)

Preparation of SAPNs was performed as mentioned in methodology. To promote differentiation in SAPNs, a total volume of 250µl per well (growth area of 2.00cm2) of a 24-well cell culture plate i.e., 200µl of diluted SAPNs and 50µl of cells in 10% sucrose solution mixed with 4µg of laminin were plated. The scaffold/cell/sucrose mixture was added to the center of the well carefully, without introducing bubbles. The gelation of the SAPNs was initiated by gently running culture media down the side of the well on top of the hydrogel. This was repeated until cells have been plated in all wells. Then the differentiation media [DMEM-F12 supplemented with 1ng/ml Brain derived neurotropic factor (BDNF), 1mM Retinoic acid, 1X B27 supplement, 2mM L-

glutamine; 1X Antibiotic and antimycotic solution] was added to the wells and cultured for 14 days at 37$^{o}$C and 5% CO2. The media was changed very gently two times over the next one hour to further equilibrate the pH of the hydrogel. Media was changed every alternate day.

### 8.2.4. Cell Proliferation (BrdU labeling) Assay:

Cell proliferation assay for the cultured neurospheres encapsulated in various concentrations of SAPNs was assessed by measuring BrdU incorporation during DNA synthesis in proliferating cultured cells. The BrdU labelling was performed as described in methodology.

### 8.2.5. Expression of Neural Stem cell markers and Retinal markers by Real Time-Polymerase Chain Reaction:

RNA isolation from CPE derived neurospheres (CPE-NS) and CPE neurosphere derived differentiated cells (CPE-DC) cultured with and without SAPNs using RNeasy mini kit as described in methodology. First-strand cDNA synthesis and amplification was performed as described in methodology.

### 8.2.6. Gene expression by focused arrays after differentiation:

The mRNA levels of 84 genes that are associated with Stem cell signalling and 84 genes that are associated with Extracellular matrix and cell adhesion molecules were also examined in CPE-DC with (test) and without SAPNs (control). The human RT2 profiler arrays were performed as described in methodology.

### 8.2.7. Immunostaining for Neural Stem cell markers and Retinal markers:

For immunocytochemistry, CPE-NS and CPE-DC cells along with the SAPN matrices were performed as described in methodology.

## 8.3. Results:

The aim of this objective was to assess the effects of the SAPNs on maintenance and proliferation of CPE derived stem/progenitor cells i.e., CPE-NS and differentiation into retinal neuronal cells i.e., CPE-DC. Therefore, this study focuses on the

morphological and genotypic characteristics of these cells including several other parameters regarding survival of cells in SAPNs.

### 8.3.1. 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 *(Figure8.1a)*. 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 *(Figure 8.1b, 1c)*. Upon induction of the dissociated neurospheres in the differentiation medium, the cells could differentiate in the SAPNs *(Figure 8.2A)*. 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 *(Figure 8.2B)*.

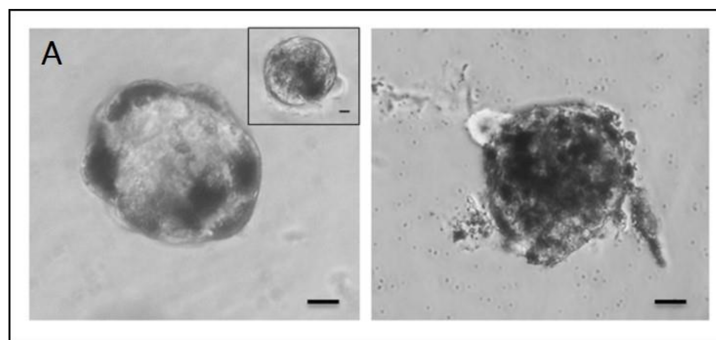



*Figure 8.1: Culturing of the CPE cells could generate neurospheres when encapsulated in 1mg/ml of SAPNs in the presence of the mitogens. A: Phase contrast images of the CPE-NS generated from the dissociated ciliary pigment epithelial cells after day 7 encapsulated in 1mg/ml of SAPNs in tissue culture. Scale bar = 10µm. Insert: Phase contrast images of the CPE-NS generated from the dissociated ciliary pigment epithelial cells after day 7 without SAPNs.*

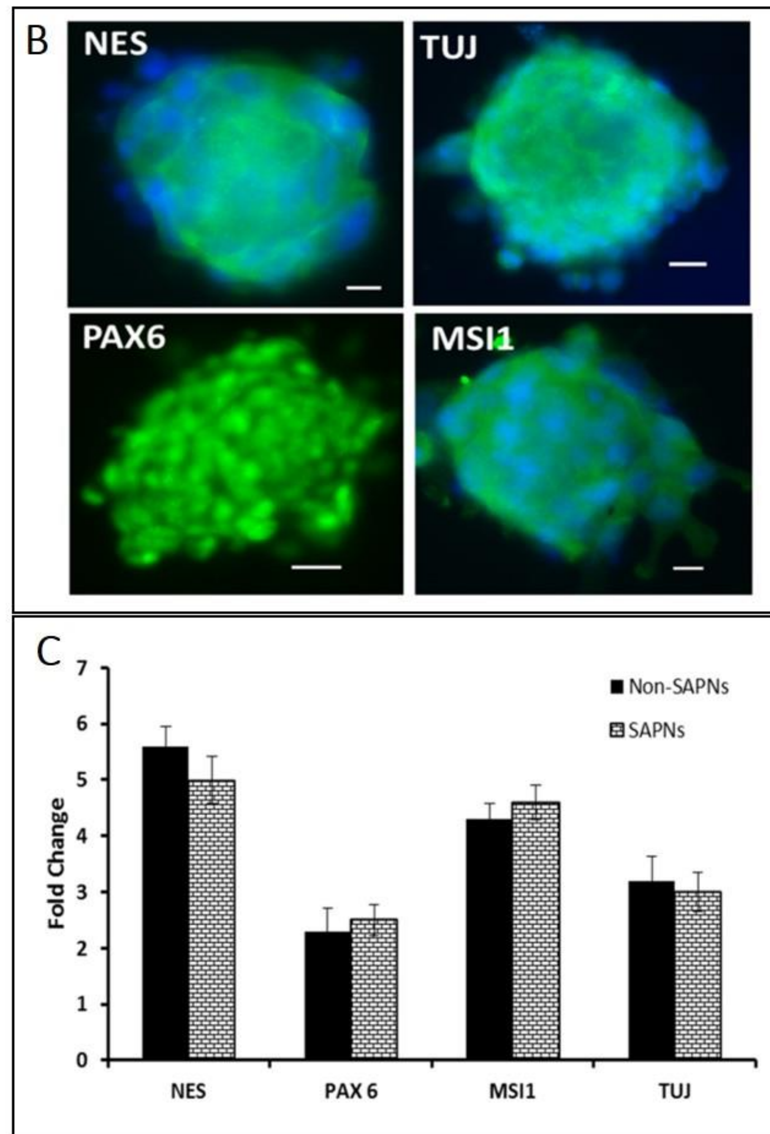



*Figure 8.1: Culturing of the CPE cells could generate neurospheres when encapsulated in 1mg/ml of SAPNs in the presence of the mitogens. B: Immunofluorescence images of CPE-NS stained for the presence of NES, PAX6, β III tubulin and MSI1 expression indicating the stem cell potential of the neurospheres encapsulated in 1mg/ml of SAPNs. Nuclei were labelled with DAPI. Scale bars 10µm. C: Real Time-PCR analysis of the progenitor markers in both the CPE-NS with and without SAPNs (n =3).* (Published in *Jasty S, Suriyanarayanan S, Krishnakumar S. Influence of self-assembling peptide nanofibre scaffolds on retinal differentiation potential of stem/progenitor cells derived from ciliary pigment epithelial cells. J Tissue Eng Regen Med. 2014 Jul 28.)*

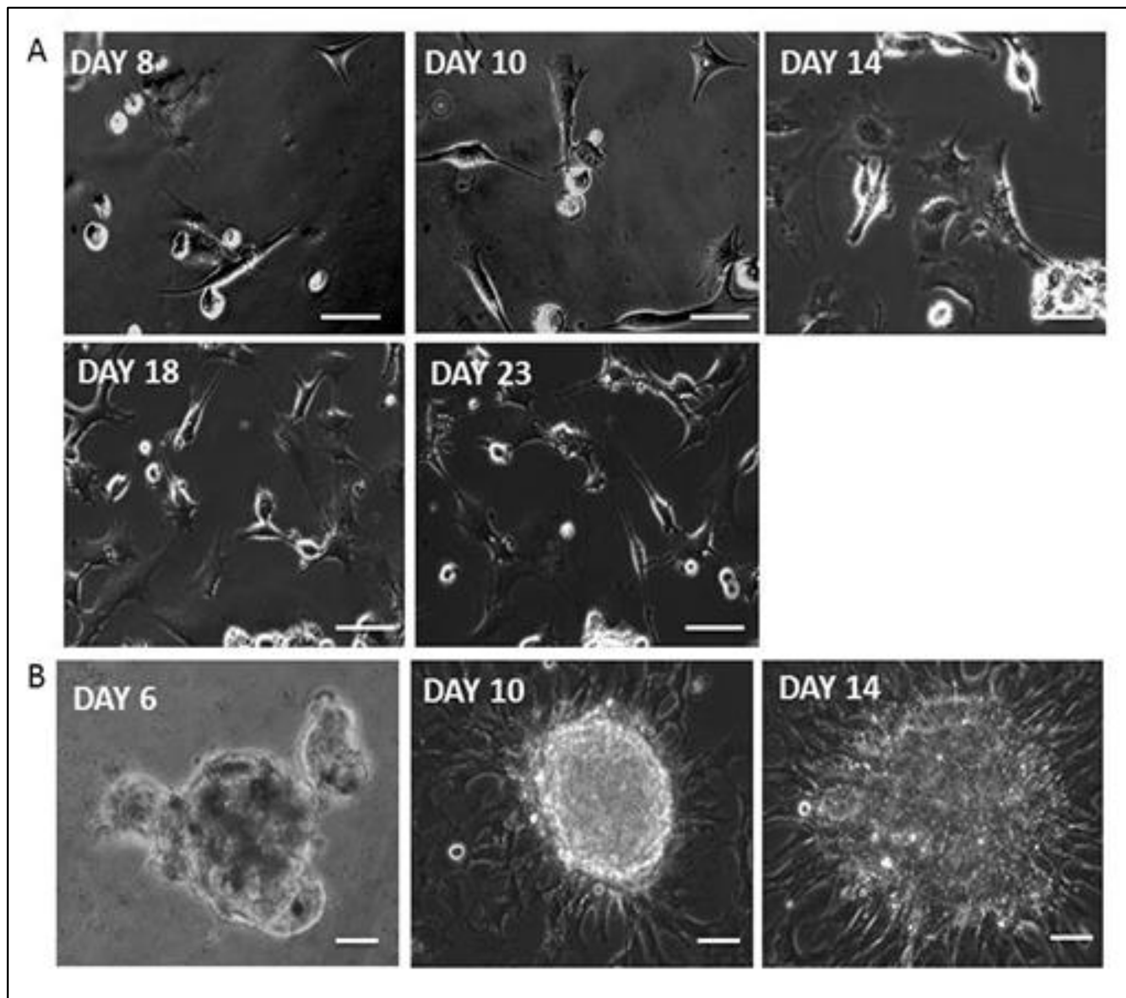



*Figure 8.2: Differentiation potential and Migration capacity of CPE-NS encapsulated in 1mg/ml of SAPNs. A: Phase contrast microscopic images of the dissociated CPE-NS exposed to differentiation conditions revealing neuronal morphology. The differentiated cells encapsulated in 1mg/ml of SAPNs at various time points - 8th Day, 10th Day, 14th Day and 23rd Day. Scale bar = 20µm. B: Migration capacity of CPE-NS after 7 days encapsulated in 1mg/ml of SAPNs. Phase-contrast images of CPE derived neurospheres at 6th days, 10th day and 14th day. A much higher proportion of migrating cells was observed from each sphere after 10th day in medium with differentiating conditions Scale bar =10µm.* (Published in *Jasty S, Suriyanarayanan S, Krishnakumar S. Influence of self-assembling peptide nanofibre scaffolds on retinal differentiation potential of stem/progenitor cells derived from ciliary pigment epithelial cells. J Tissue Eng Regen Med. 2014 Jul 28.)*

### 8.3.2. 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 *(Figure 8.3)*. 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.

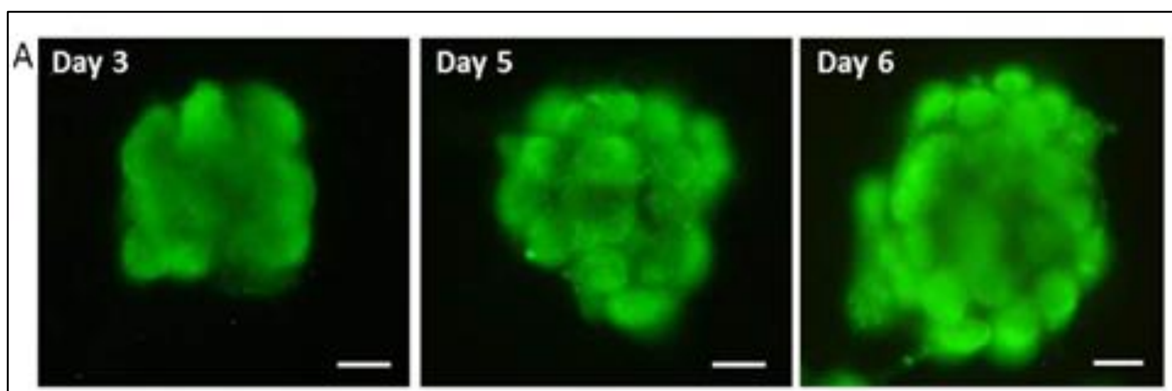



*Figure 8.3: Proliferative potential of the neurospheres cultured encapsulated in SAPNs. A: Proliferative potential as indicated by BrdU labeling immunofluorescence assay of the CPE derived neurosphere encapsulated in SAPNs at Day 3, Day 5 and Day 6.* (Published in *Jasty S, Suriyanarayanan S, Krishnakumar S. Influence of self-assembling peptide nanofibre scaffolds on retinal differentiation potential of stem/progenitor cells derived from ciliary pigment epithelial cells. J Tissue Eng Regen Med. 2014 Jul 28.)*

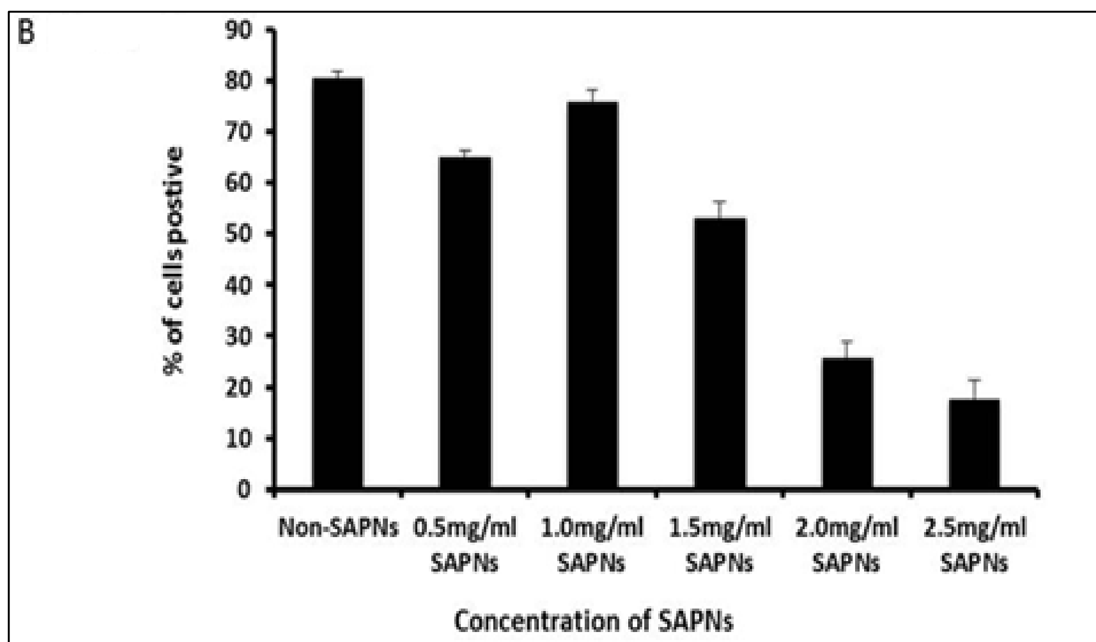



*Figure 8.3: Proliferative potential of the neurospheres cultured encapsulated in SAPNs. B: Proliferative potential as indicated by BrdU labeling of the CPE neurosphere cultured in various concentrations of the SAPNs. The Plot represents the average total number of cells that are BrdU labelled at various concentrations of the SAPNs (n=3).* (Published in *Jasty S, Suriyanarayanan S, Krishnakumar S. Influence of self-assembling peptide nanofibre scaffolds on retinal differentiation potential of stem/progenitor cells derived from ciliary pigment epithelial cells. J Tissue Eng Regen Med. 2014 Jul 28.)*

### 8.3.3. 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 *(Figure 8.2)*. 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 *(Figure 8.4 A and B)*.

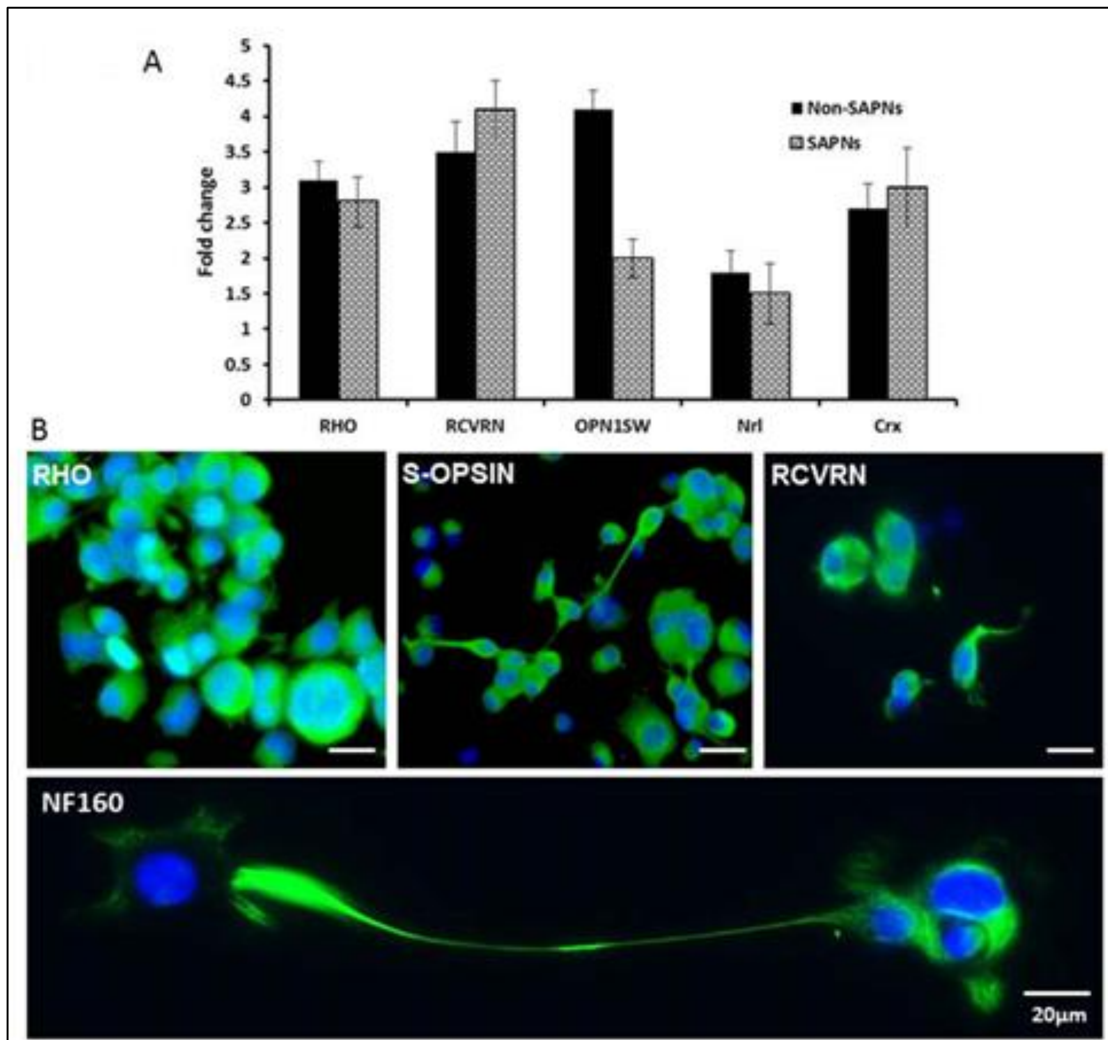



*Figure 8.4: Differentiation potential of CPE derived neurospheres encapsulated in 1mg/ml of SAPNs. CPE derived Primary neurospheres were dissociated and cultured encapsulated in SAPNs in tissue culture plates under differentiation conditions for 21 days. A: The dissociated cells expand in SAPNs which was analysed by Real time - PCR for the retinal cell markers like Rhodopsin, Recoverin, s-Opsin, Nrl and Crx (n=3) with and without SAPNs. B: The dissociated cells expand in SAPNs which was immuno-stained for the presence of Rhodopsin, s-Opsin, Recoverin, and Neural filament (NF) 160. Nuclei were labelled with DAPI. Scale bar = 10µm.* (Published in *Jasty S, Suriyanarayanan S, Krishnakumar S. Influence of self-assembling peptide nanofibre scaffolds on retinal differentiation potential of stem/progenitor cells derived from ciliary pigment epithelial cells. J Tissue Eng Regen Med. 2014 Jul 28.)*

### 8.3.4. 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 tabulated *(Table 8.1 & 8.2)* 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.

| Symbol | p-value | Fold Change | Symbol | p-value | Fold Change |
|---|---|---|---|---|---|
| LTBP3 | 0.01 | 2.40 | SP1 | 0.00 | -2.46 |
| SMAD7 | 0.01 | 2.32 | FZD7 | 0.02 | -2.53 |
| ENG | 0.01 | 2.28 | STAT3 | 0.00 | -2.71 |
| HPRT1 | 0.03 | -1.37 | GLI3 | 0.03 | -2.74 |
| E2F5 | 0.01 | -1.66 | NFATC3 | 0.01 | -2.79 |
| NFATC1 | 0.02 | -1.66 | LRP6 | 0.03 | -2.93 |
| PSENEN | 0.01 | -1.68 | SMAD2 | 0.01 | -2.94 |
| CTNNB1 | 0.01 | -1.69 | CREBBP | 0.01 | -2.94 |
| IL6ST | 0.01 | -1.70 | VANGL2 | 0.00 | -3.06 |
| NCSTN | 0.01 | -1.74 | SMAD3 | 0.03 | -3.08 |
| SMAD9 | 0.01 | -1.75 | SMAD5 | 0.00 | -3.11 |
| TCF7L2 | 0.01 | -1.81 | NFAT5 | 0.05 | -3.38 |
| PYGO2 | 0.00 | -1.82 | PTCH1 | 0.00 | -3.52 |
| LTBP1 | 0.01 | -1.85 | FZD4 | 0.00 | -3.93 |
| TGFBR2 | 0.01 | -1.85 | LIFR | 0.00 | -4.03 |
| BMPR1A | 0.01 | -1.92 | NOTCH4 | 0.00 | -4.05 |
| FGFR4 | 0.01 | -2.00 | FGFR1 | 0.00 | -4.24 |
| LTBP2 | 0.01 | -2.01 | RBL2 | 0.00 | -4.49 |
| EP300 | 0.01 | -2.04 | TGFBR3 | 0.00 | -7.45 |
| FZD3 | 0.01 | -2.05 | FZD2 | 0.00 | -7.62 |
| SMAD4 | 0.03 | -2.36 | BMPR1B | 0.00 | -7.79 |
| PSEN2 | 0.01 | -2.39 | FGFR2 | 0.00 | -11.39 |
| TGFBRAP1 | 0.01 | -2.41 | | | |

*Table 8.1: Stem cell signaling molecules gene expression profiling for of the CPE-NS in 1mg/ml of SAPNs* (Published in *Jasty S, Suriyanarayanan S, Krishnakumar S. Influence of self-assembling peptide nanofibre scaffolds on retinal differentiation potential of stem/progenitor cells derived from ciliary pigment epithelial cells. J Tissue Eng Regen Med. 2014 Jul 28.)*

| Symbol | p-value | Fold Change | Symbol | p-value | Fold Change |
|---|---|---|---|---|---|
| COL1A1 | 0.01 | 38.17 | RPL13A | 0.03 | -1.42 |
| MMP1 | 0.00 | 23.41 | SGCE | 0.05 | -1.60 |
| FN1 | 0.03 | 20.87 | VCAN | 0.05 | -1.69 |
| TNC | 0.02 | 20.55 | HPRT1 | 0.01 | -1.72 |
| TGFBI | 0.00 | 14.80 | COL8A1 | 0.03 | -1.77 |
| ITGA7 | 0.02 | 9.46 | COL12A1 | 0.02 | -2.17 |
| ITGA5 | 0.00 | 7.49 | ITGA6 | 0.05 | -2.21 |
| ITGB1 | 0.00 | 6.56 | CTNNB1 | 0.00 | -2.21 |
| ITGA3 | 0.00 | 6.32 | ITGA4 | 0.00 | -2.31 |
| MMP14 | 0.04 | 6.11 | NCAM1 | 0.04 | -2.77 |
| TIMP1 | 0.00 | 4.08 | ITGB4 | 0.01 | -2.79 |
| MMP2 | 0.01 | 3.91 | ITGA8 | 0.03 | -2.80 |
| MMP12 | 0.00 | 3.66 | SPG7 | 0.03 | -2.82 |
| MMP3 | 0.01 | 2.92 | CLEC3B | 0.04 | -3.51 |
| CD44 | 0.05 | 2.90 | CTNND2 | 0.00 | -3.64 |
| SPARC | 0.01 | 2.62 | SPP1 | 0.00 | -4.62 |
| ICAM1 | 0.01 | 2.50 | ITGB2 | 0.00 | -5.21 |
| MMP9 | 0.03 | 2.20 | VTN | 0.00 | -5.66 |
| ITGB5 | 0.02 | 1.88 | ITGA1 | 0.00 | -6.37 |
| MMP8 | 0.03 | 1.83 | SELL | 0.00 | -7.71 |
| ACTB | 0.02 | 1.83 | CTGF | 0.00 | -8.27 |
| THBS2 | 0.04 | -1.39 | SELP | 0.00 | -50.35 |

*Table 8.2: Extracellular Matrix & Adhesion Molecules gene expression profiling for CPE-DC in 1mg/ml of SAPNs.* (Published in *Jasty S, Suriyanarayanan S, Krishnakumar S. Influence of self-assembling peptide nanofibre scaffolds on retinal differentiation potential of stem/progenitor cells derived from ciliary pigment epithelial cells. J Tissue Eng Regen Med. 2014 Jul 28.)*

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.

## 8.4. Conclusion:

In conclusion, our data 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 modification of the cellular characteristics. These characteristics allow SAPN scaffolds to be the potential source for tissue engineering strategies and to meet 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 stem cell cultures.

# CHAPTER 9: 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.

# CHAPTER 10: 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.

# Future Scope of Work

The study conducted here has produced a number of interesting and novel findings and offered some valuable clues as to how iris and ciliary derived stem/progenitor proliferate and differentiate into retinal progeny *in vitro*. In addition, it has contributed much into molecular mechanisms involved in this process of differentiation, indicating the potential of the iris and ciliary derived stem/progenitor cells in treating the retinal degenerative disorders. However, application of iris and ciliary derived stem/progenitor cells in cell based therapy and regenerative medicine still rely on necessary progresses of basic research regarding the molecular basis of their stability, fate determination, integration potential and plasticity. Importantly, future work in the field may also provide knowledge relevant to targeting cancer stem cells in various ocular tumors. Significant advances are expected from relatively recent aspects of stem cell biology such as miRNA gene regulation and much details about epigenetic regulation. The study of the molecular and cellular composition of iris or ciliary stem/progenitor cell niche is also of utmost importance. Much more work on the complex 3D-culture systems and biomaterials as carriers or substrate matrices, should for instance allow to improve conditions for in vitro SCs differentiation and implantation of cellular progeny into injured or degenerating retinas. As a whole, it is hoped that this work will contribute to the current knowledge of eye development and retinal stem cell biology and that future work will unravel other unexpected aspects of iris and ciliary stem/progenitor cell biology, which will be relevant to set up therapies for retinal degenerative disorders. .

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# APPENDICES I

1. **Hanks balanced salt solution (with $Ca^{2+}$ and $Mg^{2+}$):**

   CaCl2 – 1.3mM

   MgCl2 – 0.5mM

   MgSO4 – 0.4mM

   KCl – 5.4mM

   KH2PO4 – 0.45mM

   NaHCO3 – 4.16mM

   NaCl – 138.0mM

   Na2HPO4 anhydrous – 0.33mM

   D-Glucose (Dextrose) – 5.6mM

   Phenol Red – 0.026mM

2. **Hanks balanced salt solution (without $Ca^{2+}$ and $Mg^{2+}$):**

   KCl – 5.4mM

   KH2PO4 – 0.5mM

   NaHCO3 – 4.2mM

   NaCl – 138.0mM

   Na2HPO4 anhydrous – 0.4mM

   D-Glucose (Dextrose) – 5.6mM

   Phenol Red – 0.026mM

3. **Phosphate buffered saline (with $Ca^{2+}$ and $Mg^{2+}$):**

   CaCl2 – 1.0mM

   MgCl2 – 0.5mM

   KCl – 2.67mM

   KH2PO4 – 1.5mM

   NaCl – 138.0mM

   Na2HPO4 – 8.0mM

4. **Phosphate buffered saline (without $Ca^{2+}$ and $Mg^{2+}$):**

   KCl – 2.67mM

   KH2PO4 – 1.47mM

   NaCl – 137.9mM

Na2HPO4 – 8.0mM

5. **Transport medium:**

DMEM (Dulbecco's minimum essential medium)

3% Fetal bovine serum

1X antibiotic and antimycotic mixture

6. **0.02% Trypsin solution:**

Trypsin – 0.02gms

EDTA – 0.03gms

Dextrose – 0.5gms

Dissolved in 100ml of Hanks balanced salt solution without $Ca^{2+}$ and $Mg^{2+}$or Phosphate buffered saline without $Ca^{2+}$ and $Mg^{2+}$.

7. **Neurosphere medium:**

DMEM + F12 medium

1X N2 supplement

L –Glutamine – 2mM

EGF – 20ng/mL

FGF – 10ng/mL

1X antibiotic and antimycotic mixture

8. **Differentiation medium:**

DMEM + F12 medium

1X N2 supplement

L –Glutamine – 2mM

BDNF - 1ng/ml

Retionic acid -1mM/ml

Antibiotic and antimycotic mixture – 1x concentration.

9. **4%Paraformaldehyde:**

Paraformaldehyde – 4gms

1xPhoshate buffered saline – 100ml

Heated at 70$^{o}$C in a fume hood until dissolved. Cooled to room temperature. pH adjusted to 7.4 using 0.1M NaOH or 1NHCl.

This solution was filter sterilized

10. **2% Agarose gel:**

Add 0.5g Agarose to 25 ml 1xTAE/0.5xTBE buffer.

***Tris-Acetate electrophoresis buffer (50x):***

Tris acetate - 2.0 M

EDTA - 0.05 M, pH 8.2 - 8.4

Dilute 10ml of 50x buffer with 490ml of distilled $H_2O$ to prepare 1xbuffer.

***Tris-Borate electrophoresis buffer (5x):***

Tris borate - 0.445 M

EDTA - 0.01 M, pH 8.2 - 8.4

Dilute 50ml of 5x buffer with 450ml of distilled $H_2O$ to prepare 0.5xbuffer.

**11. Bathing solution:**

KCl – 44mM

NaCl – 3mM

HEPES – 5mM

EGTA – 3mM

MgCl2 – 3mM

CaCl2 – 1mM

Glucose – 2mM

ATP – 1mM

GTP – 1mM

Reduced glutathione – 1mM, (pH 7.2)

**12. Oxygenated solution:**

NaCl – 140mM

KCl – 5mM

CaCl2 – 2mM

MgCl2 – 1mM

HEPES – 10mM

Glucose – 10mM

**13. Saline solution (Tyrode's solution):**

NaCl – 119mM

KCl – 2.5mM

$MgCl_2$ – 4mM

Glucose – 30mM

HEPES – 25mM

$CaCl_2$ – 2mM

AP-5 - 25μM

CNQX - 10μM

**14. Depolarizing solution:**

KCl – 90mM

NaCl – 29mM

$CaCl_2$ – 2mM

$MgCl_2$ – 2mM

Glucose – 30mM

HEPES - 25mM

**15. Formaldehyde Solution:**

1M HEPES-KOH, pH7.5 – 2.5ml

5M NaCl – 1.0ml

0.5M EDTA – 100.0ml

0.5M EGTA – 50.0ml

37% Formaldehyde – 14.9ml

Double distilled H2O – 31.5ml

**16. Primary fixative:**

2.5% Glutaraldehyde prepared in 0.1M Sodium cacodylate buffer at pH 7.2 to 7.4.

**17. Secondary fixative:**

0.1% Osimum tetroxide prepared in 0.1M Sodium cacodylate buffer at pH 7.2 to 7.4.

# APPENDICES II

| Sl. No. | CONSUMABLES | COMPANY |
|---|---|---|
| 1 | 0.2, 0.5, 1.5, 2.0ml vials | Axygen |
| 2 | 1000-100bp ladder | Bangalore Genei |
| 3 | 3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphoricacid (CPP) | Sigma-Aldrich |
| 4 | 5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione (MNQX) | Sigma-Aldrich |
| 5 | 5-bromo-2’-deoxy-uridine (BrdU) kit | Roche |
| 6 | 5-methylcytosin antibody | Diagnode |
| 7 | 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) | Sigma-Aldrich |
| 8 | 6well, 12well, 24well, 96well plates | BD Bioscience |
| 9 | ABCG2 antibody | Chemicon |
| 10 | Acetone | Sisco Research Laboratories Pvt. Ltd. |
| 11 | ADVASEP-7 (sulfobutyl b-cyclodextrin) | Sigma-Aldrich |
| 12 | Agarose | Sisco Research Laboratories Pvt. Ltd. |
| 13 | Anti-Mouse FITC | Sigma-Aldrich |
| 14 | Anti-Mouse TRITC | Sigma-Aldrich |
| 15 | Anti-Rabbit FITC | Sigma-Aldrich |
| 16 | Anti-Rabbit TRITC | Sigma-Aldrich |
| 17 | Beta actin antibody | Sigma-Aldrich |
| 18 | Beta III tubulin antibody | Covance |
| 19 | Bovine serum Albumin | Hi-Media |
| 20 | Brain derived neurotrophic factor (BDNF) | Sigma-Aldrich |
| 21 | Coverslips | Hi-Media |
| 22 | Cytokeratin antibody | Sigma-Aldrich |
| 23 | DAPI | Molecular probes |
| 24 | Dispase | BD Bioscience |

| | | |
|---|---|---|
| 25 | DMEM with High glucose | GIBCO |
| 26 | DMEM/F12 with High glucose | GIBCO |
| 27 | dNTPs | Bangalore Genei |
| 28 | Epidermal growth Factor (EGF) | Sigma-Aldrich |
| 29 | Ethidium bromide | Sigma-Aldrich |
| 30 | Fetal bovine serum | GIBCO |
| 31 | Fibroblast growth Factor (bFGF) | Sigma-Aldrich |
| 32 | Fluo-3AM | Molecular probes |
| 33 | FM-143 dye | Molecular probes |
| 34 | Gene chip Whole Transcript (WT) Target labeling Assay, Array, Hybridization, Washing and staining kit | Affymetrix , Inc. |
| 35 | GFAP antibody | Sigma-Aldrich |
| 36 | Glycine | Sigma-Aldrich |
| 37 | H3K27me3 antibody | Invitrogen |
| 38 | H3K4me3 antibody | Invitrogen |
| 39 | Hanks Balanced salt solution | GIBCO |
| 40 | Immunoprecipitation of Chromatin (ChIP), Array, Sample labelling, Hybridization, Wash and staining kit | Agilent technologies |
| 41 | Kainic acid | Sigma-Aldrich |
| 42 | Laminin | Sigma-Aldrich |
| 43 | Methanol | Sisco Research Laboratories Pvt. Ltd. |
| 44 | Methylated DNA Immunoprecipitation (MeDIP), Array, Sample labelling, Hybridization, wash and staining kit | Agilent technologies |
| 45 | Musashi-1 antibody | Covance |
| 46 | Nestin antibody | Chemicon |
| 47 | Neuralfilament 160 antibody | Sigma-Aldrich |
| 48 | N-methyl-D-aspartate (NMDA) | Sigma-Aldrich |
| 49 | Other organic and Inorganic chemicals | Merck |
| 50 | Paraformaldehyde | Sigma-Aldrich |

| | | |
|---|---|---|
| 51 | Pax6 antibody | Covance |
| 52 | Petriplates (30mm) | BD Bioscience |
| 53 | Phosphate buffered saline | GIBCO |
| 54 | Pluronic F127 | Sigma-Aldrich |
| 55 | Poly D-Lysine | Sigma-Aldrich |
| 56 | Primer sets | Sigma-Aldrich |
| 57 | Propidium iodide | Sigma-Aldrich |
| 58 | Puramatrix | BD Bioscience |
| 59 | QIAamp DNA Mini Kit | Qiagen |
| 60 | Recoverin antibody | Gift |
| 61 | Retinoic acid | Sigma-Aldrich |
| 62 | Rhodopsin antibody | Sigma-Aldrich |
| 63 | RNA 6000 Nano Kit | Agilant technologies |
| 64 | RNA Pol II antibody | Invitrogen |
| 65 | RNA Polymerase | Bangalore Genei |
| 66 | RNeasy mini kit | Qiagen |
| 67 | RT2 profiler arrays | SA Bioscience |
| 68 | Sensiscript RT Kit | Qiagen |
| 69 | S-opsin antibody | Abcam |
| 70 | Syber green Master mix | SA Biosciences |
| 71 | TE buffer | Bangalore Genei |
| 72 | Triton X-100 | Sigma-Aldrich |
| 73 | Trizol reagent | Qiagen |
| 74 | Trypsin | Invitrogen |
| 75 | Vimantin antibody | Sigma-Aldrich |
| 76 | α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) | Sigma-Aldrich |
| 77 | Other Plastic ware and glassware | GVI Scientific & Science House |

## APPENDICES III
### Bivalant genes (H3K4me3 and H3K27me3) enriched in the CPE-NS

| | | H3K4me3 | | | | H3K27me3 | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Primary Annotation** | **Primary Annotation Type** | **NS1** | **P[Xbar]** | **NS2** | **P[Xbar]** | **NS1** | **P[Xbar]** | **NS2** | **P[Xbar]** |
| 04-Sep | PROMOTER | 5.38 | 0.024 | 4.67 | 0.025 | 1.65 | 0.018 | 1.74 | 0.020 |
| AADACL4 | PROMOTER | 1.79 | 0.016 | 1.62 | 0.021 | 0.89 | 0.012 | 1.00 | 0.009 |
| ACTR6 | PROMOTER | 3.83 | 0.001 | 5.48 | 0.001 | 1.66 | 0.011 | 1.46 | 0.027 |
| ADCY8 | PROMOTER | 3.28 | 0.009 | 3.91 | 0.012 | 1.85 | 0.032 | 1.89 | 0.045 |
| ALAD | PROMOTER | 3.07 | 0.024 | 3.33 | 0.041 | 1.22 | 0.009 | 0.61 | 0.043 |
| AMBRA1 | PROMOTER | 2.16 | 0.005 | 2.38 | 0.006 | 1.00 | 0.019 | 0.85 | 0.022 |
| ANKRD17 | PROMOTER | 1.48 | 0.032 | 1.36 | 0.014 | 0.47 | 0.013 | 0.38 | 0.024 |
| ANKRD52 | DIVERGENT_PROMOTER | 0.67 | 0.034 | 0.68 | 0.028 | 0.57 | 0.010 | 0.53 | 0.009 |
| ARF4 | PROMOTER | 3.84 | 0.009 | 3.45 | 0.003 | 0.93 | 0.042 | 1.49 | 0.021 |
| ARL5A | PROMOTER | 2.88 | 0.024 | 2.94 | 0.024 | 0.24 | 0.042 | 0.30 | 0.046 |
| ATP1B4 | PROMOTER | 4.63 | 0.026 | 5.33 | 0.007 | 1.40 | 0.016 | 1.19 | 0.017 |
| BAI3 | PROMOTER | 1.33 | 0.024 | 1.35 | 0.028 | 0.10 | 0.034 | 0.20 | 0.028 |
| BCL6 | PROMOTER | 2.74 | 0.025 | 3.09 | 0.035 | 0.80 | 0.007 | 1.10 | 0.005 |
| BCO2 | PROMOTER | 1.92 | 0.029 | 1.88 | 0.038 | 1.26 | 0.040 | 1.21 | 0.029 |
| BIK | PROMOTER | 1.41 | 0.049 | 1.14 | 0.048 | 2.22 | 0.015 | 2.26 | 0.014 |
| BZRAP1 | PROMOTER | 2.75 | 0.013 | 2.54 | 0.030 | 0.89 | 0.009 | 1.15 | 0.005 |
| C12orf54 | PROMOTER | -0.51 | 0.040 | 0.46 | 0.013 | 1.60 | 0.050 | 1.55 | 0.036 |
| C19orf39 | PROMOTER | 2.76 | 0.020 | 3.08 | 0.019 | 2.90 | 0.008 | 3.00 | 0.014 |
| C1GALT1 | PROMOTER | 1.25 | 0.007 | 1.28 | 0.021 | 0.91 | 0.015 | 0.93 | 0.030 |
| C1orf106 | PROMOTER | 3.18 | 0.002 | 3.51 | 0.002 | 0.75 | 0.011 | 0.49 | 0.017 |
| C1orf133 | PROMOTER | 3.56 | 0.033 | 3.74 | 0.029 | 1.63 | 0.047 | 1.85 | 0.028 |
| C21orf63 | PROMOTER | 2.78 | 0.039 | 2.66 | 0.029 | 0.24 | 0.034 | 0.18 | 0.049 |
| C3orf26 | PROMOTER | 3.44 | 0.045 | 3.47 | 0.038 | 1.65 | 0.023 | 1.73 | 0.025 |
| C6orf105 | PROMOTER | 2.76 | 0.017 | 2.86 | 0.027 | 3.19 | 0.008 | 3.12 | 0.005 |
| C6orf129 | PROMOTER | 2.80 | 0.011 | 2.53 | 0.039 | 1.34 | 0.023 | 1.38 | 0.021 |
| C6orf154 | PROMOTER | 3.63 | 0.008 | 3.40 | 0.027 | 0.66 | 0.014 | 0.60 | 0.025 |
| C6orf226 | PROMOTER | 1.07 | 0.008 | 0.95 | 0.009 | 1.93 | 0.045 | 1.95 | 0.041 |
| C7orf55 | PROMOTER | 2.63 | 0.029 | 2.59 | 0.013 | 0.74 | 0.011 | 0.83 | 0.008 |
| C8orf85 | PROMOTER | 3.73 | 0.029 | 4.10 | 0.032 | 1.77 | 0.041 | 1.86 | 0.038 |
| CALCA | PROMOTER | 1.87 | 0.011 | 0.97 | 0.022 | 0.83 | 0.045 | 0.85 | 0.030 |
| CAMP | PROMOTER | 1.96 | 0.006 | 2.22 | 0.005 | 0.57 | 0.024 | 0.53 | 0.034 |
| CAPNS1 | PROMOTER | 3.11 | 0.017 | 3.68 | 0.018 | 3.50 | 0.022 | 2.22 | 0.039 |
| CARD9 | PROMOTER | 4.09 | 0.016 | 4.36 | 0.008 | 1.57 | 0.035 | 1.62 | 0.024 |
| CASD1 | PROMOTER | 5.40 | 0.002 | 5.75 | 0.004 | 6.61 | 0.015 | 2.88 | 0.027 |
| CASP14 | PROMOTER | 2.03 | 0.044 | 2.10 | 0.035 | 0.95 | 0.035 | 0.89 | 0.033 |
| CASQ2 | PROMOTER | 2.78 | 0.003 | 2.89 | 0.004 | 0.89 | 0.032 | 0.84 | 0.031 |
| CCDC136 | PROMOTER | 1.43 | 0.026 | 1.58 | 0.019 | 0.36 | 0.044 | 0.44 | 0.050 |
| CCDC28B | PROMOTER | 1.31 | 0.004 | 0.96 | 0.012 | 0.07 | 0.038 | 0.27 | 0.018 |
| CCDC42B | PROMOTER | 3.20 | 0.004 | 3.05 | 0.005 | 1.90 | 0.013 | 2.01 | 0.012 |
| CCDC71 | DIVERGENT_PROMOTER | 1.44 | 0.005 | 1.65 | 0.004 | 1.87 | 0.030 | 1.79 | 0.033 |
| CCDC99 | PROMOTER | 2.73 | 0.005 | 2.69 | 0.006 | 0.69 | 0.016 | 0.64 | 0.024 |
| CCL19 | PROMOTER | 2.56 | 0.001 | 2.85 | 0.001 | 0.65 | 0.014 | 0.74 | 0.009 |
| CCL7 | PROMOTER | 1.70 | 0.008 | 1.78 | 0.011 | 1.64 | 0.008 | 1.62 | 0.017 |
| CDX1 | DIVERGENT_PROMOTER | 2.75 | 0.019 | 2.80 | 0.017 | 0.74 | 0.038 | 0.58 | 0.038 |
| CENPM | PROMOTER | 2.17 | 0.040 | 2.17 | 0.047 | 1.80 | 0.005 | 1.61 | 0.009 |
| CEP350 | PROMOTER | 1.95 | 0.011 | 2.05 | 0.017 | 1.97 | 0.002 | 2.04 | 0.002 |
| CHIC2 | PROMOTER | 2.74 | 0.036 | 2.37 | 0.044 | 1.09 | 0.010 | 1.07 | 0.008 |
| CLDN10 | PROMOTER | 3.64 | 0.020 | 3.28 | 0.034 | 1.15 | 0.025 | 1.25 | 0.019 |
| CLDN16 | PROMOTER | 3.95 | 0.020 | 3.87 | 0.027 | 0.74 | 0.046 | 0.55 | 0.041 |
| CLDN18 | PROMOTER | 4.62 | 0.032 | 4.36 | 0.028 | 0.88 | 0.010 | 0.95 | 0.011 |
| CLEC4A | PROMOTER | 3.81 | 0.016 | 3.85 | 0.026 | 1.05 | 0.018 | 0.89 | 0.011 |
| CNKSR1 | PROMOTER | 2.42 | 0.035 | 2.71 | 0.027 | 1.12 | 0.044 | 1.19 | 0.030 |
| CNTNAP4 | PROMOTER | 1.25 | 0.049 | 1.23 | 0.013 | 1.46 | 0.047 | 1.31 | 0.048 |
| COL1A1 | PROMOTER | 2.46 | 0.004 | 2.72 | 0.004 | 1.15 | 0.015 | 1.15 | 0.029 |
| COL1A2 | PROMOTER | 0.87 | 0.004 | 1.53 | 0.002 | 1.21 | 0.018 | 1.06 | 0.019 |
| COPA | PROMOTER | 3.05 | 0.008 | 2.89 | 0.010 | 0.95 | 0.017 | 0.88 | 0.018 |
| CPNE5 | PROMOTER | 0.45 | 0.027 | 0.42 | 0.025 | 0.87 | 0.018 | 0.85 | 0.016 |
| CRB3 | DIVERGENT_PROMOTER | 1.33 | 0.030 | 1.14 | 0.045 | 1.27 | 0.025 | 1.28 | 0.022 |
| CTU1 | PROMOTER | 1.99 | 0.015 | 1.24 | 0.044 | 1.01 | 0.028 | 0.87 | 0.032 |
| CYLD | PROMOTER | 1.19 | 0.022 | 2.05 | 0.003 | 1.02 | 0.010 | 0.86 | 0.011 |
| DCN | PROMOTER | 2.93 | 0.002 | 2.86 | 0.002 | 0.91 | 0.047 | 1.09 | 0.032 |
| DDR1 | PROMOTER | 1.87 | 0.043 | 2.01 | 0.034 | 1.32 | 0.022 | 1.28 | 0.022 |
| DDX3Y | PROMOTER | 1.42 | 0.033 | 3.23 | 0.012 | 1.78 | 0.026 | 2.02 | 0.011 |
| DEDD | PROMOTER | 4.69 | 0.011 | 4.75 | 0.011 | 2.62 | 0.023 | 2.78 | 0.013 |
| DHRS11 | PROMOTER | 4.97 | 0.037 | 4.32 | 0.031 | 1.28 | 0.024 | 1.50 | 0.017 |
| DLL1 | PROMOTER | 1.49 | 0.008 | 1.63 | 0.006 | 0.78 | 0.021 | 0.66 | 0.014 |
| DNAH12 | PROMOTER | -0.40 | 0.032 | 0.27 | 0.015 | 1.52 | 0.046 | 1.29 | 0.048 |
| DNAJC22 | DIVERGENT_PROMOTER | 1.40 | 0.009 | 1.51 | 0.011 | 1.53 | 0.015 | 1.51 | 0.009 |

| DUS4L | PROMOTER | 1.79 | 0.013 | 2.42 | 0.010 | 0.92 | 0.007 | 1.29 | 0.003 |
|---|---|---|---|---|---|---|---|---|---|
| ECH1 | PROMOTER | 2.67 | 0.005 | 2.31 | 0.026 | 1.96 | 0.050 | 1.84 | 0.047 |
| EGLN1 | PROMOTER | 3.49 | 0.003 | 3.57 | 0.002 | 1.65 | 0.018 | 1.32 | 0.020 |
| EIF2S1 | DIVERGENT_PROMOTER | 2.85 | 0.036 | 2.48 | 0.033 | 1.53 | 0.006 | 1.25 | 0.009 |
| ELF1 | PROMOTER | 3.65 | 0.032 | 3.66 | 0.024 | 2.15 | 0.004 | 1.78 | 0.004 |
| EOMES | PROMOTER | 0.59 | 0.022 | 0.10 | 0.037 | 1.22 | 0.043 | 1.26 | 0.043 |
| EPN3 | PROMOTER | 3.45 | 0.006 | 3.26 | 0.011 | 1.31 | 0.018 | 1.38 | 0.020 |
| FABP12 | PROMOTER | 2.51 | 0.032 | 2.70 | 0.009 | 1.25 | 0.023 | 1.50 | 0.028 |
| FAM3B | PROMOTER | 1.43 | 0.021 | 2.07 | 0.023 | 0.69 | 0.025 | 0.86 | 0.017 |
| FBXO8 | PROMOTER | 2.20 | 0.003 | 2.24 | 0.007 | 1.10 | 0.039 | 1.45 | 0.031 |
| FEZ2 | PROMOTER | 1.74 | 0.048 | 1.81 | 0.043 | 0.68 | 0.011 | 0.89 | 0.002 |
| FGF4 | PROMOTER | 4.45 | 0.014 | 4.19 | 0.027 | 1.16 | 0.007 | 1.15 | 0.006 |
| FLCN | PROMOTER | 3.68 | 0.003 | 4.24 | 0.006 | 1.30 | 0.040 | 1.78 | 0.026 |
| GADD45A | PROMOTER | 5.01 | 0.000 | 3.93 | 0.001 | 0.65 | 0.022 | 0.44 | 0.031 |
| GATA6 | PROMOTER | 1.22 | 0.040 | 1.21 | 0.025 | 1.71 | 0.030 | 1.46 | 0.033 |
| GDA | PROMOTER | 3.27 | 0.028 | 3.20 | 0.006 | 1.74 | 0.013 | 1.92 | 0.011 |
| GEM | PROMOTER | 2.64 | 0.004 | 2.90 | 0.004 | 0.97 | 0.046 | 1.26 | 0.022 |
| GJA8 | PROMOTER | 2.14 | 0.014 | 2.05 | 0.028 | 0.63 | 0.046 | 1.04 | 0.013 |
| GLRA1 | PROMOTER | 3.86 | 0.014 | 4.56 | 0.018 | 1.23 | 0.041 | 1.69 | 0.023 |
| GP1BB | PROMOTER | 1.18 | 0.021 | 0.93 | 0.020 | 1.72 | 0.028 | 1.71 | 0.020 |
| GPR85 | PROMOTER | 2.58 | 0.004 | 2.40 | 0.013 | 0.60 | 0.031 | 0.54 | 0.046 |
| GRAMD4 | PROMOTER | 0.80 | 0.008 | 0.47 | 0.013 | 1.99 | 0.015 | 2.31 | 0.013 |
| GRAP2 | DIVERGENT_PROMOTER | 0.63 | 0.036 | 1.54 | 0.006 | 1.03 | 0.013 | 1.03 | 0.009 |
| GUCY2E | PROMOTER | 2.04 | 0.006 | 1.68 | 0.007 | 1.79 | 0.024 | 1.63 | 0.047 |
| H3F3A | PROMOTER | 2.49 | 0.014 | 2.27 | 0.012 | 1.62 | 0.033 | 1.50 | 0.033 |
| HDAC11 | PROMOTER | 3.55 | 0.015 | 3.22 | 0.008 | 1.40 | 0.012 | 1.24 | 0.024 |
| HDDC3 | DIVERGENT_PROMOTER | 1.72 | 0.010 | 1.81 | 0.009 | 1.88 | 0.011 | 2.02 | 0.006 |
| HECTD1 | PROMOTER | 1.73 | 0.001 | 1.51 | 0.002 | 2.58 | 0.004 | 1.88 | 0.004 |
| HIBADH | PROMOTER | -0.38 | 0.030 | 1.78 | 0.003 | 1.06 | 0.020 | 1.84 | 0.009 |
| HNRNPR | PROMOTER | 4.01 | 0.005 | 3.81 | 0.020 | 1.31 | 0.004 | 1.47 | 0.004 |
| HOXB9 | PROMOTER | 1.95 | 0.041 | 2.19 | 0.035 | 1.30 | 0.026 | 1.10 | 0.039 |
| HOXD3 | PROMOTER | 0.21 | 0.017 | 0.96 | 0.007 | 2.04 | 0.022 | 2.13 | 0.015 |
| HPCAL4 | PROMOTER | 0.56 | 0.015 | 0.56 | 0.014 | 1.48 | 0.002 | 1.75 | 0.001 |
| HPS4 | PROMOTER | 2.15 | 0.023 | 2.49 | 0.015 | 1.34 | 0.013 | 1.33 | 0.018 |
| HRCT1 | PROMOTER | 2.52 | 0.006 | 2.68 | 0.008 | 1.02 | 0.029 | 0.86 | 0.045 |
| HS6ST3 | PROMOTER | 2.09 | 0.006 | 1.84 | 0.011 | 1.80 | 0.008 | 1.66 | 0.009 |
| ID4 | PROMOTER | 2.82 | 0.010 | 1.91 | 0.009 | 1.13 | 0.022 | 1.55 | 0.013 |
| IFNA8 | PROMOTER | 2.90 | 0.046 | 2.60 | 0.048 | 2.26 | 0.007 | 2.45 | 0.007 |
| IFT57 | PROMOTER | 3.95 | 0.044 | 4.16 | 0.035 | 0.86 | 0.017 | 0.74 | 0.021 |
| IGFBP5 | PROMOTER | 3.48 | 0.007 | 3.42 | 0.009 | 0.90 | 0.026 | 0.75 | 0.033 |
| IGFBP7 | PROMOTER | 2.77 | 0.007 | 2.28 | 0.009 | 1.46 | 0.038 | 1.80 | 0.020 |
| IL1B | PROMOTER | 1.24 | 0.042 | 1.52 | 0.029 | 0.25 | 0.027 | -0.02 | 0.040 |
| IL1F7 | PROMOTER | 1.71 | 0.010 | 1.60 | 0.041 | -0.06 | 0.037 | 0.06 | 0.032 |
| IL5 | PROMOTER | 1.96 | 0.009 | 1.82 | 0.011 | 2.22 | 0.016 | 2.16 | 0.023 |
| INPP4B | PROMOTER | 3.52 | 0.025 | 3.54 | 0.024 | 1.94 | 0.005 | 1.53 | 0.009 |
| IRF6 | PROMOTER | 2.25 | 0.045 | 2.30 | 0.020 | 2.18 | 0.019 | 1.96 | 0.022 |
| ISCA1L | PROMOTER | 2.71 | 0.003 | 2.51 | 0.005 | 0.79 | 0.031 | 1.43 | 0.021 |
| ITGAM | PROMOTER | 1.51 | 0.005 | 0.82 | 0.018 | 1.80 | 0.013 | 1.91 | 0.017 |
| KBTBD12 | PROMOTER | 2.41 | 0.016 | 2.50 | 0.015 | 3.38 | 0.003 | 3.04 | 0.005 |
| KCNJ15 | PROMOTER | 2.26 | 0.005 | 2.24 | 0.002 | 1.69 | 0.002 | 1.58 | 0.005 |
| KCNMB1 | PROMOTER | 1.68 | 0.037 | 1.57 | 0.036 | 0.10 | 0.030 | 0.38 | 0.018 |
| KIAA1432 | PROMOTER | 1.01 | 0.010 | 1.01 | 0.010 | 0.06 | 0.038 | 0.01 | 0.041 |
| KIF16B | PROMOTER | 2.28 | 0.019 | 2.31 | 0.023 | 2.02 | 0.019 | 1.82 | 0.013 |
| KLF4 | PROMOTER | 1.67 | 0.044 | 1.83 | 0.017 | 1.09 | 0.044 | 1.16 | 0.027 |
| KLHDC7B | PROMOTER | 3.34 | 0.035 | 3.20 | 0.029 | 0.76 | 0.033 | 0.87 | 0.024 |
| KLHL38 | PROMOTER | 1.74 | 0.007 | 1.54 | 0.006 | 1.87 | 0.001 | 1.75 | 0.001 |
| LAMB3 | PROMOTER | 3.13 | 0.006 | 3.03 | 0.012 | 2.76 | 0.005 | 3.07 | 0.005 |
| LARS | PROMOTER | 3.66 | 0.003 | 3.89 | 0.003 | 0.93 | 0.036 | 1.00 | 0.047 |
| LGI2 | PROMOTER | 3.51 | 0.002 | 3.81 | 0.002 | 0.82 | 0.006 | 1.10 | 0.005 |
| LOC100101115 | PROMOTER | 2.21 | 0.024 | 2.34 | 0.018 | 1.55 | 0.006 | 1.64 | 0.008 |
| LPCAT3 | PROMOTER | 2.79 | 0.012 | 2.65 | 0.020 | 0.88 | 0.034 | 1.32 | 0.045 |
| LRP11 | PROMOTER | 2.75 | 0.023 | 2.46 | 0.033 | 0.10 | 0.020 | 0.19 | 0.022 |
| LRP4 | PROMOTER | 2.38 | 0.010 | 2.22 | 0.016 | 1.95 | 0.011 | 1.87 | 0.011 |
| LRRC30 | PROMOTER | 2.44 | 0.002 | 2.59 | 0.002 | 2.06 | 0.000 | 1.60 | 0.001 |
| LRRK1 | PROMOTER | 4.20 | 0.005 | 4.02 | 0.004 | 1.36 | 0.016 | 1.33 | 0.019 |
| LUM | PROMOTER | 4.82 | 0.011 | 5.76 | 0.006 | 2.82 | 0.011 | 2.51 | 0.023 |
| MAPKBP1 | PROMOTER | 2.77 | 0.004 | 2.92 | 0.004 | 0.60 | 0.023 | 0.50 | 0.033 |
| MBNL3 | PROMOTER | 5.23 | 0.003 | 5.50 | 0.002 | 1.62 | 0.033 | 1.62 | 0.033 |
| MCPH1 | PROMOTER | 3.68 | 0.002 | 4.37 | 0.002 | 1.74 | 0.005 | 1.98 | 0.004 |
| MIR106B | PROMOTER | 1.85 | 0.034 | 1.85 | 0.033 | 0.69 | 0.047 | 0.76 | 0.043 |
| MIR1280 | PROMOTER | 2.30 | 0.026 | 2.01 | 0.040 | 1.47 | 0.004 | 1.61 | 0.003 |
| MIR138-1 | PROMOTER | 3.22 | 0.017 | 3.32 | 0.039 | 1.80 | 0.034 | 1.54 | 0.029 |
| MIR1469 | PROMOTER | 3.52 | 0.001 | 3.32 | 0.002 | 1.67 | 0.017 | 1.85 | 0.008 |
| MIR1910 | PROMOTER | 4.11 | 0.002 | 4.01 | 0.003 | 2.59 | 0.007 | 2.38 | 0.008 |

| MIR1913 | PROMOTER | 4.76 | 0.019 | 5.97 | 0.014 | 4.99 | 0.001 | 2.43 | 0.004 |
|---|---|---|---|---|---|---|---|---|---|
| MIR200B | PROMOTER | 0.35 | 0.021 | 0.72 | 0.034 | 0.31 | 0.029 | 0.57 | 0.022 |
| MIR339 | PROMOTER | 2.52 | 0.016 | 2.50 | 0.026 | 0.63 | 0.030 | 0.33 | 0.041 |
| MIR374A | PROMOTER | 3.08 | 0.002 | 3.91 | 0.002 | 1.78 | 0.023 | 1.47 | 0.039 |
| MIR502 | PROMOTER | 1.49 | 0.014 | 1.39 | 0.006 | 0.84 | 0.017 | 1.04 | 0.013 |
| MIR942 | PROMOTER | 0.43 | 0.021 | 0.63 | 0.010 | 1.32 | 0.042 | 1.30 | 0.034 |
| MLXIPL | PROMOTER | 1.43 | 0.015 | 1.63 | 0.021 | 0.92 | 0.025 | 1.05 | 0.024 |
| MMP17 | PROMOTER | 3.12 | 0.014 | 2.58 | 0.017 | 1.45 | 0.032 | 1.85 | 0.025 |
| MOCS1 | PROMOTER | 2.99 | 0.015 | 2.62 | 0.018 | 2.37 | 0.012 | 2.52 | 0.012 |
| MORC2 | DIVERGENT_PROMOTER | 3.31 | 0.002 | 2.95 | 0.006 | 0.46 | 0.039 | 0.50 | 0.037 |
| MRAS | PROMOTER | 4.66 | 0.001 | 5.26 | 0.001 | 0.99 | 0.048 | 1.05 | 0.042 |
| MSMP | PROMOTER | 2.09 | 0.027 | 2.30 | 0.037 | 1.81 | 0.004 | 2.30 | 0.003 |
| MUS81 | DIVERGENT_PROMOTER | 1.83 | 0.024 | 1.89 | 0.011 | 0.90 | 0.039 | 1.03 | 0.025 |
| MYLK4 | PROMOTER | 3.44 | 0.030 | 2.91 | 0.036 | 0.44 | 0.026 | 1.01 | 0.008 |
| MYO18A | PROMOTER | 3.49 | 0.026 | 3.87 | 0.007 | 0.00 | 0.033 | 0.13 | 0.024 |
| NCKAP1L | PROMOTER | 2.40 | 0.010 | 1.84 | 0.021 | 1.95 | 0.012 | 4.50 | 0.010 |
| NDUFA13 | DIVERGENT_PROMOTER | 1.38 | 0.038 | 1.41 | 0.031 | 0.93 | 0.031 | 1.08 | 0.040 |
| NELL2 | PROMOTER | 6.42 | 0.043 | 5.57 | 0.017 | 0.47 | 0.038 | 0.57 | 0.035 |
| NEU3 | PROMOTER | 2.21 | 0.006 | 2.64 | 0.004 | 1.03 | 0.011 | 1.02 | 0.019 |
| NFASC | PROMOTER | 1.79 | 0.021 | 2.03 | 0.022 | 1.76 | 0.043 | 1.88 | 0.033 |
| NFE2L1 | DIVERGENT_PROMOTER | 2.31 | 0.040 | 1.96 | 0.040 | 1.77 | 0.005 | 2.33 | 0.006 |
| NFRKB | PROMOTER | 3.07 | 0.006 | 2.91 | 0.008 | 1.84 | 0.010 | 1.47 | 0.011 |
| NKX6-3 | PROMOTER | 4.03 | 0.020 | 4.06 | 0.014 | 3.12 | 0.032 | 3.64 | 0.034 |
| NLGN4Y | PROMOTER | 2.89 | 0.010 | 4.47 | 0.041 | 2.16 | 0.001 | 2.09 | 0.001 |
| NMD3 | PROMOTER | 0.80 | 0.043 | 1.54 | 0.020 | 1.13 | 0.035 | 1.03 | 0.034 |
| NME5 | PROMOTER | 1.20 | 0.022 | 2.82 | 0.007 | 1.72 | 0.039 | 1.98 | 0.037 |
| NOM1 | PROMOTER | 1.44 | 0.014 | 1.33 | 0.020 | 0.89 | 0.034 | 0.70 | 0.040 |
| NOS1AP | PROMOTER | 1.29 | 0.037 | 1.45 | 0.028 | 2.40 | 0.016 | 2.60 | 0.025 |
| NPSR1 | PROMOTER | 3.20 | 0.041 | 3.16 | 0.035 | 1.59 | 0.022 | 1.24 | 0.029 |
| NPY1R | PROMOTER | 0.48 | 0.036 | 0.40 | 0.024 | 1.98 | 0.002 | 2.17 | 0.001 |
| NRP2 | PROMOTER | 1.82 | 0.026 | 1.46 | 0.041 | 0.63 | 0.013 | 0.25 | 0.028 |
| ODZ4 | PROMOTER | 2.31 | 0.015 | 2.48 | 0.049 | 1.41 | 0.020 | 1.33 | 0.028 |
| OR10A5 | PROMOTER | 1.73 | 0.003 | 1.05 | 0.012 | 0.79 | 0.022 | 0.71 | 0.018 |
| OR10J1 | PROMOTER | 2.87 | 0.020 | 3.08 | 0.020 | 1.20 | 0.005 | 1.22 | 0.009 |
| OR10X1 | PROMOTER | 6.29 | 0.019 | 6.11 | 0.033 | 1.94 | 0.020 | 1.83 | 0.034 |
| OR13G1 | PROMOTER | 6.35 | 0.003 | 6.06 | 0.009 | 1.96 | 0.034 | 2.07 | 0.023 |
| OR56A3 | PROMOTER | 1.08 | 0.016 | 1.33 | 0.028 | 2.27 | 0.004 | 0.75 | 0.013 |
| OR5AU1 | PROMOTER | 3.05 | 0.014 | 0.85 | 0.037 | 0.47 | 0.045 | 0.81 | 0.018 |
| OR6S1 | PROMOTER | 0.66 | 0.038 | 1.09 | 0.028 | 1.96 | 0.029 | 1.80 | 0.027 |
| PCDH12 | DIVERGENT_PROMOTER | 5.83 | 0.000 | 5.13 | 0.000 | 0.97 | 0.026 | 1.00 | 0.025 |
| PCDH7 | PROMOTER | 1.01 | 0.012 | 0.97 | 0.012 | 0.81 | 0.004 | 0.65 | 0.005 |
| PCMTD1 | PROMOTER | 3.08 | 0.010 | 3.37 | 0.028 | 1.59 | 0.009 | 1.53 | 0.014 |
| PCP4 | PROMOTER | 0.79 | 0.035 | 2.79 | 0.010 | 0.98 | 0.037 | 1.06 | 0.036 |
| PDE12 | PROMOTER | 0.68 | 0.036 | 0.64 | 0.045 | 0.72 | 0.047 | 0.78 | 0.035 |
| PDE8A | PROMOTER | 1.98 | 0.003 | 1.95 | 0.004 | 0.80 | 0.030 | 0.84 | 0.032 |
| PGF | PROMOTER | 4.31 | 0.009 | 3.95 | 0.044 | 3.53 | 0.015 | 3.49 | 0.008 |
| PHLDA3 | PROMOTER | 1.94 | 0.022 | 1.83 | 0.030 | 0.89 | 0.011 | 0.69 | 0.021 |
| PHLDB1 | PROMOTER | 2.83 | 0.004 | 2.52 | 0.005 | 0.44 | 0.050 | 0.84 | 0.021 |
| PISRT1 | PROMOTER | 3.81 | 0.001 | 4.49 | 0.002 | 1.95 | 0.032 | 1.93 | 0.033 |
| PIWIL1 | PROMOTER | 2.02 | 0.011 | 0.51 | 0.039 | 2.04 | 0.020 | 2.34 | 0.043 |
| PLEC1 | PROMOTER | 2.97 | 0.003 | 2.94 | 0.003 | 1.51 | 0.003 | 1.54 | 0.003 |
| PMM1 | PROMOTER | 2.59 | 0.048 | 2.80 | 0.044 | 0.17 | 0.035 | 0.08 | 0.037 |
| PMS1 | PROMOTER | 1.89 | 0.032 | 1.71 | 0.015 | 0.91 | 0.033 | 0.71 | 0.044 |
| PODN | PROMOTER | 2.44 | 0.021 | 3.98 | 0.024 | 0.56 | 0.032 | 0.80 | 0.028 |
| PPFIBP1 | PROMOTER | 2.07 | 0.023 | 2.12 | 0.026 | -0.12 | 0.035 | -0.03 | 0.035 |
| PPP1R2 | PROMOTER | 1.61 | 0.021 | 1.65 | 0.040 | 1.38 | 0.011 | 1.51 | 0.021 |
| PRNT | PROMOTER | 3.81 | 0.007 | 3.58 | 0.015 | 0.67 | 0.027 | 0.91 | 0.017 |
| PRUNE2 | PROMOTER | 2.35 | 0.002 | 2.35 | 0.002 | 1.98 | 0.031 | 2.22 | 0.043 |
| PTGS2 | PROMOTER | 3.14 | 0.015 | 3.44 | 0.009 | 2.44 | 0.036 | 3.01 | 0.041 |
| PTPRE | PROMOTER | 0.99 | 0.047 | 1.06 | 0.045 | 1.45 | 0.024 | 1.42 | 0.029 |
| PVALB | PROMOTER | 3.56 | 0.006 | 3.45 | 0.003 | 3.02 | 0.001 | 2.78 | 0.001 |
| PVRL1 | PROMOTER | 0.29 | 0.031 | 0.56 | 0.022 | 1.72 | 0.022 | 1.59 | 0.027 |
| PXDNL | PROMOTER | 4.07 | 0.007 | 3.71 | 0.016 | 1.40 | 0.005 | 0.77 | 0.014 |
| QRICH1 | PROMOTER | 0.12 | 0.031 | 0.12 | 0.045 | 0.48 | 0.033 | 0.68 | 0.032 |
| RAB11FIP5 | PROMOTER | 2.08 | 0.048 | 2.30 | 0.015 | 0.43 | 0.034 | 0.40 | 0.041 |
| RAB1A | PROMOTER | 3.85 | 0.024 | 3.81 | 0.045 | 1.09 | 0.022 | 1.17 | 0.024 |
| RAB32 | PROMOTER | 0.69 | 0.029 | 1.22 | 0.024 | 0.83 | 0.031 | 0.71 | 0.033 |
| RAB43 | PROMOTER | 2.07 | 0.033 | 2.75 | 0.013 | 1.89 | 0.014 | 1.97 | 0.008 |
| RAB6B | PROMOTER | 2.65 | 0.010 | 2.40 | 0.010 | 2.14 | 0.016 | 2.27 | 0.016 |
| RAB9B | PROMOTER | 4.33 | 0.025 | 2.22 | 0.011 | 2.77 | 0.013 | 2.74 | 0.018 |
| REPIN1 | PROMOTER | 2.40 | 0.010 | 2.17 | 0.011 | 0.90 | 0.013 | 0.71 | 0.019 |
| RGS12 | PROMOTER | 3.59 | 0.015 | 3.50 | 0.011 | 0.77 | 0.004 | 0.75 | 0.003 |
| RNU4ATAC | PROMOTER | 3.57 | 0.027 | 3.19 | 0.042 | 1.87 | 0.004 | 1.83 | 0.005 |
| RPL27A | PROMOTER | 1.10 | 0.036 | 1.46 | 0.033 | 2.53 | 0.016 | 2.73 | 0.009 |

| RPS8 | DOWNSTREAM | 4.05 | 0.013 | 3.65 | 0.016 | 2.64 | 0.004 | 2.33 | 0.006 |
|---|---|---|---|---|---|---|---|---|---|
| RUNX1 | PROMOTER | 3.32 | 0.002 | 3.82 | 0.001 | 2.35 | 0.005 | 1.58 | 0.016 |
| S1PR1 | PROMOTER | 1.35 | 0.043 | 1.07 | 0.028 | 1.01 | 0.011 | 1.30 | 0.011 |
| SCARNA21 | PROMOTER | 1.69 | 0.006 | 1.20 | 0.014 | 0.88 | 0.044 | 0.95 | 0.021 |
| SCD | PROMOTER | 2.23 | 0.035 | 2.29 | 0.044 | 1.79 | 0.008 | 1.56 | 0.013 |
| SCN2A | PROMOTER | 4.60 | 0.003 | 4.98 | 0.002 | 0.78 | 0.008 | 1.19 | 0.006 |
| SCNN1A | DIVERGENT_PROMOTER | 2.90 | 0.004 | 2.89 | 0.004 | 1.07 | 0.029 | 1.14 | 0.025 |
| SCRN1 | PROMOTER | 2.24 | 0.010 | 2.47 | 0.002 | 0.49 | 0.041 | 0.88 | 0.014 |
| SDK1 | PROMOTER | 3.21 | 0.021 | 3.48 | 0.021 | 1.09 | 0.033 | 1.05 | 0.046 |
| SDR42E1 | PROMOTER | 3.06 | 0.007 | 4.15 | 0.002 | 1.41 | 0.026 | 1.43 | 0.018 |
| SEC61A1 | PROMOTER | 3.07 | 0.020 | 2.78 | 0.047 | 1.75 | 0.004 | 1.70 | 0.006 |
| SEMA4D | PROMOTER | 3.91 | 0.029 | 3.66 | 0.032 | 1.14 | 0.033 | 1.63 | 0.020 |
| SERPINA10 | PROMOTER | 3.83 | 0.006 | 3.76 | 0.004 | 2.79 | 0.021 | 3.46 | 0.018 |
| SFRS9 | DIVERGENT_PROMOTER | 5.60 | 0.001 | 4.90 | 0.001 | 1.44 | 0.034 | 1.29 | 0.034 |
| SHC1 | PROMOTER | 1.21 | 0.007 | 1.11 | 0.007 | 0.92 | 0.004 | 0.68 | 0.006 |
| SHISA4 | PROMOTER | 1.58 | 0.032 | 1.53 | 0.043 | 0.62 | 0.039 | 0.57 | 0.029 |
| SLC12A6 | PROMOTER | 4.18 | 0.012 | 3.89 | 0.016 | 1.85 | 0.004 | 1.88 | 0.005 |
| SLC26A1 | PROMOTER | 1.54 | 0.028 | 1.21 | 0.039 | 1.07 | 0.044 | 1.21 | 0.048 |
| SLC39A14 | PROMOTER | 2.14 | 0.045 | 1.79 | 0.038 | 0.92 | 0.025 | 1.09 | 0.029 |
| SMNDC1 | PROMOTER | 5.38 | 0.005 | 5.95 | 0.003 | 1.55 | 0.048 | 2.00 | 0.030 |
| SMTNL2 | PROMOTER | 2.65 | 0.016 | 2.69 | 0.040 | 1.29 | 0.039 | 1.06 | 0.029 |
| SNAPC3 | PROMOTER | 0.73 | 0.024 | 0.59 | 0.032 | 1.52 | 0.041 | 1.60 | 0.044 |
| SNORA48 | PROMOTER | 2.40 | 0.041 | 2.47 | 0.037 | 0.88 | 0.015 | 0.84 | 0.014 |
| SNORA76 | DOWNSTREAM | 1.08 | 0.008 | 0.88 | 0.011 | 0.13 | 0.034 | 0.08 | 0.037 |
| SNORD114-6 | PROMOTER | 3.86 | 0.018 | 3.42 | 0.031 | 1.93 | 0.014 | 1.70 | 0.021 |
| SNORD116-28 | PROMOTER | 3.00 | 0.001 | 2.91 | 0.029 | 2.49 | 0.027 | 1.69 | 0.043 |
| SNORD19 | PROMOTER | 1.09 | 0.015 | 0.48 | 0.048 | 1.03 | 0.040 | 1.17 | 0.033 |
| SNORD51 | PROMOTER | 2.54 | 0.024 | 2.71 | 0.021 | 1.92 | 0.019 | 1.87 | 0.017 |
| SNORD94 | PROMOTER | 3.13 | 0.002 | 3.26 | 0.003 | 2.07 | 0.003 | 2.16 | 0.007 |
| SPOCK2 | PROMOTER | 3.74 | 0.008 | 4.48 | 0.002 | 1.54 | 0.005 | 1.54 | 0.005 |
| SPRY1 | PROMOTER | 1.43 | 0.033 | 1.95 | 0.047 | 0.93 | 0.013 | 1.07 | 0.013 |
| SRCIN1 | PROMOTER | 4.25 | 0.024 | 4.71 | 0.016 | 1.00 | 0.034 | 0.94 | 0.040 |
| STAT6 | PROMOTER | 1.81 | 0.024 | 1.90 | 0.026 | 0.82 | 0.048 | 0.88 | 0.032 |
| SYT13 | PROMOTER | 3.43 | 0.040 | 3.48 | 0.034 | 2.48 | 0.000 | 2.43 | 0.000 |
| TAGLN3 | PROMOTER | 0.91 | 0.048 | 1.14 | 0.043 | 1.90 | 0.035 | 1.72 | 0.031 |
| TBCC | PROMOTER | 2.81 | 0.007 | 2.62 | 0.010 | 1.47 | 0.025 | 1.80 | 0.009 |
| TEF | PROMOTER | 2.63 | 0.005 | 2.55 | 0.007 | 1.17 | 0.016 | 1.26 | 0.025 |
| TFAP2A | PROMOTER | 2.12 | 0.032 | 2.27 | 0.009 | 0.89 | 0.012 | 1.14 | 0.006 |
| TGFB3 | DIVERGENT_PROMOTER | 1.72 | 0.002 | 1.23 | 0.004 | 1.82 | 0.045 | 1.96 | 0.049 |
| TGIF2LY | PROMOTER | 2.61 | 0.021 | 2.48 | 0.017 | 2.64 | 0.008 | 2.66 | 0.006 |
| THAP11 | PROMOTER | 4.53 | 0.010 | 2.93 | 0.008 | 0.45 | 0.020 | 0.79 | 0.022 |
| TINAGL1 | PROMOTER | 1.60 | 0.003 | 1.53 | 0.004 | 1.25 | 0.013 | 1.39 | 0.013 |
| TM9SF2 | PROMOTER | 0.97 | 0.012 | 1.05 | 0.016 | 1.30 | 0.021 | 1.29 | 0.023 |
| TMEM125 | PROMOTER | 0.85 | 0.012 | 0.92 | 0.017 | 1.05 | 0.031 | 1.09 | 0.024 |
| TMEM55B | DIVERGENT_PROMOTER | 1.86 | 0.027 | 2.03 | 0.032 | 1.15 | 0.009 | 1.08 | 0.018 |
| TMPRSS8 | PROMOTER | 3.14 | 0.009 | 2.91 | 0.026 | 0.26 | 0.022 | 0.58 | 0.014 |
| TRAF5 | PROMOTER | 1.79 | 0.044 | 3.04 | 0.030 | 0.78 | 0.015 | 0.91 | 0.013 |
| TREML1 | PROMOTER | 2.43 | 0.010 | 2.65 | 0.007 | 0.82 | 0.031 | 1.07 | 0.018 |
| TRH | PROMOTER | 2.03 | 0.018 | 1.62 | 0.030 | 1.18 | 0.005 | 1.19 | 0.007 |
| TRIM11 | PROMOTER | 2.85 | 0.008 | 2.50 | 0.011 | 0.85 | 0.039 | 0.89 | 0.031 |
| TRIM26 | PROMOTER | 1.95 | 0.019 | 1.74 | 0.029 | 0.57 | 0.022 | 0.70 | 0.023 |
| TRIM3 | PROMOTER | 4.11 | 0.006 | 4.14 | 0.013 | 1.73 | 0.022 | 1.76 | 0.039 |
| TTC32 | PROMOTER | 4.45 | 0.012 | 4.17 | 0.014 | 1.64 | 0.017 | 1.53 | 0.026 |
| TTPAL | PROMOTER | 1.22 | 0.012 | 1.13 | 0.009 | 0.08 | 0.022 | 0.11 | 0.022 |
| TTTY10 | PROMOTER | 1.63 | 0.014 | 1.68 | 0.029 | 0.91 | 0.001 | 0.71 | 0.002 |
| TTTY6B | PROMOTER | 1.85 | 0.009 | 1.77 | 0.016 | 1.81 | 0.002 | 1.61 | 0.002 |
| TTTY8 | PROMOTER | 0.93 | 0.039 | 0.90 | 0.035 | 0.99 | 0.031 | 0.95 | 0.038 |
| TUSC2 | PROMOTER | 3.25 | 0.004 | 3.20 | 0.008 | 1.38 | 0.024 | 1.43 | 0.019 |
| VIM | PROMOTER | 2.39 | 0.018 | 2.47 | 0.017 | 1.14 | 0.017 | 1.19 | 0.033 |
| WAPAL | PROMOTER | 1.77 | 0.021 | 2.03 | 0.020 | 1.73 | 0.025 | 1.42 | 0.030 |
| WDR37 | DIVERGENT_PROMOTER | 2.24 | 0.032 | 2.30 | 0.045 | 2.29 | 0.011 | 2.12 | 0.016 |
| WNT10A | PROMOTER | 1.07 | 0.009 | 1.01 | 0.010 | 1.26 | 0.042 | 1.39 | 0.029 |
| WNT2B | PROMOTER | 1.63 | 0.045 | 1.93 | 0.044 | 1.21 | 0.040 | 1.37 | 0.029 |
| XPO7 | DIVERGENT_PROMOTER | 1.10 | 0.012 | 1.22 | 0.019 | 1.70 | 0.006 | 2.20 | 0.002 |
| ZNF133 | PROMOTER | 3.80 | 0.003 | 3.95 | 0.004 | 2.26 | 0.004 | 2.34 | 0.012 |
| ZNF16 | PROMOTER | 2.57 | 0.017 | 2.57 | 0.027 | 0.89 | 0.010 | 0.95 | 0.018 |
| ZNF165 | PROMOTER | 3.93 | 0.011 | 4.17 | 0.009 | 1.68 | 0.045 | 1.58 | 0.042 |
| ZNF335 | PROMOTER | 1.93 | 0.004 | 2.17 | 0.003 | 0.33 | 0.041 | 0.49 | 0.040 |
| ZNF33B | PROMOTER | 4.09 | 0.018 | 4.32 | 0.018 | 0.72 | 0.026 | 0.81 | 0.009 |
| ZNF639 | PROMOTER | 1.93 | 0.017 | 1.43 | 0.028 | 3.02 | 0.031 | 3.01 | 0.046 |

# APPENDCES IV
## Bivalant genes (H3K4me3 and H3K27me3) enriched in the CPE-DC

| | | H3K4me3 | | | | H3K27me3 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Primary Annotation | Primary Annotation Type | ND1 | P[Xbar] | ND2 | P[Xbar] | ND1 | P[Xbar] | ND2 | P[Xbar] |
| 06-Mar | PROMOTER | 2.40 | 0.005 | 2.50 | 0.004 | 1.80 | 0.024 | 1.74 | 0.025 |
| A2BP1 | PROMOTER | 3.53 | 0.044 | 3.99 | 0.035 | 2.99 | 0.007 | 2.81 | 0.010 |
| ABCB4 | PROMOTER | 2.11 | 0.030 | 1.77 | 0.049 | 1.69 | 0.018 | 1.69 | 0.026 |
| ABCB9 | PROMOTER | 0.17 | 0.023 | 0.11 | 0.030 | 2.87 | 0.021 | 2.71 | 0.026 |
| ABLIM1 | PROMOTER | 0.62 | 0.044 | 0.40 | 0.017 | 2.08 | 0.020 | 2.33 | 0.021 |
| ACTR5 | PROMOTER | 5.11 | 0.010 | 5.55 | 0.004 | 1.82 | 0.029 | 1.75 | 0.039 |
| ADAM30 | PROMOTER | 3.49 | 0.002 | 3.40 | 0.005 | -0.21 | 0.047 | 0.17 | 0.042 |
| AGR3 | PROMOTER | 1.87 | 0.006 | 2.39 | 0.005 | 1.23 | 0.026 | 1.19 | 0.022 |
| AGTR2 | PROMOTER | 6.44 | 0.002 | 4.43 | 0.003 | 3.45 | 0.009 | 2.98 | 0.011 |
| AKR1B1 | PROMOTER | 2.86 | 0.022 | 2.80 | 0.050 | 1.86 | 0.016 | 1.87 | 0.011 |
| ALCAM | PROMOTER | 4.12 | 0.038 | 4.07 | 0.040 | 2.03 | 0.009 | 1.84 | 0.033 |
| ALG13 | PROMOTER | 3.17 | 0.026 | 2.85 | 0.028 | 2.00 | 0.016 | 2.65 | 0.008 |
| AMPH | PROMOTER | 2.33 | 0.048 | 2.64 | 0.017 | 1.73 | 0.017 | 1.48 | 0.032 |
| ANGPTL3 | PROMOTER | 4.12 | 0.033 | 3.97 | 0.043 | 2.65 | 0.011 | 2.39 | 0.012 |
| ANKRD33 | PROMOTER | 2.53 | 0.004 | 2.64 | 0.003 | 0.34 | 0.045 | 0.83 | 0.036 |
| ANLN | PROMOTER | -0.17 | 0.041 | -0.22 | 0.039 | 2.70 | 0.023 | 3.41 | 0.026 |
| ANO5 | PROMOTER | 0.94 | 0.049 | 3.08 | 0.040 | 3.28 | 0.007 | 2.70 | 0.033 |
| ANXA3 | PROMOTER | 1.71 | 0.042 | 1.26 | 0.045 | 2.25 | 0.041 | 2.43 | 0.026 |
| APEX2 | DIVERGENT_PROMOTER | 4.47 | 0.042 | 4.20 | 0.040 | 4.14 | 0.005 | 0.00 | 0.043 |
| APOBEC4 | PROMOTER | 2.45 | 0.002 | 2.59 | 0.001 | 2.98 | 0.014 | 2.70 | 0.023 |
| APOL6 | PROMOTER | 4.18 | 0.002 | 3.81 | 0.002 | 1.49 | 0.023 | 1.64 | 0.018 |
| AQP4 | PROMOTER | 3.73 | 0.005 | 3.66 | 0.005 | 4.62 | 0.002 | 5.29 | 0.003 |
| ARHGAP6 | PROMOTER | 2.42 | 0.026 | 2.49 | 0.034 | 2.57 | 0.011 | 2.32 | 0.009 |
| ARVCF | DIVERGENT_PROMOTER | 1.82 | 0.009 | 1.78 | 0.018 | 1.05 | 0.016 | 0.47 | 0.034 |
| ATP10A | PROMOTER | 0.93 | 0.024 | 0.93 | 0.023 | 1.28 | 0.010 | 1.34 | 0.012 |
| ATP2B2 | PROMOTER | 3.05 | 0.002 | 2.92 | 0.002 | 1.48 | 0.033 | 1.02 | 0.037 |
| ATP4B | DIVERGENT_PROMOTER | 2.82 | 0.006 | 2.85 | 0.005 | 1.58 | 0.028 | 1.66 | 0.022 |
| ATP8A1 | PROMOTER | 1.79 | 0.009 | 1.05 | 0.017 | 0.95 | 0.030 | 1.20 | 0.031 |
| ATP9A | PROMOTER | 3.64 | 0.039 | 3.64 | 0.013 | 2.25 | 0.032 | 2.59 | 0.023 |
| AXIN2 | PROMOTER | 6.20 | 0.001 | 5.72 | 0.001 | 2.10 | 0.036 | 1.84 | 0.047 |
| B3GALT1 | PROMOTER | 3.02 | 0.011 | 3.09 | 0.006 | 2.23 | 0.010 | 1.99 | 0.007 |
| BAI1 | PROMOTER | 1.22 | 0.044 | 1.42 | 0.033 | 4.11 | 0.042 | 4.46 | 0.030 |
| BATF3 | PROMOTER | 2.47 | 0.004 | 2.29 | 0.004 | 1.02 | 0.026 | 0.96 | 0.035 |
| BHLHB9 | PROMOTER | 1.67 | 0.043 | 1.63 | 0.044 | 1.50 | 0.019 | 1.12 | 0.025 |
| BMP15 | PROMOTER | 2.55 | 0.038 | 2.23 | 0.045 | 0.77 | 0.008 | 0.75 | 0.012 |
| BOLL | PROMOTER | 1.90 | 0.013 | 1.80 | 0.019 | 1.55 | 0.006 | 0.61 | 0.012 |
| C10orf10 | PROMOTER | -0.03 | 0.041 | 0.73 | 0.015 | -0.09 | 0.049 | 0.22 | 0.029 |
| C10orf107 | PROMOTER | 3.56 | 0.016 | 3.81 | 0.008 | 2.29 | 0.012 | 2.22 | 0.005 |
| C11orf42 | PROMOTER | 3.27 | 0.016 | 2.96 | 0.010 | 1.40 | 0.044 | 1.42 | 0.032 |
| C11orf83 | PROMOTER | 2.58 | 0.025 | 2.84 | 0.014 | 1.61 | 0.037 | 1.67 | 0.043 |
| C13orf26 | PROMOTER | 2.50 | 0.004 | 1.99 | 0.038 | 2.15 | 0.002 | 2.08 | 0.002 |
| C14orf23 | PROMOTER | 2.43 | 0.002 | 2.52 | 0.001 | 3.27 | 0.033 | 3.54 | 0.027 |
| C14orf48 | PROMOTER | 1.07 | 0.030 | 1.38 | 0.026 | 1.06 | 0.014 | 1.12 | 0.012 |
| C15orf27 | PROMOTER | 2.24 | 0.002 | 2.08 | 0.002 | 0.29 | 0.026 | 0.49 | 0.019 |
| C19orf18 | PROMOTER | 2.95 | 0.003 | 2.73 | 0.004 | 1.81 | 0.023 | 1.94 | 0.020 |
| C20orf70 | PROMOTER | 2.30 | 0.008 | 2.37 | 0.028 | 0.98 | 0.034 | 0.87 | 0.032 |
| C4orf7 | PROMOTER | 4.50 | 0.013 | 5.11 | 0.011 | 1.50 | 0.037 | 3.53 | 0.023 |
| C9orf21 | PROMOTER | 1.85 | 0.005 | 1.74 | 0.006 | 2.19 | 0.006 | 2.21 | 0.006 |
| CACNG3 | PROMOTER | 1.23 | 0.011 | 1.55 | 0.006 | 2.22 | 0.014 | 2.60 | 0.007 |
| CAPN13 | PROMOTER | 4.52 | 0.014 | 4.40 | 0.011 | 3.02 | 0.050 | 3.16 | 0.030 |
| CASP10 | PROMOTER | 4.49 | 0.034 | 3.24 | 0.015 | 2.26 | 0.025 | 1.41 | 0.047 |
| CAV2 | PROMOTER | 2.13 | 0.020 | 2.05 | 0.019 | 2.43 | 0.015 | 1.83 | 0.036 |
| CBX3 | DIVERGENT_PROMOTER | 0.73 | 0.043 | 0.86 | 0.040 | 2.09 | 0.004 | 2.13 | 0.003 |
| CCBE1 | PROMOTER | 1.37 | 0.020 | 1.49 | 0.009 | 2.25 | 0.043 | 2.07 | 0.039 |
| CCDC110 | PROMOTER | 3.47 | 0.013 | 3.46 | 0.006 | 2.19 | 0.013 | 1.56 | 0.016 |
| CCDC155 | PROMOTER | 2.90 | 0.010 | 2.71 | 0.021 | 0.77 | 0.042 | 0.76 | 0.030 |
| CCDC160 | PROMOTER | 4.21 | 0.017 | 4.02 | 0.013 | 2.60 | 0.009 | 1.96 | 0.022 |
| CCND2 | PROMOTER | 3.02 | 0.003 | 0.49 | 0.016 | 2.67 | 0.034 | 2.42 | 0.022 |
| CD109 | PROMOTER | 0.93 | 0.021 | 0.55 | 0.031 | 3.08 | 0.024 | 3.33 | 0.017 |
| CD22 | PROMOTER | 2.71 | 0.001 | 2.76 | 0.002 | 0.69 | 0.028 | 0.44 | 0.037 |
| CDGAP | PROMOTER | 1.34 | 0.015 | 0.90 | 0.025 | 4.98 | 0.001 | 4.39 | 0.001 |
| CDH19 | PROMOTER | 1.61 | 0.003 | 1.10 | 0.004 | 2.20 | 0.037 | 1.93 | 0.019 |
| CDH22 | PROMOTER | 3.01 | 0.021 | 3.29 | 0.020 | 2.82 | 0.043 | 2.59 | 0.045 |
| CDK15 | PROMOTER | 2.04 | 0.038 | 2.02 | 0.023 | 2.75 | 0.002 | 2.92 | 0.002 |
| CDKN1B | PROMOTER | 0.08 | 0.020 | 1.91 | 0.002 | 2.02 | 0.011 | 2.59 | 0.010 |
| CHCHD2 | PROMOTER | 1.31 | 0.008 | 1.13 | 0.011 | 1.45 | 0.027 | 1.39 | 0.025 |
| CHEK1 | PROMOTER | 2.72 | 0.026 | 2.87 | 0.022 | 1.77 | 0.025 | 1.20 | 0.011 |
| CHMP1B | PROMOTER | 1.05 | 0.023 | 0.77 | 0.032 | 3.54 | 0.034 | 3.51 | 0.039 |

| CHN2 | PROMOTER | 2.32 | 0.027 | 2.11 | 0.041 | 1.81 | 0.014 | 2.39 | 0.010 |
|---|---|---|---|---|---|---|---|---|---|
| CKAP2L | PROMOTER | 1.70 | 0.027 | 1.79 | 0.034 | 2.50 | 0.012 | 2.50 | 0.017 |
| CLMN | PROMOTER | 3.67 | 0.018 | 3.34 | 0.034 | 2.57 | 0.003 | 2.29 | 0.005 |
| CNR2 | PROMOTER | 0.51 | 0.029 | 0.79 | 0.024 | 4.23 | 0.012 | 4.09 | 0.014 |
| COPB2 | PROMOTER | 2.47 | 0.003 | 1.07 | 0.007 | 1.98 | 0.015 | 1.80 | 0.018 |
| COX18 | PROMOTER | 2.43 | 0.016 | 3.45 | 0.011 | 1.54 | 0.049 | 1.30 | 0.026 |
| COX7A2L | PROMOTER | 2.59 | 0.042 | 2.48 | 0.037 | 1.60 | 0.048 | 1.67 | 0.035 |
| CPEB2 | PROMOTER | 3.93 | 0.005 | 3.94 | 0.005 | 0.91 | 0.032 | 0.37 | 0.035 |
| CPXCR1 | PROMOTER | 2.86 | 0.004 | 2.62 | 0.006 | 3.56 | 0.001 | 2.95 | 0.002 |
| CPZ | PROMOTER | 3.37 | 0.024 | 3.22 | 0.029 | 0.31 | 0.047 | 0.63 | 0.031 |
| CRHBP | PROMOTER | 2.39 | 0.016 | 2.92 | 0.013 | 1.61 | 0.033 | 1.55 | 0.029 |
| CSRNP1 | PROMOTER | 3.56 | 0.011 | 3.55 | 0.011 | 0.72 | 0.012 | 0.83 | 0.011 |
| CST9 | PROMOTER | 1.47 | 0.002 | 1.53 | 0.002 | 2.45 | 0.007 | 2.41 | 0.011 |
| CXorf39 | DIVERGENT_PROMOTER | 2.06 | 0.007 | 1.06 | 0.011 | 2.04 | 0.003 | 2.35 | 0.003 |
| CYP20A1 | PROMOTER | 2.30 | 0.038 | 1.74 | 0.044 | 2.94 | 0.029 | 2.31 | 0.035 |
| CYP24A1 | PROMOTER | 1.54 | 0.004 | 1.95 | 0.002 | 2.13 | 0.032 | 2.35 | 0.030 |
| DCX | PROMOTER | 0.51 | 0.045 | 0.71 | 0.022 | 3.55 | 0.008 | 2.25 | 0.012 |
| DDX31 | PROMOTER | 3.40 | 0.006 | 3.74 | 0.009 | 2.80 | 0.013 | 3.41 | 0.018 |
| DEFB127 | PROMOTER | 0.38 | 0.023 | -0.31 | 0.025 | 3.24 | 0.011 | 1.86 | 0.033 |
| DHRS3 | PROMOTER | 1.87 | 0.014 | 1.89 | 0.009 | 1.23 | 0.043 | 1.57 | 0.027 |
| DLK1 | PROMOTER | 2.49 | 0.019 | 3.18 | 0.044 | 3.93 | 0.004 | 3.02 | 0.004 |
| DMD | PROMOTER | 2.55 | 0.024 | 1.79 | 0.043 | 4.05 | 0.009 | 5.18 | 0.016 |
| DNAJC3 | PROMOTER | 1.49 | 0.019 | 1.28 | 0.027 | 3.82 | 0.010 | 3.85 | 0.015 |
| DOK2 | DIVERGENT_PROMOTER | 1.87 | 0.015 | 1.79 | 0.020 | 1.50 | 0.020 | 1.67 | 0.015 |
| DRG1 | PROMOTER | 1.89 | 0.013 | 1.83 | 0.013 | 3.37 | 0.021 | 3.07 | 0.036 |
| DUSP4 | PROMOTER | 1.70 | 0.020 | 1.50 | 0.018 | 2.27 | 0.028 | 2.24 | 0.035 |
| DYRK2 | PROMOTER | 1.92 | 0.008 | 0.00 | 0.050 | 2.52 | 0.032 | 2.39 | 0.011 |
| EEF2 | PROMOTER | 1.70 | 0.030 | 2.47 | 0.049 | 3.33 | 0.009 | 3.07 | 0.016 |
| EGLN2 | PROMOTER | 1.67 | 0.009 | 1.47 | 0.010 | 1.90 | 0.050 | 2.03 | 0.048 |
| EIF2C4 | PROMOTER | 0.84 | 0.022 | 1.01 | 0.028 | 2.01 | 0.031 | 2.25 | 0.019 |
| EIF6 | PROMOTER | 3.67 | 0.040 | 3.48 | 0.044 | 1.76 | 0.049 | 1.84 | 0.046 |
| EMID1 | PROMOTER | 2.21 | 0.026 | 2.45 | 0.001 | 1.53 | 0.008 | 1.68 | 0.005 |
| ERG | PROMOTER | 3.28 | 0.001 | 3.38 | 0.001 | 1.37 | 0.021 | 1.31 | 0.024 |
| ESAM | PROMOTER | 3.02 | 0.006 | 3.00 | 0.010 | 1.30 | 0.015 | 1.08 | 0.021 |
| EXOC5 | PROMOTER | 3.06 | 0.010 | 3.30 | 0.028 | 3.08 | 0.032 | 2.84 | 0.042 |
| FAM123B | PROMOTER | 0.49 | 0.033 | 0.98 | 0.012 | -0.08 | 0.029 | -0.03 | 0.029 |
| FAM155A | PROMOTER | 3.14 | 0.009 | 2.76 | 0.009 | 1.84 | 0.017 | 1.64 | 0.014 |
| FAM190B | PROMOTER | 2.35 | 0.006 | 0.90 | 0.014 | 1.65 | 0.042 | 1.55 | 0.037 |
| FBXL14 | PROMOTER | 2.58 | 0.035 | 2.64 | 0.026 | 1.33 | 0.049 | 1.64 | 0.045 |
| FBXO34 | PROMOTER | -0.13 | 0.032 | -0.04 | 0.028 | 0.36 | 0.044 | 0.48 | 0.036 |
| FHL2 | PROMOTER | 0.36 | 0.010 | 0.25 | 0.011 | 1.07 | 0.029 | 1.26 | 0.048 |
| FLRT3 | PROMOTER | 1.48 | 0.019 | 3.64 | 0.038 | 0.74 | 0.036 | 0.69 | 0.038 |
| GALNT1 | PROMOTER | 1.14 | 0.012 | 0.49 | 0.023 | 0.42 | 0.032 | 0.28 | 0.043 |
| GBA3 | PROMOTER | 1.64 | 0.050 | 2.01 | 0.049 | 2.40 | 0.045 | 2.17 | 0.036 |
| GFM1 | PROMOTER | 2.18 | 0.022 | 1.86 | 0.025 | 4.01 | 0.029 | 2.56 | 0.032 |
| GFM2 | DIVERGENT_PROMOTER | 1.88 | 0.041 | 2.34 | 0.031 | 1.32 | 0.043 | 0.98 | 0.047 |
| GIPC1 | PROMOTER | 1.78 | 0.021 | 1.93 | 0.022 | 1.41 | 0.024 | 0.65 | 0.039 |
| GLT1D1 | PROMOTER | 1.55 | 0.029 | 2.06 | 0.026 | 1.29 | 0.041 | 1.40 | 0.028 |
| GNASAS | DIVERGENT_PROMOTER | 2.44 | 0.020 | 2.28 | 0.031 | 2.49 | 0.003 | 1.43 | 0.006 |
| GPC4 | PROMOTER | 1.18 | 0.032 | 2.12 | 0.037 | 2.50 | 0.044 | 2.22 | 0.047 |
| GPR153 | PROMOTER | 6.00 | 0.024 | 6.17 | 0.022 | 1.64 | 0.026 | 1.49 | 0.037 |
| GPR174 | PROMOTER | 3.34 | 0.001 | 3.21 | 0.002 | -2.14 | 0.029 | 0.82 | 0.025 |
| GPR50 | PROMOTER | 1.56 | 0.035 | 1.35 | 0.038 | 0.83 | 0.028 | 1.08 | 0.027 |
| GPSM3 | PROMOTER | 2.67 | 0.005 | 2.66 | 0.006 | 0.87 | 0.034 | 0.78 | 0.032 |
| GPX4 | DIVERGENT_PROMOTER | 2.46 | 0.022 | 2.68 | 0.026 | 3.13 | 0.006 | 2.96 | 0.004 |
| GRIA3 | PROMOTER | 0.25 | 0.022 | 0.47 | 0.015 | 4.42 | 0.029 | 4.25 | 0.049 |
| GSDMB | PROMOTER | 3.20 | 0.004 | 3.67 | 0.003 | 1.48 | 0.041 | 1.80 | 0.038 |
| GSG1 | PROMOTER | 2.19 | 0.012 | 2.26 | 0.020 | 1.63 | 0.006 | 1.36 | 0.015 |
| H2AFY2 | PROMOTER | 1.35 | 0.007 | 0.00 | 0.041 | 1.86 | 0.004 | 2.07 | 0.007 |
| HBP1 | PROMOTER | 3.79 | 0.001 | 3.71 | 0.001 | 2.29 | 0.041 | 2.37 | 0.032 |
| HDAC6 | PROMOTER | 3.46 | 0.004 | 3.73 | 0.004 | 1.45 | 0.034 | 1.88 | 0.033 |
| HMHA1 | PROMOTER | 1.26 | 0.005 | 1.28 | 0.005 | 1.71 | 0.017 | 1.70 | 0.017 |
| HPSE2 | PROMOTER | 2.99 | 0.003 | 3.16 | 0.002 | 1.34 | 0.036 | 1.11 | 0.046 |
| HSF4 | PROMOTER | 1.03 | 0.008 | 1.26 | 0.007 | 2.26 | 0.010 | 2.02 | 0.013 |
| HSPA13 | PROMOTER | 3.78 | 0.019 | 3.01 | 0.031 | 2.48 | 0.032 | 1.10 | 0.046 |
| HTN3 | PROMOTER | 3.23 | 0.007 | 3.11 | 0.013 | 1.37 | 0.033 | 2.49 | 0.012 |
| HTR2B | PROMOTER | 3.73 | 0.001 | 0.00 | 0.031 | 0.00 | 0.039 | 3.48 | 0.007 |
| HTR2C | PROMOTER | 2.16 | 0.029 | 1.86 | 0.043 | 1.92 | 0.007 | 2.00 | 0.019 |
| HUWE1 | PROMOTER | 4.48 | 0.001 | 0.00 | 0.042 | 2.10 | 0.007 | 2.55 | 0.004 |
| HVCN1 | PROMOTER | 1.49 | 0.044 | 1.38 | 0.046 | 3.38 | 0.012 | 3.12 | 0.013 |
| IAH1 | PROMOTER | 3.40 | 0.046 | 3.20 | 0.021 | 1.75 | 0.013 | 1.78 | 0.016 |
| ID1 | PROMOTER | 1.03 | 0.039 | 0.81 | 0.015 | 0.90 | 0.028 | 1.16 | 0.033 |
| IKBKB | PROMOTER | 1.01 | 0.010 | 1.18 | 0.011 | 0.52 | 0.038 | 0.73 | 0.039 |
| IL1R1 | PROMOTER | 2.73 | 0.023 | 2.72 | 0.047 | 1.77 | 0.029 | 1.89 | 0.029 |

| IL1RAPL1 | PROMOTER | 1.22 | 0.015 | 1.10 | 0.023 | 2.94 | 0.039 | 2.98 | 0.030 |
|---|---|---|---|---|---|---|---|---|---|
| IL8 | PROMOTER | 2.78 | 0.008 | 2.74 | 0.007 | 2.71 | 0.008 | 2.26 | 0.015 |
| IMP3 | DIVERGENT_PROMOTER | 4.12 | 0.001 | 4.12 | 0.001 | 2.46 | 0.010 | 2.71 | 0.009 |
| IRX4 | PROMOTER | 0.95 | 0.004 | -0.21 | 0.017 | 1.14 | 0.041 | 1.27 | 0.038 |
| IRX5 | PROMOTER | 2.36 | 0.012 | 2.94 | 0.009 | 1.83 | 0.023 | 2.13 | 0.017 |
| KCMF1 | PROMOTER | -0.10 | 0.018 | -0.06 | 0.016 | 0.57 | 0.029 | 0.89 | 0.018 |
| KCNIP2 | PROMOTER | 2.05 | 0.024 | 2.80 | 0.023 | 2.13 | 0.046 | 2.18 | 0.028 |
| KDM5D | PROMOTER | 2.25 | 0.004 | 0.00 | 0.029 | 3.95 | 0.004 | 2.90 | 0.009 |
| KIAA0240 | PROMOTER | 2.14 | 0.013 | 0.05 | 0.050 | 2.69 | 0.030 | 4.67 | 0.013 |
| KIAA0467 | PROMOTER | -0.40 | 0.050 | -0.32 | 0.035 | 1.28 | 0.036 | 1.30 | 0.043 |
| KIAA0922 | PROMOTER | 1.54 | 0.003 | 1.52 | 0.003 | 1.97 | 0.025 | 1.60 | 0.041 |
| KIAA1026 | PROMOTER | 2.80 | 0.029 | 2.75 | 0.044 | 1.51 | 0.042 | 2.06 | 0.032 |
| KIAA1147 | PROMOTER | 3.39 | 0.017 | 3.40 | 0.006 | 3.91 | 0.044 | 3.86 | 0.041 |
| KIAA1217 | PROMOTER | 4.91 | 0.001 | 4.92 | 0.001 | 2.48 | 0.003 | 1.80 | 0.004 |
| KIAA1370 | PROMOTER | 0.53 | 0.035 | 0.36 | 0.035 | 7.35 | 0.006 | 5.58 | 0.007 |
| KIAA2022 | PROMOTER | 2.87 | 0.004 | 3.11 | 0.003 | 4.37 | 0.020 | 3.44 | 0.040 |
| KIF5C | PROMOTER | 0.89 | 0.007 | 0.54 | 0.008 | 2.39 | 0.038 | 2.08 | 0.035 |
| KLHL18 | PROMOTER | 2.34 | 0.015 | 2.60 | 0.012 | 1.02 | 0.045 | 1.39 | 0.041 |
| KRTAP10-10 | PROMOTER | 2.57 | 0.002 | 2.62 | 0.002 | 0.28 | 0.029 | 0.59 | 0.024 |
| KRTAP19-2 | PROMOTER | 3.31 | 0.013 | 3.27 | 0.007 | 2.10 | 0.009 | 1.61 | 0.014 |
| KRTAP20-2 | PROMOTER | 0.38 | 0.016 | 0.00 | 0.032 | 1.32 | 0.009 | 2.47 | 0.002 |
| LEAP2 | PROMOTER | 1.57 | 0.014 | 1.76 | 0.011 | 2.59 | 0.041 | 2.25 | 0.049 |
| LGALS1 | PROMOTER | 2.19 | 0.034 | 2.62 | 0.024 | 1.73 | 0.047 | 1.65 | 0.043 |
| LHX1 | PROMOTER | 2.28 | 0.002 | 2.21 | 0.004 | 1.25 | 0.035 | 1.30 | 0.033 |
| LILRP2 | PROMOTER | 0.86 | 0.023 | 1.21 | 0.013 | 4.33 | 0.011 | 3.73 | 0.028 |
| LIN28 | PROMOTER | 3.01 | 0.033 | 3.06 | 0.035 | 2.20 | 0.036 | 1.95 | 0.047 |
| LOC148824 | PROMOTER | 1.61 | 0.016 | 1.39 | 0.005 | 1.40 | 0.023 | 1.33 | 0.022 |
| LOC642587 | DOWNSTREAM | 4.85 | 0.003 | 4.43 | 0.001 | 2.42 | 0.005 | 2.24 | 0.011 |
| LRCH3 | PROMOTER | 2.24 | 0.006 | 2.38 | 0.006 | 0.67 | 0.034 | 1.25 | 0.013 |
| LRRK1 | PROMOTER | 3.69 | 0.013 | 3.55 | 0.021 | 1.56 | 0.021 | 1.23 | 0.035 |
| MAP6D1 | PROMOTER | 1.86 | 0.016 | 1.62 | 0.016 | 1.16 | 0.020 | 0.93 | 0.025 |
| MAPK8 | PROMOTER | 1.95 | 0.016 | 2.51 | 0.011 | 1.72 | 0.037 | 2.07 | 0.026 |
| MFNG | PROMOTER | 2.67 | 0.034 | 2.78 | 0.034 | 2.54 | 0.044 | 2.34 | 0.048 |
| MGST1 | PROMOTER | 1.50 | 0.031 | 1.77 | 0.037 | 1.25 | 0.018 | 1.37 | 0.020 |
| MIR103-2 | PROMOTER | 1.46 | 0.024 | 1.01 | 0.021 | 1.96 | 0.031 | 2.00 | 0.047 |
| MIR122 | PROMOTER | 2.82 | 0.006 | 2.71 | 0.009 | 1.08 | 0.024 | 0.98 | 0.030 |
| MIR1256 | DIVERGENT_PROMOTER | 3.73 | 0.001 | 3.93 | 0.001 | 1.42 | 0.022 | 1.40 | 0.013 |
| MIR1277 | PROMOTER | 0.92 | 0.007 | 1.02 | 0.007 | 2.49 | 0.020 | 3.19 | 0.015 |
| MIR1286 | PROMOTER | 2.77 | 0.003 | 2.39 | 0.003 | 1.52 | 0.017 | 1.17 | 0.022 |
| MIR130B | DOWNSTREAM | 0.85 | 0.005 | 0.82 | 0.005 | 0.53 | 0.050 | 0.53 | 0.038 |
| MIR183 | PROMOTER | 0.34 | 0.015 | 0.11 | 0.025 | 2.03 | 0.007 | 1.97 | 0.011 |
| MIR26B | PROMOTER | 2.30 | 0.031 | 2.21 | 0.028 | 1.58 | 0.010 | 1.78 | 0.008 |
| MIR449C | PROMOTER | 7.47 | 0.000 | 7.22 | 0.000 | 1.56 | 0.035 | 2.04 | 0.011 |
| MIR490 | PROMOTER | 1.23 | 0.026 | 1.06 | 0.032 | 3.54 | 0.028 | 2.32 | 0.042 |
| MIR542 | PROMOTER | 1.38 | 0.011 | 1.65 | 0.016 | 1.70 | 0.021 | 2.47 | 0.021 |
| MIR548B | PROMOTER | 0.61 | 0.021 | 0.39 | 0.029 | 0.82 | 0.032 | 1.14 | 0.016 |
| MIR548K | PROMOTER | 0.41 | 0.047 | 0.67 | 0.034 | 0.38 | 0.024 | 0.29 | 0.027 |
| MIR583 | PROMOTER | 3.02 | 0.017 | 0.00 | 0.024 | 0.00 | 0.024 | 0.00 | 0.024 |
| MIR617 | PROMOTER | 2.38 | 0.015 | 2.47 | 0.025 | 2.75 | 0.015 | 1.78 | 0.013 |
| MIR939 | PROMOTER | 2.07 | 0.010 | 1.72 | 0.016 | 1.14 | 0.033 | 0.91 | 0.044 |
| MIRLET7D | DOWNSTREAM | 1.78 | 0.018 | 1.94 | 0.015 | 1.17 | 0.035 | 1.31 | 0.012 |
| MLST8 | DIVERGENT_PROMOTER | 0.84 | 0.007 | 0.29 | 0.009 | 1.75 | 0.029 | 1.76 | 0.027 |
| MORC1 | PROMOTER | 3.24 | 0.001 | 3.54 | 0.001 | 4.30 | 0.017 | 3.81 | 0.001 |
| MRAS | PROMOTER | 1.51 | 0.032 | 1.77 | 0.029 | 1.69 | 0.019 | 2.18 | 0.021 |
| MREG | PROMOTER | 1.35 | 0.006 | 1.17 | 0.007 | 1.67 | 0.014 | 1.62 | 0.026 |
| MRPL17 | PROMOTER | 1.52 | 0.008 | 1.20 | 0.008 | 1.17 | 0.020 | 1.37 | 0.015 |
| MRPL54 | DIVERGENT_PROMOTER | 2.40 | 0.023 | 2.24 | 0.023 | 3.35 | 0.025 | 3.13 | 0.030 |
| MXD4 | PROMOTER | 4.02 | 0.003 | 3.76 | 0.005 | 1.69 | 0.024 | 1.17 | 0.045 |
| NCRNA00120 | PROMOTER | 2.29 | 0.002 | 2.18 | 0.003 | 1.49 | 0.009 | 1.68 | 0.006 |
| NEFH | PROMOTER | 0.51 | 0.046 | 2.34 | 0.013 | 1.11 | 0.027 | 1.32 | 0.022 |
| NEFL | PROMOTER | 1.90 | 0.009 | 1.63 | 0.011 | 1.95 | 0.021 | 1.96 | 0.017 |
| NFIL3 | PROMOTER | 4.29 | 0.000 | 4.49 | 0.000 | 1.71 | 0.015 | 1.93 | 0.025 |
| NLRP12 | PROMOTER | 3.43 | 0.005 | 3.21 | 0.009 | 1.56 | 0.040 | 1.13 | 0.050 |
| NOM1 | PROMOTER | 2.67 | 0.004 | 3.24 | 0.011 | 2.04 | 0.029 | 2.16 | 0.017 |
| NPY1R | PROMOTER | 2.45 | 0.002 | 2.18 | 0.003 | 1.78 | 0.049 | 1.89 | 0.031 |
| NUMB | PROMOTER | 3.10 | 0.033 | 3.20 | 0.041 | 2.64 | 0.049 | 2.55 | 0.036 |
| NXT1 | PROMOTER | 1.04 | 0.009 | 0.32 | 0.035 | 2.59 | 0.006 | 2.72 | 0.006 |
| OR52A5 | PROMOTER | 1.76 | 0.049 | 2.69 | 0.033 | 2.15 | 0.016 | 1.79 | 0.007 |
| OR52B2 | PROMOTER | 3.56 | 0.007 | 3.79 | 0.005 | 3.48 | 0.006 | 2.49 | 0.008 |
| OR52K1 | PROMOTER | 1.84 | 0.047 | 2.07 | 0.034 | 1.92 | 0.014 | 1.94 | 0.016 |
| OR6A2 | PROMOTER | 3.56 | 0.019 | 3.53 | 0.028 | 1.77 | 0.012 | 1.76 | 0.009 |
| OR6C74 | PROMOTER | 3.87 | 0.002 | 4.44 | 0.001 | 4.64 | 0.041 | 4.15 | 0.024 |
| OR6T1 | PROMOTER | 0.16 | 0.013 | 0.10 | 0.012 | 3.99 | 0.016 | 3.88 | 0.021 |
| ORC5L | PROMOTER | 2.29 | 0.011 | 3.08 | 0.006 | 1.40 | 0.011 | 1.86 | 0.008 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| P2RY13 | PROMOTER | 4.26 | 0.001 | 4.28 | 0.000 | 1.80 | 0.029 | 1.76 | 0.037 |
| PAGE5 | PROMOTER | 2.81 | 0.025 | 3.27 | 0.013 | 0.59 | 0.030 | 1.10 | 0.034 |
| PAX8 | PROMOTER | 1.07 | 0.029 | 1.08 | 0.048 | 2.47 | 0.011 | 2.69 | 0.009 |
| PCDH19 | PROMOTER | 1.34 | 0.024 | 1.47 | 0.019 | 2.10 | 0.007 | 1.99 | 0.009 |
| PCDH7 | PROMOTER | 4.32 | 0.002 | 4.28 | 0.003 | 0.64 | 0.035 | 0.72 | 0.033 |
| PCYT1B | PROMOTER | 4.86 | 0.015 | 5.19 | 0.012 | 2.66 | 0.003 | 2.65 | 0.005 |
| PDIK1L | PROMOTER | -0.10 | 0.032 | 0.13 | 0.021 | 3.12 | 0.014 | 2.49 | 0.017 |
| PDK4 | PROMOTER | 2.30 | 0.005 | 2.44 | 0.006 | 4.56 | 0.021 | 3.69 | 0.032 |
| PELI2 | PROMOTER | 4.36 | 0.046 | 4.12 | 0.034 | 0.77 | 0.026 | 0.18 | 0.049 |
| PEX10 | PROMOTER | 1.27 | 0.021 | 1.19 | 0.015 | 1.06 | 0.044 | 1.25 | 0.042 |
| PFKFB1 | DIVERGENT_PROMOTER | 1.79 | 0.003 | 1.76 | 0.004 | 4.57 | 0.003 | 4.63 | 0.004 |
| PHF15 | PROMOTER | 0.89 | 0.023 | 1.23 | 0.022 | 0.31 | 0.013 | 0.30 | 0.016 |
| PHF8 | PROMOTER | 4.89 | 0.011 | 4.70 | 0.011 | 1.49 | 0.009 | 1.36 | 0.008 |
| PHPT1 | PROMOTER | 2.02 | 0.018 | 1.68 | 0.017 | 1.60 | 0.026 | 1.26 | 0.030 |
| PIP5KL1 | PROMOTER | 1.76 | 0.013 | 1.97 | 0.010 | 2.14 | 0.036 | 2.64 | 0.033 |
| PITPNC1 | PROMOTER | 3.46 | 0.003 | 3.11 | 0.005 | 2.14 | 0.009 | 0.93 | 0.035 |
| PLA2G4A | PROMOTER | 2.53 | 0.021 | 3.06 | 0.009 | 3.93 | 0.041 | 3.68 | 0.005 |
| PLCL2 | PROMOTER | 2.65 | 0.002 | 3.03 | 0.002 | 2.73 | 0.049 | 2.85 | 0.041 |
| PLEC1 | PROMOTER | 2.27 | 0.004 | 1.40 | 0.009 | 1.51 | 0.022 | 1.28 | 0.047 |
| POF1B | PROMOTER | 3.98 | 0.021 | 3.96 | 0.046 | 1.78 | 0.004 | 1.54 | 0.008 |
| PON3 | PROMOTER | 3.37 | 0.002 | 3.64 | 0.028 | 1.10 | 0.015 | 1.37 | 0.006 |
| POT1 | PROMOTER | 1.53 | 0.039 | 1.68 | 0.048 | 4.77 | 0.043 | 5.34 | 0.032 |
| PPP1R3C | PROMOTER | 2.76 | 0.001 | 2.70 | 0.001 | 1.26 | 0.009 | 1.37 | 0.007 |
| PRAGMIN | PROMOTER | 0.51 | 0.034 | 0.42 | 0.037 | 1.83 | 0.050 | 1.64 | 0.036 |
| PRDM2 | PROMOTER | 2.84 | 0.033 | 2.32 | 0.030 | 1.63 | 0.039 | 1.86 | 0.027 |
| PRKACB | PROMOTER | 1.08 | 0.002 | 1.00 | 0.003 | 1.10 | 0.030 | 1.51 | 0.022 |
| PRKD1 | PROMOTER | 2.35 | 0.008 | 2.55 | 0.009 | 3.03 | 0.029 | 2.94 | 0.027 |
| PROKR2 | PROMOTER | 2.65 | 0.035 | 2.20 | 0.045 | 1.68 | 0.024 | 1.38 | 0.028 |
| PRPS2 | PROMOTER | 2.90 | 0.019 | 2.14 | 0.027 | 3.63 | 0.016 | 3.18 | 0.020 |
| PRSS50 | PROMOTER | 2.15 | 0.029 | 2.11 | 0.043 | 3.38 | 0.037 | 3.43 | 0.039 |
| PRSS8 | PROMOTER | 1.66 | 0.002 | 1.79 | 0.002 | 2.24 | 0.005 | 2.25 | 0.005 |
| PTHLH | PROMOTER | 1.80 | 0.017 | 1.97 | 0.014 | 1.40 | 0.009 | 1.34 | 0.011 |
| RAB30 | PROMOTER | 1.05 | 0.024 | 0.92 | 0.036 | 2.20 | 0.032 | 1.82 | 0.029 |
| RAB33A | DIVERGENT_PROMOTER | 4.45 | 0.004 | 4.86 | 0.006 | 3.59 | 0.026 | 3.90 | 0.030 |
| RALGAPB | PROMOTER | 3.86 | 0.002 | 4.34 | 0.001 | 3.08 | 0.013 | 3.67 | 0.012 |
| RANBP3 | PROMOTER | 3.23 | 0.013 | 2.74 | 0.033 | 1.46 | 0.027 | 1.68 | 0.029 |
| RASGRP3 | PROMOTER | 3.15 | 0.027 | 3.36 | 0.020 | 2.19 | 0.035 | 2.35 | 0.038 |
| REG3A | PROMOTER | 3.61 | 0.029 | 3.51 | 0.048 | 0.85 | 0.035 | 0.97 | 0.028 |
| RFX5 | PROMOTER | 3.75 | 0.017 | 3.31 | 0.010 | 2.29 | 0.028 | 2.33 | 0.031 |
| RGAG1 | PROMOTER | 0.17 | 0.030 | 0.37 | 0.027 | 2.03 | 0.043 | 2.13 | 0.047 |
| RGS20 | PROMOTER | 1.95 | 0.017 | 1.33 | 0.031 | 1.90 | 0.012 | 1.02 | 0.021 |
| RGS5 | PROMOTER | 3.09 | 0.008 | 3.04 | 0.016 | 4.69 | 0.030 | 4.63 | 0.019 |
| RNF144B | PROMOTER | 2.53 | 0.017 | 2.91 | 0.012 | 1.33 | 0.028 | 1.33 | 0.027 |
| RPS19BP1 | PROMOTER | 1.10 | 0.016 | 1.16 | 0.019 | 1.10 | 0.018 | 0.97 | 0.022 |
| RPS6KB2 | PROMOTER | 0.54 | 0.019 | 0.58 | 0.021 | 1.80 | 0.025 | 1.56 | 0.031 |
| RREB1 | PROMOTER | 1.41 | 0.010 | 1.64 | 0.009 | 0.60 | 0.021 | 0.52 | 0.025 |
| RTN1 | PROMOTER | 2.67 | 0.001 | 0.00 | 0.017 | 3.72 | 0.020 | 4.96 | 0.020 |
| RUNX2 | PROMOTER | 0.32 | 0.027 | 0.36 | 0.038 | 1.15 | 0.011 | 1.34 | 0.008 |
| S100A3 | PROMOTER | 2.35 | 0.013 | 2.24 | 0.022 | 2.56 | 0.006 | 2.51 | 0.006 |
| SBF1 | DIVERGENT_PROMOTER | 1.91 | 0.019 | 1.67 | 0.027 | 2.00 | 0.044 | 2.04 | 0.048 |
| SCARNA27 | PROMOTER | 2.32 | 0.028 | 2.35 | 0.012 | 1.21 | 0.023 | 0.86 | 0.035 |
| SCGB2A2 | PROMOTER | 2.09 | 0.022 | 1.80 | 0.011 | 1.69 | 0.037 | 1.61 | 0.033 |
| SEMA6D | PROMOTER | 1.68 | 0.041 | 1.50 | 0.034 | 1.27 | 0.017 | 1.06 | 0.026 |
| SERINC3 | DIVERGENT_PROMOTER | 3.20 | 0.019 | 3.16 | 0.016 | 3.95 | 0.033 | 3.04 | 0.045 |
| SERTAD2 | PROMOTER | 0.86 | 0.006 | 0.75 | 0.007 | 3.05 | 0.016 | 2.37 | 0.031 |
| SGIP1 | PROMOTER | 1.47 | 0.014 | 1.55 | 0.011 | 0.78 | 0.039 | 1.25 | 0.018 |
| SIX6 | PROMOTER | 1.82 | 0.002 | 1.80 | 0.002 | 1.03 | 0.020 | 0.87 | 0.025 |
| SKP1 | PROMOTER | 2.95 | 0.004 | 2.88 | 0.003 | 3.67 | 0.001 | 3.70 | 0.001 |
| SLC1A6 | PROMOTER | 1.96 | 0.005 | 1.87 | 0.009 | 0.61 | 0.020 | 0.60 | 0.023 |
| SLC41A3 | PROMOTER | 4.20 | 0.019 | 4.09 | 0.010 | 3.43 | 0.034 | 3.28 | 0.041 |
| SNORD116-12 | PROMOTER | 2.57 | 0.026 | 2.59 | 0.038 | 1.23 | 0.035 | 1.29 | 0.039 |
| SNORD116-27 | PROMOTER | -0.13 | 0.016 | 1.29 | 0.003 | 2.05 | 0.033 | 2.30 | 0.021 |
| SOBP | PROMOTER | 6.23 | 0.002 | 5.93 | 0.002 | 1.38 | 0.038 | 1.15 | 0.046 |
| SOX3 | PROMOTER | 0.65 | 0.026 | 0.78 | 0.018 | 1.32 | 0.014 | 1.37 | 0.010 |
| SP9 | PROMOTER | -0.16 | 0.029 | -0.22 | 0.036 | 2.88 | 0.033 | 2.28 | 0.040 |
| SPANXB1 | PROMOTER | 2.25 | 0.038 | 2.40 | 0.041 | 1.47 | 0.016 | 1.66 | 0.011 |
| SPANXN3 | PROMOTER | 1.46 | 0.013 | 1.33 | 0.011 | 3.80 | 0.022 | 2.77 | 0.043 |
| SPG21 | PROMOTER | 2.54 | 0.016 | 2.96 | 0.027 | 2.15 | 0.038 | 2.13 | 0.025 |
| SSRP1 | DIVERGENT_PROMOTER | 2.04 | 0.016 | 1.72 | 0.029 | 1.93 | 0.028 | 3.19 | 0.038 |
| ST3GAL3 | PROMOTER | 1.91 | 0.002 | 2.44 | 0.001 | 1.90 | 0.030 | 1.18 | 0.042 |
| ST6GALNAC5 | PROMOTER | 2.60 | 0.028 | 2.77 | 0.012 | 6.00 | 0.032 | 6.33 | 0.032 |
| STIP1 | PROMOTER | 3.81 | 0.013 | 3.73 | 0.013 | 1.71 | 0.048 | 1.68 | 0.048 |
| STRN4 | PROMOTER | 4.43 | 0.033 | 4.62 | 0.048 | 1.31 | 0.036 | 1.63 | 0.033 |
| STX16 | PROMOTER | 3.16 | 0.012 | 3.15 | 0.020 | 3.11 | 0.010 | 2.48 | 0.021 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SYNRG | PROMOTER | 2.80 | 0.049 | 2.83 | 0.017 | 1.63 | 0.024 | 1.16 | 0.029 |
| TAAR3 | PROMOTER | 0.43 | 0.010 | 0.47 | 0.007 | 2.04 | 0.045 | 2.12 | 0.006 |
| TAL1 | PROMOTER | 2.88 | 0.035 | 2.73 | 0.046 | 1.99 | 0.035 | 1.95 | 0.034 |
| TAS2R5 | PROMOTER | 4.55 | 0.003 | 4.32 | 0.003 | 3.05 | 0.015 | 2.76 | 0.016 |
| TBX22 | PROMOTER | -0.10 | 0.026 | -0.57 | 0.050 | 4.91 | 0.027 | 4.50 | 0.024 |
| TCEB3C | PROMOTER | 1.55 | 0.036 | 1.44 | 0.032 | 0.41 | 0.020 | 0.70 | 0.026 |
| TCP11L1 | PROMOTER | 1.54 | 0.047 | 1.65 | 0.046 | 2.24 | 0.048 | 1.69 | 0.033 |
| TCTN1 | PROMOTER | 4.45 | 0.010 | 4.54 | 0.033 | 1.94 | 0.040 | 1.20 | 0.044 |
| THOC6 | PROMOTER | 0.99 | 0.031 | 0.98 | 0.036 | 1.75 | 0.013 | 1.84 | 0.014 |
| TIAL1 | PROMOTER | 1.54 | 0.025 | 1.84 | 0.046 | 0.47 | 0.030 | 0.69 | 0.036 |
| TIMM8A | PROMOTER | 1.88 | 0.027 | 1.64 | 0.035 | 3.21 | 0.017 | 3.23 | 0.021 |
| TIMP4 | PROMOTER | 1.73 | 0.017 | 1.21 | 0.019 | 2.92 | 0.031 | 2.33 | 0.024 |
| TMED6 | PROMOTER | 6.34 | 0.003 | 6.34 | 0.002 | 7.81 | 0.004 | 2.68 | 0.007 |
| TMEM150C | PROMOTER | 2.17 | 0.017 | 2.55 | 0.028 | 1.32 | 0.012 | 1.39 | 0.006 |
| TMEM178 | PROMOTER | 0.00 | 0.049 | 1.10 | 0.016 | 2.72 | 0.007 | 3.38 | 0.008 |
| TMEM92 | PROMOTER | 1.06 | 0.032 | 1.10 | 0.032 | 1.46 | 0.006 | 1.18 | 0.009 |
| TMX4 | PROMOTER | 3.35 | 0.021 | 3.34 | 0.035 | 1.10 | 0.032 | 1.13 | 0.048 |
| TNFSF18 | PROMOTER | 3.55 | 0.006 | 3.53 | 0.003 | 2.51 | 0.023 | 1.70 | 0.049 |
| TRPC6 | PROMOTER | 1.72 | 0.012 | 1.72 | 0.041 | 0.37 | 0.029 | 0.39 | 0.023 |
| TSC22D1 | PROMOTER | 0.00 | 0.042 | 4.10 | 0.004 | 5.82 | 0.010 | 5.56 | 0.005 |
| TSC22D3 | PROMOTER | 2.19 | 0.011 | 2.46 | 0.010 | 2.97 | 0.001 | 2.96 | 0.001 |
| TSHZ3 | PROMOTER | 2.01 | 0.002 | 0.00 | 0.020 | 2.01 | 0.013 | 1.88 | 0.023 |
| TTL | PROMOTER | 0.47 | 0.008 | -0.95 | 0.048 | 3.13 | 0.034 | 2.83 | 0.036 |
| TTLL10 | PROMOTER | 4.28 | 0.019 | 4.44 | 0.013 | 1.54 | 0.019 | 1.46 | 0.017 |
| TTPAL | PROMOTER | 0.01 | 0.029 | -0.62 | 0.047 | 0.93 | 0.028 | 0.55 | 0.037 |
| TTTY19 | PROMOTER | 5.20 | 0.016 | 3.55 | 0.013 | 2.90 | 0.030 | 2.47 | 0.047 |
| TXNDC17 | PROMOTER | 1.15 | 0.009 | 1.22 | 0.014 | 0.99 | 0.026 | 0.81 | 0.041 |
| UNC80 | PROMOTER | 4.29 | 0.045 | 4.72 | 0.037 | 1.38 | 0.048 | 1.15 | 0.041 |
| USH1C | PROMOTER | 3.50 | 0.001 | 3.50 | 0.001 | 2.26 | 0.016 | 2.59 | 0.015 |
| USP26 | PROMOTER | 3.90 | 0.006 | 4.38 | 0.005 | 2.00 | 0.042 | 2.03 | 0.036 |
| UTS2D | PROMOTER | 2.32 | 0.005 | 2.77 | 0.048 | 1.30 | 0.012 | 2.16 | 0.004 |
| VAPB | PROMOTER | 0.97 | 0.017 | 0.88 | 0.022 | 4.94 | 0.042 | 3.31 | 0.019 |
| VEZF1 | PROMOTER | 1.51 | 0.007 | 1.39 | 0.006 | 2.15 | 0.009 | 2.12 | 0.009 |
| VOPP1 | PROMOTER | 6.11 | 0.024 | 6.28 | 0.033 | 2.59 | 0.021 | 2.29 | 0.029 |
| VSTM2L | PROMOTER | 1.95 | 0.011 | 1.90 | 0.013 | 0.57 | 0.018 | 0.24 | 0.032 |
| VWA5B2 | PROMOTER | 4.92 | 0.002 | 4.36 | 0.003 | 1.92 | 0.015 | 1.75 | 0.013 |
| WNT10A | PROMOTER | 2.39 | 0.007 | 2.22 | 0.016 | 0.57 | 0.026 | 0.79 | 0.026 |
| XK | PROMOTER | 1.41 | 0.019 | 1.91 | 0.009 | 2.59 | 0.017 | 3.01 | 0.015 |
| ZBTB41 | PROMOTER | 2.36 | 0.019 | 2.82 | 0.015 | 2.11 | 0.019 | 2.29 | 0.037 |
| ZBTB7C | PROMOTER | 2.22 | 0.020 | 2.61 | 0.018 | 1.08 | 0.008 | 1.16 | 0.008 |
| ZC3H7A | PROMOTER | 1.23 | 0.008 | 1.46 | 0.016 | 2.69 | 0.046 | 6.27 | 0.016 |
| ZDHHC18 | PROMOTER | 0.75 | 0.018 | 0.80 | 0.019 | 1.45 | 0.016 | 1.31 | 0.014 |
| ZFP42 | PROMOTER | 2.36 | 0.013 | 2.36 | 0.008 | 2.97 | 0.026 | 2.67 | 0.048 |
| ZNF41 | PROMOTER | 2.51 | 0.047 | 2.60 | 0.041 | 0.55 | 0.015 | 0.74 | 0.011 |
| ZNF480 | PROMOTER | 2.22 | 0.034 | 2.34 | 0.015 | 1.76 | 0.005 | 1.57 | 0.009 |
| ZNF74 | PROMOTER | 1.70 | 0.012 | 1.28 | 0.007 | 3.19 | 0.045 | 3.17 | 0.035 |
| ZNF782 | PROMOTER | 5.01 | 0.000 | 4.66 | 0.000 | 2.60 | 0.023 | 2.53 | 0.022 |

# APPENDICES V
## Bivalent genes CPE-NS

| Gene Symbol | Full Name |
|---|---|
| ADCY8 | adenylate cyclase 8 (brain) |
| BAI3 | brain-specific angiogenesis inhibitor 3 |
| BIK | BCL2-interacting killer (apoptosis-inducing) |
| CALCA | calcitonin-related polypeptide alpha |
| CCDC71 | coiled-coil domain containing 71 |
| CDX1 | caudal type homeobox 1 |
| Cnksr1 | connector enhancer of kinase suppressor of Ras 1 |
| COL1A1 | collagen, type I, alpha 1 |
| Col1a2 | collagen, type I, alpha 2 |
| CPNE5 | copine V |
| DLL1 | delta-like 1 (Drosophila) |
| DNAJC22 | DnaJ (Hsp40) homolog, subfamily C, member 22 |
| Egln1 | EGL nine homolog 1 (C. elegans) |
| EOMES | eomesodermin |
| EPN3 | epsin 3 |
| FAM3B | family with sequence similarity 3, member B |
| FGF4 | fibroblast growth factor 4 |
| GATA6 | GATA binding protein 6 |
| GDA | guanine deaminase |
| GEM | GTP binding protein overexpressed in skeletal muscle |
| GLRA1 | glycine receptor, alpha 1 |
| GP1BB | glycoprotein Ib (platelet), beta polypeptide |
| GPR85 | G protein-coupled receptor 85 |
| Gucy2e | guanylate cyclase 2e |
| HDAC11 | histone deacetylase 11 |
| HOXB9 | homeobox B9 |
| HOXD3 | homeobox D3 |
| HPCAL4 | hippocalcin like 4 |
| HS6ST3 | heparan sulfate 6-O-sulfotransferase 3 |
| Id4 | inhibitor of DNA binding 4 |
| IGFBP5 | insulin-like growth factor binding protein 5 |
| IGFBP7 | insulin-like growth factor binding protein 7 |
| INPP4B | inositol polyphosphate-4-phosphatase, type II, 105kDa |
| KLF4 | Kruppel-like factor 4 (gut) |
| KLHDC7B | kelch domain containing 7B |
| Lamb3 | laminin, beta 3 |
| LGI2 | leucine-rich repeat LGI family, member 2 |
| LRP11 | low density lipoprotein receptor-related protein 11 |
| LRP4 | low density lipoprotein receptor-related protein 4 |
| LRRK1 | leucine-rich repeat kinase 1 |
| MLXIPL | MLX interacting protein-like |
| MMP17 | matrix metallopeptidase 17 (membrane-inserted) |
| Mras | muscle and microspikes RAS |
| MYO18A | myosin XVIIIA |
| Nell2 | NEL-like 2 |
| Nfasc | neurofascin |
| NKX6-3 | NK6 homeobox 3 |
| NPY1R | neuropeptide Y receptor Y1 |
| NRP2 | neuropilin 2 |
| Odz4 | teneurin transmembrane protein 4 |
| PCDH7 | protocadherin 7 |
| PDE8A | phosphodiesterase 8A |
| PGF | placental growth factor |
| PHLDA3 | pleckstrin homology-like domain, family A, member 3 |

| | |
|---|---|
| PHLDB1 | pleckstrin homology-like domain, family B, member 1 |
| Plec1 | plectin |
| PODN | podocan |
| PPP1R2 | protein phosphatase 1, regulatory (inhibitor) subunit 2 |
| PTGS2 | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) |
| PTPRE | protein tyrosine phosphatase, receptor type, E |
| PVALB | parvalbumin |
| Pvrl1 | poliovirus receptor-related 1 |
| Rab11fip5 | RAB11 family interacting protein 5 (class I) |
| Rab32 | RAB32, member RAS oncogene family |
| Rab43 | RAB43, member RAS oncogene family |
| RAB6B | RAB6B, member RAS oncogene family |
| RAB9B | RAB9B, member RAS oncogene family |
| Repin1 | replication initiator 1 |
| RUNX1 | runt-related transcription factor 1 |
| S1PR1 | sphingosine-1-phosphate receptor 1 |
| Scnn1a | sodium channel, nonvoltage-gated 1 alpha |
| SDK1 | sidekick cell adhesion molecule 1 |
| Sema4d | sema domain, immunoglobulin domain (Ig), transmembrane domain |
| SMTNL2 | smoothelin-like 2 |
| SPOCK2 | sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2 |
| SRCIN1 | SRC kinase signaling inhibitor 1 |
| SYT13 | synaptotagmin XIII |
| TAGLN3 | transgelin 3 |
| Tef | thyrotroph embryonic factor |
| TFAP2A | transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha) |
| TGFB3 | transforming growth factor, beta 3 |
| Traf5 | TNF receptor-associated factor 5 |
| TRH | thyrotropin-releasing hormone |
| TRIM11 | tripartite motif containing 11 |
| VIM | vimentin |
| WNT10A | wingless-type MMTV integration site family, member 10A |
| WNT2B | wingless-type MMTV integration site family, member 2B |

| APPENDICES VI<br>Bivalent genes CPE-DC | |
|---|---|
| **Gene Symbol** | **Full Name** |
| A2bp1 | RNA binding protein, fox-1 homolog (C. elegans) 1 |
| ABCB4 | ATP-binding cassette, sub-family B (MDR/TAP), member 4 |
| ABCB9 | ATP-binding cassette, sub-family B (MDR/TAP), member 9 |
| Ablim1 | actin-binding LIM protein 1 |
| ALCAM | activated leukocyte cell adhesion molecule |
| AMPH | amphiphysin |
| ANKRD33 | ankyrin repeat domain 33 |
| AQP4 | aquaporin 4 |
| ARHGAP6 | Rho GTPase activating protein 6 |
| ARVCF | armadillo repeat gene deleted in velocardiofacial syndrome |
| ATP10A | ATPase, class V, type 10A |
| Atp2b2 | ATPase, Ca++ transporting, plasma membrane 2 |
| ATP8A1 | ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1 |
| Axin2 | axin2 |
| BAI1 | brain-specific angiogenesis inhibitor 1 |
| BATF3 | basic leucine zipper transcription factor, ATF-like 3 |
| BOLL | bol, boule-like (Drosophila) |
| C15orf27 | chromosome 15 open reading frame 27 |
| CACNG3 | calcium channel, voltage-dependent, gamma subunit 3 |
| CAV2 | caveolin 2 |
| CCBE1 | collagen and calcium binding EGF domains 1 |
| CCDC110 | coiled-coil domain containing 110 |
| CCND2 | cyclin D2 |
| Cd109 | CD109 antigen |
| Cdgap | Rho GTPase activating protein 31 |
| CDH22 | cadherin 22, type 2 |
| CHN2 | chimerin 2 |
| CLMN | calmin (calponin-like, transmembrane) |
| CPEB2 | cytoplasmic polyadenylation element binding protein 2 |
| CPZ | carboxypeptidase Z |
| CRHBP | corticotropin releasing hormone binding protein |
| CYP24A1 | cytochrome P450, family 24, subfamily A, polypeptide 1 |
| CYTH3 | cytohesin 3 |
| Dhrs3 | dehydrogenase/reductase (SDR family) member 3 |
| DLK1 | delta-like 1 homolog (Drosophila) |
| DUSP4 | dual specificity phosphatase 4 |
| Eif2c4 | eukaryotic translation initiation factor 2C, 4 |
| Emid1 | EMI domain containing 1 |
| ERG | v-ets erythroblastosis virus E26 oncogene homolog (avian) |
| ESAM | endothelial cell adhesion molecule |
| FAM155A | family with sequence similarity 155, member A |
| FBXL14 | F-box and leucine-rich repeat protein 14 |
| FHL2 | four and a half LIM domains 2 |
| GLT1D1 | glycosyltransferase 1 domain containing 1 |
| Gpc4 | glypican 4 |
| GPR153 | G protein-coupled receptor 153 |
| GPR50 | G protein-coupled receptor 50 |
| Gria3 | glutamate receptor, ionotropic, AMPA3 (alpha 3) |
| H2AFY2 | H2A histone family, member Y2 |
| HPSE2 | heparanase 2 |
| HSF4 | heat shock transcription factor 4 |
| HTR2C | 5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled |
| HVCN1 | hydrogen voltage-gated channel 1 |
| IRX4 | iroquois homeobox 4 |

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| IRX5 | iroquois homeobox 5 |
| KCNIP2 | Kv channel interacting protein 2 |
| KIAA1026 | kazrin, periplakin interacting protein |
| KIAA1217 | KIAA1217 |
| KIAA2022 | KIAA2022 |
| Kif5c | kinesin family member 5C |
| LHX1 | LIM homeobox 1 |
| LRRK1 | leucine-rich repeat kinase 1 |
| MAP6D1 | MAP6 domain containing 1 |
| Mras | muscle and microspikes RAS |
| MXD4 | MAX dimerization protein 4 |
| NEFH | neurofilament, heavy polypeptide |
| NEFL | neurofilament, light polypeptide |
| NFIL3 | nuclear factor, interleukin 3 regulated |
| NPY1R | neuropeptide Y receptor Y1 |
| PAX8 | paired box 8 |
| PCDH19 | protocadherin 19 |
| PCDH7 | protocadherin 7 |
| PDK4 | pyruvate dehydrogenase kinase, isozyme 4 |
| Peli2 | pellino 2 |
| PIP5KL1 | phosphatidylinositol-4-phosphate 5-kinase-like 1 |
| Plcl2 | phospholipase C-like 2 |
| Plec1 | plectin |
| PPP1R3C | protein phosphatase 1, regulatory subunit 3C |
| Prdm2 | PR domain containing 2, with ZNF domain |
| Prkar1b | protein kinase, cAMP dependent regulatory, type I beta |
| PRKD1 | protein kinase D1 |
| PROKR2 | prokineticin receptor 2 |
| PRPS2 | phosphoribosyl pyrophosphate synthetase 2 |
| PTHLH | parathyroid hormone-like hormone |
| RAB33A | RAB33A, member RAS oncogene family |
| RGS20 | regulator of G-protein signaling 20 |
| Rreb1 | ras responsive element binding protein 1 |
| RTN1 | reticulon 1 |
| RUNX2 | runt-related transcription factor 2 |
| SEMA6D | sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D |
| SERTAD2 | SERTA domain containing 2 |
| Sgip1 | SH3-domain GRB2-like (endophilin) interacting protein 1 |
| SIX6 | SIX homeobox 6 |
| Slc1a6 | solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6 |
| SOBP | sine oculis binding protein homolog (Drosophila) |
| SOX3 | SRY (sex determining region Y)-box 3 |
| Sp9 | trans-acting transcription factor 9 |
| ST6GALNAC5 | ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltran |
| TAL1 | T-cell acute lymphocytic leukemia 1 |
| TBX22 | T-box 22 |
| TMEM150C | transmembrane protein 150C |
| Tmem178 | transmembrane protein 178 |
| TRPC6 | transient receptor potential cation channel, subfamily C, member 6 |
| TSC22D3 | TSC22 domain family, member 3 |
| TSHZ3 | teashirt zinc finger homeobox 3 |
| USH1C | Usher syndrome 1C (autosomal recessive, severe) |
| WNT10A | wingless-type MMTV integration site family, member 10A |
| XK | X-linked Kx blood group (McLeod syndrome) |
| Zbtb7c | zinc finger and BTB domain containing 7C |

# LIST OF PUBLICATIONS AND PRESENTATIONS

**Publications:**

1. Balasubramanian S, **Srilatha Jasty**, Sitalakshmi G, Madhavan HN, Krishnakumar S. Influence of feeder layer on the expression of stem cell markers in cultured limbal corneal epithelial cells. **Indian J Med Res. 2008 Nov; 128(5):616-22**.
2. Sudha B, **Srilatha Jasty**, Krishnan S, Krishnakumar S. Signal transduction pathway involved in the ex vivo expansion of limbal epithelial cells cultured on various substrates. **Indian J Med Res. 2009 Apr; 129(4):382-9**.
3. **Srilatha Jasty**, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. **Stem Cell Reviews and Reports. 2012 Dec; 8(4):1163-77**.
4. **Srilatha Jasty,** Saranya Suriyanarayanan, Krishnakumar Subramanian. Influence of self-assembling peptide nanofibre scaffold on retinal differentiation potential of Stem/progenitor cells derived from ciliary pigment epithelial cells. **Journal of Tissue Engineering and Regenerative Medicine. 2014 Jul 28.**
5. **Srilatha Jasty,** Subramanian Krishnakumar. Profiling of DNA and histone methylation reveals epigenetic-based regulation of gene expression during retinal differentiation of Stem/progenitor cells isolated from ciliary pigment epithelium of Human cadaveric eyes. **Manuscript submitted to Stem cell therapy and regenerative medicine.**

**Presentations:**

1. **Srilatha Jasty**, Gunisha Pasricha, Archana N and S. Krishnakumar. Isolation and Characterization of retinal stem/progenitor cells from human iris and

ciliary body. **Oral presentation in IERG 2007 on July 28th to 29th held at LVPI Hyderabad**. (Best paper presentation)

2. S. Krishnakumar, **Srilatha Jasty**, Gunisha Pasricha, and Archana N. Are there Retinal stem/progenitor cells in the ciliary body and iris – A preliminary Report. **Poster presentation at Frontier conclave 2007 an International conference on Stem cell, tissue engineering and regenerative medicine held in 30th Nov to December 2nd 2007, Chennai.**
3. **Srilatha Jasty**, Gunisha Pasricha, Archana N and S. Krishnakumar. Isolation and Characterization of retinal stem/progenitor cells from human ciliary body. **Oral presentation in National seminar on cord blood stem cells and their potential clinical applications at NRI Academy**, **Guntur held in 12th and 13th Nov 2007** (Best paper presentation).
4. **Srilatha J**, Krishnakumar S, Gunisha P, Archana N, Suganeswari G, Gopal L. A study on neural stem/progenitor cells from human cadaveric eyes – A Report. **Poster presentation at 12th ADNAT symposium on Biology of Embryonic and Adult stem cells, CCMB, Hyderabad held in February 23rd and 24th 2007.**
5. **Srilatha J**, Krishnakumar S, Gunisha P, Archana N, Suganeswari G, Gopal L. Retinal stem/progenitor properties of Ciliary Epithelial and Iris pigment epithelial cells from the Adult human cadaveric eye. **Oral presentation at ARVO 2008 held in Florida USA on 27th to 1st May** (Awarded international travel grant).
6. **J. Srilatha**, P. Gunisha, C. Nivedita, S. Krishnakumar. The Influence of cataract surgery on the Adult Retinal progenitors from Iris and Ciliary body. **Poster presentation at ASIA ARVO held in Hyderabad on 15-18 January 2009** (Awarded travel grant).
7. 8th Indo-Australia Biotechnology Conference on Stem Cell Biology held at **Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India on December 7-9, 2011.**

# BRIEF BIODATA OF CANDIDATE

Ms. Srilatha Jasty obtained her B.Sc. degree from TSR and TBK degree college, Affiliated to Andhra university in 2004. She completed her MS in medical laboratory technology in 2007 from Birla institute of Technology & Science – Pilani in collaboration with Medical research foundation. She did MS internship in L&T Department of Ocular pathology on the topic of "Proliferation, expansion and characterization of limbal stem cells" which was published in Indian Journal of Medical Research 2008. She later joined the department as a Research fellow in 2007. She worked under Dr. S. Krishnakumar in Department of Biotechnology funded project "To identify and characterize the retinal stem/progenitor properties of human iris pigment epithelial cells and ciliary epithelial cells *in vitro*". Later she registered for PhD in 2008 under the guidance of Dr. S. Krishna Kumar. During which she made four poster presentations, two oral presentations and attended one workshop. She has published two papers in her PhD topic, one manuscript under review and other manuscripts are under preparation. She has standardized the culture protocols for iris and ciliary pigment epithelial cells as well as retinal cells in this department. She also handled several cell lines like MIOM1 (Muller glial cell line), 3T3 (fibroblast cell line), CC (corneal endothelial cell line), HEK293 (human embryonic kidney cell line), HT29 (Human Colorectal Adenocarcinoma Cell Line), Y79 (Retinoblastoma cell line), and WERI (Retinoblastoma cell line) and she even handled the human amniotic membrane (denudation). Her research interest involves comprehensive understanding the key mechanisms involved in the proliferation and differentiations of adult stem cells. Her academic training and research experience have provided an excellent background in multiple biological disciplines including molecular biology, microbiology, biochemistry, and genetics.

# BRIEF BIODATA OF SUPERVISER

Dr. S. Krishnakumar (Deputy Director, Vision Research Foundation, Sankara Nethralaya) has been involved in teaching and research activities since 2000. He also serves as Ocular Pathologist, Medical Research Foundation, Sankara Nethralaya and contributes his service to the pathological diagnostics (Ocular).

His area of research focuses on diagnostic implications in cancer biology and stem cell biology. He has published about 130 peer-review articles till date with one of his landmark works is identifying blood based markers for retinoblastoma, a childhood eye cancer. He has established his international research collaborations with Deakin University, Australia; University of Missouri, Columbia and Johns Hopkins University School of Medicine, United States of America. He has earned his MD (Haematology and Ocular Pathology) in 1998, Research Fellowship (Opthalmic Pathology) at Doheny Eye Institute, Keck School of Medicine, University of Southern California (USA) in 2000, ICMR Research Fellowship for Ocular drug delivery at Durham's Research Center, Omaha in 2007 and DBT Nanotechnology Fellowship at Department of Radiology, University of Missouri, Columbia in 2007. He has supervised five Ph.D. students and currently 10 students are pursuing Ph.D. under his supervision.