

**Characterisation of Neural Progenitors  
from the Adult Retina and Ciliary  
Epithelium**

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## **Declaration**

I, Ioannis Kokkinopoulos, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. A few figures combine data from experiments conducted by myself and Rachael Pearson and those have been published in a manuscript included in the Appendix of this thesis.

The intraperitoneal BrdU injections (Chapter 6) and intraocular stem cell injections (Chapter 5) were performed by Rachael Pearson and Robert MacLaren, respectively.

## **Abstract**

The mammalian central neural retina (CNR) lacks the capability to regenerate, a phenomenon retained by lower vertebrates. However, retinal stem cells have been isolated from the ciliary epithelium of the mammalian retina. *Chx10* is a paired-like homeobox transcription factor gene expressed in the presumptive neural retina of the invaginating optic vesicle. The *Chx10* gene is expressed in the proliferating retinal progenitor cell population throughout retinal development hence is one of the earliest characterised RPC-specific markers. Mutations in the *Chx10* homeobox gene cause reduced proliferation of retinal progenitor cells during development, leading to microphthalmia. Recently, it was showed that in the ocular retardation mouse model lacking Chx10 (*Chx10<sup>orJ/orJ</sup>*), dividing cells persist in the adult CNR, suggesting the existence of a dormant stem/progenitor population.

The neurosphere-forming assay is a tool which has allowed scientists to study the behaviour of neural stem/progenitor cells *in vitro*. Here, I show that cells deriving from the CNR of the adult microphthalmic retina are proliferative and give rise to neurospheres *in vitro*, a characteristic of neural stem cells. However, these adult-derived CNR progenitors differ from those of the wildtype CE, leading to de-pigmented, larger and more numerous neurospheres expressing Müller glial cell markers. My results suggest that lack of Chx10 leads to maintenance of a dormant neural progenitor population in the adult CNR possible deriving from the abnormal appearance of GFAP<sup>pos</sup> Müller glia in late embryonic stages of the *Chx10<sup>orJ/orJ</sup>* retina. Furthermore, Chx10 is not required for *in vitro* proliferation of these progenitors.

One of the cardinal features of stem cells is their differentiation potential and multipotency. My experiments illustrate that *Chx10<sup>orJ/orJ</sup>* CNR-derived neurospheres are able to differentiate in a similar fashion to wildtype CE-derived neurospheres. Furthermore, when neurospheres lacking *Chx10* are

placed in conditions that promote differentiation, they significantly up-regulate the expression of photoreceptor genes in comparison to wildtype.

Hitherto, the developmental origin of CE-derived neurosphere-forming retinal stem cell is unclear. The ciliary body, where the CE is located in adult mammals, includes cells of mesodermal, neural crest and neural ectodermal origin. Here, data collected from lineage tracing analysis and *in vivo* BrdU-tagging experiments suggest that neurospheres are formed from BrdU<sup>pos</sup> cells observed *in vivo*, and that these cells originate from the embryonic anterior forebrain.

The comparative analysis of the microphthalmic CNR retinal progenitors and CE-derived progenitors provides valuable information on cell properties relevant for potential cell-based replacement therapies, as well for retinal regeneration potential in mammals.



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## **List of Abbreviations**

AP	alkaline phosphatase
ApoE	Apolipoprotein E
bHLH	basic helix-loop-helix
AMP	adenosine monophosphate
Bmp	bone morphogenetic protein
bp	base pairs
BrdU	bromodeoxyuridine
BrdU <sup>pos</sup>	Bromodeoxyuridine positive
BSA	bovine serum albumin
cfu	colony forming unit
CDK	cyclin-dependent kinase
CE	ciliary epithelium
CB	ciliary body
CNS	central nervous system
CDKI	cyclin-dependent kinase inhibitor
CRALBP	Cellular Retinaldehyde-Binding Protein
D	dorsal
DEPC	diethylpyrocarbonate
dH <sub>2</sub> O	distilled H <sub>2</sub> O
DIC	differential interference contrast
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
dNTP	deoxyribonucleotide triphosphate
D-V	dorso-ventral
EDTA	ethylenediamine tetraacetic acid
EB	embryonic body

eGFP	enhanced green fluorescent protein
hES	human embryonic stem cell
HSC	haematopoietic stem cell
FCS	fetal calf serum
Fgf- $\beta$	fibroblast growth factor basic
FITC	fluorescein isothiocyanate dye
Cy3	Fluorescent cyanine dye
g	gravity
Gas1	growth arrest specific gene 1
GFP	green fluorescent protein
GS	glutamine synthetase
GM-CSF	granulocyte macrophage colony stimulating factor
GFAP	glial fibrillary acidic protein
H&E	haematoxylin and eosin
Hh	hedgehog
IRES	internal ribosomal entry site
ICM	inner cell mass
IL	Interleukin
kb	kilo bases
kDa	kilo Daltons
Log	Log <sub>10</sub>
LIF	leukaemia inhibition factor
mAb	monoclonal antibody
Min	minute
MHC	myosin heavy chain
MLC	myosin light chain
mES	murine embryonic stem cell
Mitf	microphthalmia associated transcription factor
MHC I	major histocompatibility complex I
mm	millimetre

µm	micrometre
NCAM	neural cell adhesion molecule
NE	neuroepithelium
NK	natural killer
nm	nanometre
NFL	nerve fibre layer
NGM	neurosphere growth medium
NDM	neurosphere differentiation medium
CNR	central neural retina
NSC	neural stem cells
NR	neural retina
OC	optic cup
OS	optic stalk
OV	optic vesicle
PBS	phosphate buffered saline
PFA	paraformaldehyde
pH3	phosphorylated histone H3
pCB	presumptive ciliary body
pRPE	presumptive retinal pigmented epithelium
pNR	presumptive neural retina
pAb	polyclonal antibody
RA	retinoic acid
PTEN	phosphatase and tensin homolog deleted on chromosome 10
Re-Ti	real-time polymerase chain reaction
PCR	
RGC	retinal ganglion cell
RPE65	retinal pigmented epithelium 65Kda protein
RPE	retinal pigmented epithelium
RPC	retinal progenitor cell

RSC	retinal stem cell
Rho <sup>-/-</sup>	rhodopsin knockout
rpm	revolutions per minute
Rds	retinal degeneration slow
SDS	sodium dodecyl sulphate
SE	surface ectoderm
Sec	second
Shh	sonic hedgehog
SS	somite stage
TGF- $\beta$	transforming growth factor-beta
SVZ	subventricular zone
UV	ultra violet
v/v	volume per volume
w/v	weight per volume

## **Chapter 1 – General Introduction**

The eyes may be the window to the soul for poets, but for neuroscientists and developmental biologists, they serve a more practical purpose. One of the key concepts of embryology derived from examination of ocular development from different species showing the striking developmental similarity between them (Spemann H 1923). The human brain involves around 100 billion cells dedicated to its operation and structure. Due to this high complexity, the vertebrate retina is a relatively simple part of the central nervous system (CNS). It is an ideal model for study of cell development and physiology of CNS structures. By means of investigating the ever-expanding universe of stem cells and progenitor cells in humans and other species, there is now considerable evidence indicating that many organs in our body contain a small population of cells able to proliferate and differentiate giving rise to different cell types including neurons (Gottlieb DI 2002).

## **1.1 Overview of eye development**

The morphological description below is primarily based on current knowledge about murine eye development, which is the basic experimental model used in this thesis. Morphogenetic processes are conserved among species, in general. Human developmental guidelines are mentioned, where appropriate for comparative purposes.

### **1.1.1 Early eye development**

The eye develops from the embryonic forebrain in distinct serial morphological stages (Figure 1.1). Cells deriving from the neural ectoderm, surface ectoderm and cells from the periocular mesenchyme, containing neural crest and mesoderm-derived cells (Gage PJ 2005) contribute to eye formation. Its

development is initiated during neural tube formation, a stage called neurulation, when the diencephalon bulges out as optic placodes giving rise to bilateral evaginations, in the early neurula stage of the vertebrate embryo [in mouse this is stage 5-7 pairs of somites, or embryonic day 8.5 (E8.5), day 22 in humans] (Coulombre AJ 1965; Kaufman MH 1992). The optic placode will enlarge and form the optic vesicle (day 24 in human, E9.5 in mouse), in proximity to the overlying surface ectoderm. Direct contact will occur later, at mouse stage of 20-25 pairs of somites. This contact is essential for the induction of the lens placode, by signals deriving from the optic vesicle, leading to the formation of the lens vesicle. In mouse embryos with 25-30 pair of somites (E10), invagination of the optic vesicle occurs forming the bilayered optic cup. This transition is completed when the mouse embryo has 35-40 pairs of somites.

At this stage, the lens vesicle has formed the lens vesicle, with epithelial cells forming the primary and secondary lens fibers. Corneal formation is one of the last structures formed in ocular morphogenesis, mainly involving the two waves of mesenchymal cell migration between the lens and the primary stroma, a multilayered structure deriving from the basal surface. Once they have settled these cells secrete Collagen Type I and hyaluronidase, eventually becoming the transparent cornea (Coulombre AJ 1965).

While the cornea and lens are being formed, the optic cup undergoes thorough and precise modifications. During invagination, the innermost layer of the optic cup, closest to the lens vesicle, will thicken due to rapid proliferation and give rise to the formation of the neural retina, while the outer one remains a thin epithelial monolayer that will form the Retinal Pigment Epithelium (RPE). At the same time, the outer regions of the optic cup, the so-called peripheral margin of the optic cup, where the presumptive neural retina and RPE layers meet, give rise to the ciliary body (CB) and part of the iris (Beebe DC 1986). The CB controls ocular muscles and secretes aqueous humour.

Image removed for copyright reasons

*Figure 1.1 Schematic view of the timeline of developing murine eye [adapted from (Endo et al. 2007)]*

### **1.1.2 Late eye development – retinogenesis**

The RPC of the inner layer of the optic cup in the presumptive neural retina undergo extensive proliferation and differentiation, generating interneurons, ganglion cells, photoreceptors and glial cells, leading to the formation of a demarcated and multilayered neural retina. Pioneering studies from Camillo Golgi and Santiago Ramon y Cajal led to the re-evaluation and abandonment of the reticular theory for the neuron theory (Piccolino M 1989). The former was introduced before the 1880's indicating of a collective nervous impulse based on a holistic response of the nervous system. At the end of the 19<sup>th</sup> century, Ramon y Cajal proposed and proved that “the nervous system is made up of the sum of just so many neurons, each of which is an anatomical, functional, and embryological individuality and not merely a transit station in a network of nervous filaments”.

Vertebrate retinas consist of six major classes of neuronal cell types and one glial cell type. A more rigorous examination reveals that within each major class



are numerous minor cell subtypes bringing the total number of cell types to more than 50 (Masland RH 2001). Experiments attempting to trace the cell fate of these populations using [<sup>3</sup>H]-Thymidine labelling and autoradiography, revealed an extended evolutionary conserved mechanism of cell fate determination (Cepko CL 1996). Retinal cells are generated in sequence, by initiating their first mitotic events before optic cup formation. In mammals, the first cell type being produced in the developing retina is the retinal ganglion cell (RGC) followed by cone photoreceptor and amacrine cells (Figure 1.2). Horizontal and rod photoreceptor cells are born later. Retinal histogenesis ends with the formation of bipolar and Müller glia cells. This stereotypic sequence of cellular retinogenesis contains considerable overlap of different cell types being formed in order to construct the laminated structure of the adult retina after postnatal day 11. This hierarchy points to the assumption that a retinal progenitor cell (RPC) population exists having the ability to precisely respond to complex positive-negative extrinsic cues leading to the creation of different retinal cell types (Holt et al. 1988; Wetts and Fraser 1988; Wetts et al. 1989). In a simplistic model, one can hypothesise that extracellular signalling from newly-developed cells might provide the inhibiting factors for halting RPC differentiation towards one cell population leading to the creation of other, lateral cell types. This would also lead to the assumption that if postnatal RPC were cultured with early embryonic retinal progenitors, the former would adopt cell fates found in earlier developmental stages. But experimental data from Cepko and colleagues (Belliveau MJ 2000) revealed that intrinsic “barriers” in RPC halt their re-specification capability, if any. In contrast, culturing cells from postnatal retina with an excess of embryonic retinal cells led to an inhibition of later-produced retinal cell types but without promoting early-produced cell types. In a similar manner, embryonic RPC failed to adopt later cell fates when cultured in excess with postnatal RPC. Therefore, intracellular signalling pathways are vital for RPC differentiation and “stemness” preservation. The above observations propose that RPC switch between different competence stages during retinal

development. Thus, embryonic and postnatal RPC respond differently to stimuli (Ahmad I 2004).

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*Figure 1.2 Retinal histogenesis proceeds in a predetermined order across species [adapted from (Marquardt T 2002)]*

## **1.2 Stem Cells**

### **1.2.1 Principals of stem cells**

This thesis is focuses on the characterisation of RPC and retinal stem cells (RSC) in the adult mammalian retina. It is essential to establish knowledge for the ongoing stem cell research, as well as for advances in this vast evolving

field. Understanding the mechanisms controlling cell fate and proliferation will provide us with tools for cell-based therapies and inducing retinal regeneration in humans. Below, I give an overview of embryonic stem as well as adult stem cells focusing on well-studied organs and systems that are known for their regenerative capabilities. Finally, I focus on the main subject of my study, neural stem cells, extending to retinal stem/progenitor cells. In that way, I am hoping to bridge views and aspects of established stem cell niches with the newly discovered adult retinal stem cell niche.

### 1.2.2 Embryonic Stem Cells

In many aspects of life there is a hierarchical structure. Not surprisingly, that also exists in the formation of a living organism and is one of potential. At the top of the pyramid is the fertilised oocyte (egg), which will perform its first mitotic event giving rise to two totipotent cells (Thomson et al. 1998) (Figure 1.3). These cells will form the embryo and the trophoblast of the placenta. At E4 in humans, the formation of the blastocyst occurs comprising of the trophoblast cells and the inner cell mass (ICM). The cells of the ICM are considered pluripotent deriving from the zygote (fertilised egg), which is totipotent until the eight-cell stage of the morula stage. Cells of the ICM, upon gastrulation, are able to differentiate into cells of the three germ layers, endoderm, mesoderm and ectoderm, and germ cells *in vitro* and *in vivo* (Bradley et al. 1984; Toyooka et al. 2003). These are called embryonic stem (ES) cells and are considered immortal with indefinite self-renewal capacity *in vitro*. Each germ layer possesses cells with a different potential able to generate tissue-specific cell types; thus they are termed multipotent (Evans and Kaufman 1981). The first mammalian ES cells were extracted from the murine ICM in 1981 (Evans and Kaufman 1981), while in 1995, primate ES cells were cultured using mouse embryonic fibroblasts as feeder cells (Thomson et al. 1995). Human ES cells were first cultured in 1998

using the same technique as for murine ES cells (Thomson, Itskovitz-Eldor et al. 1998). In essence, the three distinct germ layers of the embryo give rise to specific pluripotent cell populations.

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*Figure 1.3 Path from totipotency to organogenesis [modified from (Wobus and Boheler 2005)]*

### **1.2.2.1 Endoderm-derived stem cells**

Vertebrate endoderm consists at first of flattened cells, which subsequently become columnar. After gastrulation, it is a 500-cell thick layer in mouse embryos that eventually will give rise to the epithelial lining of the whole of the digestive tube (excluding the mouth, the pharynx and part of the rectum), stomach and the intestine. It will also give rise to the lungs and is an essential component of glands found in the thymus, pancreas and liver (Wells JM 1999). Liver- and pancreas-differentiated cells can be obtained from murine ES (mES) cells by selecting Nestin<sup>pos</sup> cells and using B27-rich N2 medium supplemented with Fibroblast Growth Factor 2 (FGF-2) for progenitor expansion, while for terminal differentiation, albumin, alpha-1-antitrypsin, cytokeratin 8 and 18 as well as insulin and c-protein but not alpha-fetoprotein are being used (Lumelsky et al. 2001; Rambhatla et al. 2003). There are also traces of neuronal ( $\beta$ -III tubulin) fate mixed with islet-like cells, leading to the assumption that an ectodermal fate might be a default one (Schuldiner et al. 2001). Combination of Fgf4, Activin A, bone morphogenic protein 4 (Bmp4), retinoic acid (RA) and cyclopamine showed that human ES (hES) cells potentially can be driven towards an endodermal fate giving rise to insulin-secreting cells and/or  $\beta$ -like cells (Assady et al. 2001; Sharon D 2002; Serafimidis et al. 2007).

### **1.2.2.2 Mesoderm-derived stem cells**

The haematopoietic, vascular and cardiac lineages derive from the mesoderm. It is the most researched progenitor system since it gives rise to hematopoietic cells and blood vessels. The common progenitor giving rise to both systems is called a hemangioblast, typically expressing flk-1, and generally blood islands can be generated by mES cells along with erythrocytes and macrophages

(Doetschman et al. 1985; Wiles and Keller; Nakano et al. 1994). Upon differentiation by addition of Fetal Calf Serum (FCS), interleukin (IL) 1, granulocyte macrophage colony stimulating factor (GM-CSF), IL-3 or feeder cell layer such as OP9 [a line of bone marrow-derived mouse stromal cells, (Kitajima et al. 2003)] leads to the development of hematopoietic cell types from the erythroid, myeloid, and lymphoid lineages, and of natural killer (NK) cells. In high amounts of FCS-containing (>15%) medium, mES and hES cells can differentiate to cardiomyocytes (Boheler et al. 2002; Reppel et al. 2004), having a characteristic profile expressing  $\beta$ -tubulin,  $\alpha$ -myosin heavy chain (MHC) and  $\beta$ -MHC, myosin light chain (MLC) 2v mRNA transcripts, strongly immunopositive for  $\alpha$ -troponin-T. Electrophysiological experiments showed a potential similar to that of primary myocardium which subsequently differentiate to atrial-, ventricle-, Purkinje-, and pacemaker-like cardiomyocytes while hES cells showed a lower potential in terms of number of cardiomyocytes and beats per minute potential while their  $\beta$ -adrenergic and muscarinic receptors potential was similar for frequency and voltage manipulation. Other cell types that can be formed from the mesoderm layer of mES cells are adipogenic, chondrogenic, osteoblast and myogenic cells upon addition of growth factors such as RA and di-butyl cyclic adenosine monophosphate (AMP) in successive steps (Drab et al. 1997).

### 1.2.2.3 Primordial germ cells

The most recent characteristic of ES cells was the production of germ-line cells *in vitro* by embryoid bodies (EBs) (Hubner et al. 2003; Toyooka, Tsunekawa et al. 2003; Geijsen et al. 2004). Three independent groups using germ cell-specific gene tags, detection of germ-cell-specific protein by immunolabelling and functional analysis, showed that after mES and hES cell expansion with leukaemia inhibition factor (LIF) and formation of EBs, it was feasible to obtain

germ-like cells after intermediate culture periods (~12-14 days *in vitro*). These cell aggregates expressed characteristic proteins, such as Oct3 and Oct4, the post-migratory germ cell marker Vasa, estradiol synthesis, a typical function of somatic cells, and expression of *zona pellucida* proteins (ZP) 2 and 3, but not ZP 1 accounting for insufficient *in vitro* conditions for obtaining fully undifferentiated germ cells. In addition, classical methylation imprints were absent for the Igf2r and H19 set of meiotic genes in those cells.

#### 1.2.2.4 Ectoderm-derived stem cells

The ectodermal lineage can give rise to cells of the neuro-ectodermal lineage such as those of the peripheral nervous system (PNS) and CNS, to epithelial cells and some vascular smooth muscle cells. The neurogenic potential of ectoderm-derived murine ES cells became evident in 1995, when independent research groups (Bain et al. 1995; Fraichard et al. 1995; Strubing et al. 1995) identified cell populations that upon RA treatment expressed high levels of Nestin, glial fibrillary acidic protein (GFAP), O4,  $\beta$ -III tubulin and Brn-3. Also, electrophysiological analysis showed possible GABAergic and/or cholinergic neuronal fates. The isolated cells were able to grow as EBs or cell aggregates. After introduction to different substances such as RA ( $10^{-6}$ - $10^{-7}$  M) (Rohwedel et al. 1999; Rolletschek et al. 2001), and the use of lineage-tracing analysis, like  $\beta$ -gal ( $\beta$ geo) insertion to SRY-box containing gene 2 (Sox2) gene or GFP insertion to Sox1 gene (the earliest neuron-ectoderm murine marker) (Li et al. 1998; Guan et al. 2001; Li et al. 2001; Aubert et al. 2002; Aubert et al. 2003; Ying et al. 2003) or co-culturing with stromal cells, like PA6 cells (Kawasaki et al. 2000; Gottlieb et al. 2002) it was shown that the above-mentioned cells were able to differentiate, or rather to be specified to the neuronal lineage. Note, that this pathway is not considered the default one and could be inhibited by factors such as transforming growth factor-beta (TGF- $\beta$ ) (Tropepe et al. 2001), and BMP-4

(Rohwedel et al. 1998). To date, conflicting evidence exist in terms of which conditions are optimal for neuronal differentiation and an optimal neuronal differentiation “cocktail” is still elusive. Nonetheless, a clear pathway is being constructed pinpointing which factors are to be avoided and which to be included in order to obtain homogeneous neural populations *in vitro* (Figure 1.4). After EBs have formed, either large amounts of RA are introduced to the culture, or FCS is removed with addition of FGF-2 and Epidermal Growth Factor (EGF) in order to obtain neuro-ectodermal cells (Lee et al. 2000). After that step, neuronal differentiation will again occur by addition of Poly-L-Lysine (PLL), Poly-Ornithine (PO), Laminin and re-introduction of FCS.



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*Figure 1.4 Lineage selection conditions used for ES induced differentiation [modified from (Guan, Chang et al. 2001)]. EG; embryonic germ cells.*

The potential for neuro-ectodermal derivatives from human ES cells was first demonstrated in 2001 (Reubinoff et al. 2001), forming EB *in vitro*, expressing Neural Cell Adhesion Molecule (N-CAM), Nestin, A2B5 and Vimentin (Carpenter

et al. 2001). Upon differentiation in serum-free conditions and withdrawal of growth factors, these cells were able to give rise to astrocytes, oligodendrocytes, and mature neurons. Finally, several factors (RA, Nerve Growth Factor  $\beta$ , FGF-2 and EGF) (Schuldiner, Eiges et al. 2001), substrates like Matrigel<sup>TM</sup> and laminin (Xu et al. 2001), as well as feeder layer cells expressing Wnt-1 (Perrier et al. 2004) have been applied to hES cells for differentiation induction showing upregulation of neuron-specific genes, neuronal morphology and neuronal-specific protein expression *in vitro*.

For this project, importance was given to the ectoderm-derived neural cells, taking into consideration the factors need to be taken on board for obtaining ectoderm-derived cells while avoiding the ones that might give rise to mesoderm- or endoderm-derived cells.

### **1.2.3 Adult stem cells**

#### **1.2.3.1 Lessons from the haematopoietic system**

Stem cells are defined as single cells with the ability to form clonal and differentiated progenies. The first stem cell was discovered in the haematopoietic system in 1961 (Till JE 1961; Till JE 1961) and since then it is considered as a model for studying stem cell properties and characteristics in organs and systems newly found to possess progenitor populations, including the CNS. In these experiments, colonies of progeny cells were found in the spleens (spleen colony-forming assays, CFU-S) of rodents that received radiation and therefore their bone marrow cells were diminished. These colonies were able to reconstitute the whole blood-cell lineage (Becker KL 1963; Wu AM 1968; Wu AM 1968). Following that work, the first haematopoietic stem cell (HSC) was isolated by developing assays for the identification of B- and T-

lymphocytes as well as myeloerythroid cells (Ezine S 1984; Muller-Sieburg CE 1986). Two additional properties that were able to elucidate further these stem cells was the identification of cell surface markers as well as ability to sort the cells that possess or lack specific surface “stem” cell markers (Spangrude GJ 1988). Currently, HSC are lineage negative ( $\text{Lin}^-$ ) meaning that they do not express markers found on T cells, B cells, macrophages and other white- or red-blood cells. In contrast, they express markers that are not readily found on their progenies such as Sca-1 and Thy-1 and c-kit receptor. Furthermore,  $\text{Lin}^{\text{neg}}$ ,  $\text{Sca-1}^{\text{pos}}$ ,  $\text{Thy-1}^{\text{pos}}$ ,  $\text{c-kit}^{\text{pos}}$  cells can be subdivided into populations that express different levels of additional surface markers such as CD34 and/or Mac-1 show different levels of self-renewal (Morrison SJ 1994).

The same approach was used for isolating oligolineage progenitors of HSC such as common myeloid progenitors (CMP) and common lymphocyte progenitors (CLP), with the former expressing granulocyte macrophage cell-stimulating factor (GM-CSF) receptors but not interleukin 7 (IL-7) receptor, while the latter having the opposite expression profile. These lineage-restricted progenitors can also give rise to even more restricted progenies expressing a different set of receptor markers (Akashi K 2000). Finally, these will give rise to non self-renewal cells that will be considered as end-lineage precursors (Metcalf D 1991). This hierarchy is believed to exist for all stem cells residing in different tissues in mammals. The lesson learned from HSC is that it is vital to identify surface markers able to distinguish between progenitors in different states of commitment in any organ examined.

### **1.2.3.2 The stem cell niche**

As mentioned previously, spleen CFU introduced the concept of locations where stem cells reside (Schofield R 1978). These locations should possess properties

essential for stem cell maintenance and self-renewal. The cellular and molecular microenvironment is paramount and the 3-dimensional structure seems to play a vital role in stem cell quiescence and cell survival. Often, the niche is composed of stromal cells where possibly stem cells anchor, receiving all the appropriate signals that direct growth and inhibit differentiation (Pinto D 2003). The niche's properties and structures are different and are closely related with the organ in which they reside. Studies performed in *Drosophila melanogaster* indicate that stem cell niches have the power to maintain exogenously introduced stem cells even when there are no stem cells inside them, thus being “empty” (Ohlstein B 2004).

Stem cell gain and loss seems to be an insurance mechanism for sustainable and healthy supply of stem daughter cells. Studies performed in the fruit flies' ovary have shown that there is continuous replacement of stem cells, and this also might occur in mammals, such as in the case of HSC in the bone marrow. More complicated niches such as those residing in the subventricular zone (SVZ) of the brain, might need a complicated cyto-architecture compiled of astrocytes, ependymal and endothelial cells and a basal lamina for replenishing and sustaining a stem cell population (Doetsch 2003; Pardal 2007) .

### **1.2.3.3 Cancer stem cells**

The concept of cancerous stem cells arise from the pioneering studies of KJ Anlyan in leukemic cells and the levels of expression of Beta-glucuronidase (Anlyan 1954). Although the field of stem cell biology then lacked the technological advancements of today, the concept of a cell being unable to differentiate and instead continuing to undergo multiple rounds of cell divisions emerged. Because normal stem cells and cancer cells have the ability to self-renew, the former is considered an excellent candidate for the origin of a cancer

population in solid and blood-borne tumours (Reya et al. 2001). Evidence showed that a number of signalling pathways might be affected upon malignant transformation of stem cells. Of interest, the sonic hedgehog (Shh), the wingless and Int (Wnt) and Notch pathways that are activated already in normal stem cells might be involved in the creation of several types of cancer stem cells (Taipale and Beachy 2001; Reya and Clevers 2005). Abnormal activation of these pathways has shown a shift from differentiation of an over-activation of the self-renewal machinery that can also affect progenitor or more transient-amplifying cells as well, by transforming them to stem-like cells able to self-renew more potently.

#### **1.2.4 Overview of stem cell markers**

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 bone-marrow derived stem cell lineages, the expression levels and combination of a number of these markers need to be investigated for identifying a stem cell. Table 1.1 summarises the markers to date for stem/progenitor cell characterisation [for review see (Alison MR et al. 2002)].

*Table 1.1 Summary of commonly used markers for the identification of various murine stem cell lineages.*

<b><i>Tissue or Organ of interest</i></b>	<b><i>Identification factors</i></b>
<b>Bone Marrow and Blood</b>	Bone morphogenetic protein receptor (BMPR), CD4, CD8, CD38, CD34, CD44, CD45, c-Kit, Lin, Mac-1, Muc-18, Sca-1, Thy-1, Stro-1, Colony-forming unit (CFU), Fibroblast colony-forming unit (CFU-F), Hoechst dye efflux assay
<b>Nervous System</b>	CD133, MAP-2, MPB, Nestin, NF, Neurosphere-forming assay, Noggin, O4, O1, Synaptophysin, Tau
<b>Pancreas</b>	Cytokeratin 19, Glucagon, Insulin, PDX-1, Nestin, somatostatin
<b>Pluripotent Stem Cells, ES cells</b>	Alpha-fetoprotein (AFP), Alkaline phosphatase, BMP-4, CD30, TDGF-1, GATA-4 gene, Nestin, N-CAM, Oct-4, Pax6, SSEA-3, SSEA-4, Telomerase, TRA-1-60, TRA-1-81, Vimentin
<b>Skeletal Muscle/Cardiac/Smooth Muscle</b>	MyoD, Pax7, Myogenin, MR4, Myosin heavy and light chain

## **1.3 Adult Neural Stem Cells**

### **1.3.1 Neurospheres of the brain**

Neural stem cells (NSC) were originally isolated from the adult mammalian CNS by Reynolds and Weiss in 1992 (Reynolds BA 1992). Cells isolated from the striatum of the adult brain were able to proliferate in the presence of EGF, forming floating clonal colonies that became known as “neurospheres”. The two cardinal properties of stem cells that of immortality and multipotentiality were demonstrated by neurosphere cultures; when neurospheres are dissociated to single cells, some of these cells are able to give rise to secondary neurosphere colonies, thus demonstrating self-renewal, whereas neurosphere cells exhibit multipotentiality when cultured under differentiating conditions (Potten CS 1990; Reynolds BA 1996). In essence, each neurosphere contains a mixed population of neural progenitor and stem cells (Bjornson CR 1999). Since it has been shown that progenitors divide more rapidly than stem cells, this heterogeneity could explain the robustness in proliferation observed in neural stem/progenitor *in vitro* assays.

What is the correlation between a neurosphere and a stem cell? Cells isolated from subependyma (SE) of adult brains revealed that only a proportion (~3%) of those were “unidentified” and thus probably stem cells (Doetsch F 1997). Further, with the use of a transgenic mouse model expressing the herpes virus thymidine kinase (HSV-tk) from the GFAP promoter and exposure to an antiviral agent ganciclovir, it was possible to distinguish proliferating cells. This agent is taken up by all cells but retained only in cells expressing Tk (Frank KB 1984). It was shown that culturing dividing cells from this transgene in the presence of ganciclovir kinase resulted in the complete abolishment of neurosphere formation *in vitro*.

One of the drawbacks of the neurosphere-formation *in vitro* assay as a neural stem cell indicator is the absence of clonally-derived aggregates from a tissue/region of interest. Inability to observe them might be primarily due to the absence of a stem cell; another possibility is stem cells might be present but in a quiescent state since *de novo* or inhibitory signals are present. Finally, stem cell progeny (progenitors) might not survive in the same culture conditions that favour stem cell division and therefore die [A cells (immature neuroblasts), B cells (astrocyte-like stem cells) and C cells (transient amplifying cells)]. The HSV-tk experiment showed that stem cells are not simply inhibited from dividing. Experiments injecting high doses of [<sup>3</sup>H]-Thymidine or using a mitotic inhibitor, cytosine-beta-D-arabinofuronaside (AraC) into mice showed that even in the absence of mitotically active cells, there was proliferation after 2 days *in vitro* (Doetsch F 1999; Weissman IL 2000; Gritti A 2002). These experiments state that there is a population of stem cells that is probably quiescent *in vivo*.

However, reports challenged the above experiments (Gritti A 1999), mentioning that in high doses of thymidine, 50% of neurospheres were present in the SVZ of mice leading to the assumption that some, but not all, neurospheres derived from transiently proliferating cells. Also, the authors of the contradicting reports used different cell densities and it is well established that non-cell autonomous properties influence proliferation of cells *in vitro* (Chiasson BJ 1999; Tropepe V 1999; Song H 2002). Nevertheless, it is essential to understand how the endogenous NSC population is maintained and functions and how is it possible to be activated for regeneration purposes (Pardal 2007).

### **1.3.2 Relationship between NSC and cancer stem cells**



The presence of dividing neural cell populations within the brain provided a launching pad for investigating the possibility of these stem/progenitor cells to form brain tumours in children and adults. The neurosphere-forming assay has provided researchers with a tool to further investigate this link since formation of a neurosphere is due to clonal expansion resembling that of a brain tumour such as medulloblastoma. Indeed, in 1972 it was reported that these tumours might have arisen from transformed neural progenitors or even stem cells (Rubinstein 1972). Several lines of more recent evidence showed that brain tumours are composed from different neural cell types many of them expressing the stem/progenitor marker Nestin (Dahlstrand et al. 1992; Dahlstrand et al. 1992; Dahlstrand et al. 1995), show a high degree of heterogeneity (Tohyama et al. 1992; Trojanowski et al. 1992), and are comprised of cells expressing phenotypes of more than one neural lineage, implicating a multipotential cell of origin (Lendahl et al. 1990).

### **1.3.3 Retinal stem cell niche and retinal progenitors**

Lower vertebrates display the capacity to generate new retinal neurons in response to injury (Hollyfield JG 1971; Reh TA 1998). New neurons arise from a region called the ciliary marginal zone (CMZ) located around the anterior margin of the retina, which maintains a population of retinal stem cells throughout life. Regenerative potential in the central retina is more limited. A population of rod precursor cells exists in teleost fish that can regenerate all retinal neuronal types after damage. [Figure 1.5, for review, see (Raymond 1991)]. In embryonic chicks and rodents, RPE is capable of transdifferentiation to an inverted neural retina *in vivo* and generating new neurons *in vitro* (Zhao et al. 1995; Liang et al. 2006).

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*Figure 1.5 retinal stem cell niche in amphibians and vertebrates [adapted from (Reh TA 2001)].*

Similarly, recent work showed that in zebrafish, the Müller glial cells function as multipotent retinal stem cells that respond to loss of photoreceptors by specifically regenerating the missing neurons (Bernardos et al. 2007). Conversely, acute damage of the postnatal chick retina induced Müller glial cells to undergo limited cell division and rarely to express markers characteristic of retinal neurons (Reh TA 1998; Dyer MA 2000; Fischer AJ 2001). Although the adult mammalian eye lacks these regenerative capabilities, recent evidence has demonstrated that the ciliary epithelium (CE, Figure 1.6), part of the ciliary body (CB), a structure analogous to the lower vertebrate CMZ but not part of the neural retina, contains a population of retinal stem cells (RSC) (Ahmad I 2000; Tropepe V 2000). Currently, their *in vivo* function is unclear (Das AV 2005). While quiescent during adult life, *in vitro* these cells demonstrate characteristics typical of neural stem cells, including multipotentiality and self-renewal (Ahmad I 2000; Tropepe V 2000). Whether the mammalian central neural retina (CNR) contains a dormant stem cell population, similar to the Müller-like progenitor cell observed in lower vertebrates, remains an important question. Characterisation of adult stem cells offers insight into the regenerative potential in the mammalian nervous system and may have applications in the development of novel therapies for the treatment of retinal disease.

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*Figure 1.6 Intraocular view of the CE (modified from [www.bouldereyesurgeons.com/.../page54.html](http://www.bouldereyesurgeons.com/.../page54.html))*

Recent literature describes two different cell populations; retinal stem cells (RSC) and retinal progenitor cells (RPC) (Cepko CL 1996; Reh TA 1998; Ahmad I 2000; Tropepe V 2000; James J 2004; Zaghoul NA 2005). The latter is purported to derive from asymmetric proliferation of the former (Zhong W 2003). During retinal histogenesis, RPC undergo a finite number of divisions to generate the full complement of retinal neurons and Müller glia, which is complete by around post-natal day 11 in the mouse (Young 1985). By contrast, RSCs appear to persist during adulthood in the CE and when stimulated by growth factors *in vitro*, proliferate and show properties of multipotentiality and self-renewal. However, there is limited evidence of either specific cell markers or assays that permit the distinction between these two cell types (Bhattacharya S 2003; Das AV 2005). For this reason, here I am using the term progenitor cell to describe stem-like cells in the adult neural retina with the ability to proliferate and form neurospheres *in vitro* and to differentiate to express retinal cell markers.

During optic cup formation the presumptive neural retina contains multipotential RPC. It is those that will generate the various cell types that will migrate to either the developing outer nuclear layer (ONL) such as photoreceptors (PR), inner nuclear layer (INL) comprised of bipolar cells (BP), horizontal cells (HC), amacrine cells (AC) and Müller glia (MC) or the Ganglion Cell Layer (GCL) comprising or retinal ganglion cells (RGC). The basic scheme of histogenesis has been revealed by studies performed by Ramon y Cajal (Piccolino M 1988; Piccolino M 1989) (Figure 1.7).

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*Figure 1.7 The adult murine retinal layers and cells comprising them [provided by Dr. D. Zack (Gouras et al. 1991)]*

Embryonic retinal progenitors can be categorised based on their responsiveness to growth factors and tendency to generate neurons or glia (James J 2004). In many warm-blooded species early RPC give rise to early histogenesis-produced

cells such as RGC, cones, horizontal cells and amacrine cells, while late RPC differentiate to rods, bipolar cell, Müller glia and the remainder of the amacrine population (James J 2004). The mouse embryonic gestation period is normally 19-23 days. The optic cup begins to develop at day 9, thus enabling any RPC in the optic pit area to initiate their retinal specification events. After E9.5, RPC continually give rise to different cell populations. Experiments performed on RPC isolated from E14 (early) and E18 (late) embryos show distinct responses to mitogens and growth factors leading to different outcomes (James J 2004). Transcriptional profiling indicates the usage of signalling pathways for different objectives. For instance, *Notch* is a highly conserved gene among species producing Notch protein, which in mammals, has similar expression patterns (Klassen HJ 2004). Briefly, proteolytic cleavage of Notch protein releases Notch intracellular domain (NICD) that translocates to the nucleus, acting as part of a transcription complex initiating a cascade that induces genes involved in survival, proliferation and differentiation. One report showed that CNS stem cells use the Notch pathway to delay their onset of differentiation during development. In contrast, analysis of embryonic RPC shows that while early RPC use the Notch pathway for stemness maintenance, in late RPC it is utilised for generation of glia (James J 2004).

The differences in the exploitation of extracellular signalling networks might occur due to changes in three parameters; a) changes in extracellular ligands concentration, b) upregulation and/or downregulation of membrane receptors and c) recruitment of different intracellular cues such as cell cycle proteins. As aforementioned, the microenvironment plays a vital but not absolute part in RPC's behaviour. Genetic expression analysis showed that proliferation and differentiation potential exists due to changes in membrane receptor gene expression like Fibroblast Growth Factor Receptor (FGFR) 1 and Epidermal Growth Factor Receptor (EGFR). Therefore, early RPC respond better to FGF2, an FGFR1 ligand, preferentially generating neurons, while EGF favours late

RPC, an EGFR ligand, biasing them towards glial differentiation (James J 2004). For late stem cell maintenance Insulin-like Growth Factor (IGF) II and Stem Cell Factor (SCF) are recruited for stemness and activation of anti-apoptotic pathways. The above-mentioned temporal regulation by growth factors would lead to the assumption that inhibition of certain signals could influence the bias of RPC to specific cell fates (Davis AA 2000; McAvoy JW 2000); that was not the case due to recent evidence showing that EGF treatment or inhibition of EGFR signalling does not lead RPC to specifically generate neurons or glia (Klassen HJ 2004). That indicates the recruitment of different and/or alternate extracellular molecules leading to differential cascades of i.e. Notch signalling that may explain a “preference” for specific cell types.

Embryonic retinal stem cells/progenitors can be influenced by neonatal retinal cultures and vice-versa (Belliveau MJ 2000). However, the former are only theoretically considered as stem cells since solid evidence is yet to arise supporting their *in vitro* self-renewal capability. In respect to the above observations, RPC might switch through different competence states, during retinogenesis (Marquardt T 2002). This transition may be influenced by the increased expression of EGFR in the early postnatal stages in comparison to late embryonic stages. It can be hypothesised that the leap from embryonic to the postnatal developmental stage, ignites a chain of events to produce altered RPC, possibly further away from the uncommitted stage. In conclusion, postnatal RPC show a better self-renewal potential than embryonic ones, but their differentiation niche is considerably narrower than that of their counterparts (Engelhardt M 2004). To date, the distinction between RPC potential of young and adult mammals has not been appreciated and my hypothesis is if there is a window after gestation in which RPC, if any, found in the retina can be efficiently manipulated or exploited to induce favourable outcomes.



### 1.3.4 Neurosphere-forming assay and retinal progenitors

In this project, the neurosphere forming assay is one of the basic tools used for the characterisation of retinal progenitors. Using a controlled *in vitro* environment it is possible to study the multipotentiality of adult retinal cells from the murine retina. The principle behind this assay is the fact that when neuronal cells are dissociated from a given tissue, in this case the neural retina, and placed in an environment enriched with growth factors, the non-proliferating neurons will perish and undergo apoptosis or necrosis due to lack of energy resources (Reynolds BA 1992). Neuronal cells that acquire or have the ability to re-enter the cell cycle (i.e including de-differentiation events), are the only ones surviving and thus after several rounds of cells division these are the ones that will give rise to neurospheres. This colloquial term encapsulates the three-dimensional spheroid vehicles produced by a mixture of mitotic neural stem and progenitor cells of the CNS *in vitro*. The assay for obtaining retinal neurospheres uses EGF and FGF-2 (Gritti A 1999) plus N2 supplement containing recombinant insulin, human transferrin, sodium selenite, putrescine and progesterone. This supplement has shown to increase the yield of neural stem/progenitor cell proliferation but cannot induce cell proliferation without growth factors.

Following cell proliferation, neurospheres are removed from the growth factor-enriched environment and introduced to high amount of FCS to induce arrest of mitosis and morphological differentiation (Ahmad I 2000; Tropepe V 2000; Yin et al. 2004). After 14-21 days in these conditions retinal progenitors can give rise to retinal neurons and glia as previously reported (Ahmad I 2000; Tropepe V 2000). The combination of different growth factors and conditions in this assay allows a researcher to finely tune the behaviour of these progenitors to obtain a certain partially or fully differentiated population. In this project one of the main focuses, as will be shown in Chapter 5, is to obtain rod photoreceptor precursors

following a recent publication indicating that fully differentiated photoreceptors or RPC are not optimal for retinal transplantation in mammals (MacLaren et al. 2006).

In addition, the neurosphere assay can also be used for characterising an *in vivo* progenitor population. Note that, this characterisation is based on functional criteria of assessing how the progenitor or stem cell populations behave in a certain way upon specific culture conditions, but without reflecting their *in vivo* behaviour. Saying that, it is feasible to quantify some aspects of their proliferation rate, such as neurosphere number and diameter and compare them with a known retinal progenitor population, in this case neurospheres deriving from the adult murine CE (Ahmad I 2000; Tropepe V 2000). It is important to note that until today there has not been an assay distinguishing between retinal stem and progenitor cells *in vitro*. Essentially, the presence of neurospheres indicates the existence of neural progenitors and neurosphere characteristics can be compared between two distinct populations assessing proliferation rates, self-renewal ability and multipotentiality.

An additional advantage of the neurosphere assay is that mitotic cells can be traced back and their developmental origin revealed if a certain gene expressed only in a certain developmental population is probed. In this case, pigmented cells that comprise some retinal neurospheres also act as indicators of the nature of the progenitors either acquiring characteristics *in vitro* or originate from RPE or neural retina.

### **1.3.5 Muller glia as retinal progenitors**

Glial cells are non-neuronal cells that provide support and nutrition in the CNS. They are comprised of microglia and macroglia and have been shown to

possess a number of functions. Microglia have important developmental and phagocytic roles in the brain, while macroglia have structural roles in the CNS architecture. The former derive from haemopoietic precursors while the latter derive from ectodermal tissue. In the brain, the main classes of macroglia are oligodendrocytes and astrocytes. The former transfer neural impulses along their cell body and are myelinated. The latter provide structural and metabolic support to neurons throughout the CNS. In the SVZ and dentate gyrus (DG), it has been demonstrated that cell populations with glial characteristics are latent neural stem/progenitor cells (Lois and Alvarez-Buylla 1993).

In the postnatal chick retina, Müller glia constitute the vast majority of radial glia in the retina spanning the whole length of the retina along with a small population of astrocytes, confined to the nerve fibre layer (NFL). Upon injury, Müller glia re-enter the cell cycle, and co-express retinal progenitor proteins such as Chx10, Pax6 and CASH-1 (Fischer and Reh 2001), a characteristic of retinal progenitors. In addition they show signs of differentiation into new neurons and glia. This induced neurogenesis in the vertebrate retina is also a natural property of adult fish that re-activate an endogenous retinal population that gives rise to new differentiated cells, upon injury (Bernardos, Barthel et al. 2007). Interestingly, these studies have pointed to a glial cell population being activated upon injury possessing Müller glia characteristics. Importantly, it was recently reported that the adult mammalian retina re-activates its Müller glia population upon neurotoxic injury (Dyer MA 2000, Ooto S, 2004 #85). According to these studies, a retinal progenitor population residing either in the CNR or in the CE of adult mammals should express neural stem and Müller glia markers, upon activation. In this Thesis, this Müller/progenitor population is going to be examined in a mouse model of a small eye phenotype.

## **1. 4 Eye disease**

Congenital ocular defects, which are present at birth, occur relatively frequently in humans and can often cause blindness due to abnormal eye development in terms of structural or functional defects. Causality can be either genetic or environmental. Common congenital defects include the small eye phenotype called microphthalmia, the lack of the eye named anophthalmia and coloboma, characterised by gaps in several regions of the eye such as the iris or the eyelid. Eye defects also have environmental causes, such as viral infections that could cause cataracts.

Twenty million children under the age of 16 suffer from cataracts, while 1.4 million are blind (Johnson GJ 2003). Half of those cases have an underlying genetic cause which is a significant cause of childhood blindness according to the World Health Organisation ([www.who.int/blindness/causes](http://www.who.int/blindness/causes)). Specifically, when genes encoding transcription factors (TFs) and signalling molecules are affected by mutations during early eye development can cause structural congenital eye defects (see Table 1.1). However, the underlying aetiology of these genetic defects is largely unknown. The realisation of the effect of visual impairment, especially on children's well-being and social development (Rahi et al. 1999) has initiated a global World Health Organisation partnership scheme called VISION 2020 (<http://www.who.int/blindness/partnerships/vision2020/en/>) involving more than 20 private organisations comprising the International Agency for the Prevention of Blindness (IAPB) with an ultimate goal of preventing avoidable blindness by the year 2020.

By elucidating the genetic basis of eye development it would be possible to acquire knowledge for the mechanisms involved in the development of congenital eye defects. Although today the gap between the successful identification of genes and mechanisms that cause abnormal eye development

and the treatment or prevention of congenital eye defects is large, better understanding of eye development is essential, and in combination with the robust technological advancements will be the only combination for treating such defects in the future.

Several mutations have been identified in genes affecting correct eye development. Here, I will focus on genetic cues known to affect retinal development. Of course, in such a complicated system as the eye, the function of multiple factors overlaps and contributes to the normal eye development.

### **1.4.1 Retinal disease**

The eye is a very complex structure originating from the surface ectoderm, the mesenchyme, the wall of the diencephalon and migrating neural crest cells. Using several mouse mutants as indicators, it is clear that an extremely complicated system of genes, particularly those encoding TFs are important for appropriate eye development. The retina has been branded as an approachable part of the brain because of its accessibility in both embryonic and adult tissues. It is one of the most highly conserved systems of the CNS, conveying similar cell types across species from *Drosophila* to humans. A large number of human and mouse eye mutations have been observed and since the eye is a non-essential organ, it can be readily studied *in vivo*. Biochemical and genetic analysis are available while mutations affecting the eye are readily recognised and thus any abnormality is reported. In a survey conducted in 1985 (Costa T 1985), it was shown that the eye is affected in approx. 1 in 4 genetic diseases in humans (2811 phenotypes recorded, 27% of them scored for vision abnormalities). The rest of the CNS and muscle tissue is considered the fourth most common organ being affected by genetic abnormalities in humans. Ocular diseases in *Homo sapiens* have been mapped in over 50 loci by 1994 (Rosenfeld PJ 1994) and in

*Mus musculus*, more than 110 eye mutations have been identified by 1996 (The Jackson Laboratory 1996). Many more have been also identified in the last decade.

### 1.4.2 Master genes

Over the last decade several genes sitting on the top of the hierarchy of eye development have been identified. These genes are called master genes and are primarily expressed during early embryogenesis leading to ocular cell-lineage commitment. Paired box 6 (Pax6), a homeodomain TF, is the master control gene in eye development. Loss of function causes severe ocular defects in fruit flies and mammals (Gehring WJ 2002; Niimi T 2002). Ectopic expression of the murine Pax6 induces eye formation (ommatidal eye) in *Drosophila* (Halder G 1995; Halder G 1995). Heterozygous mutants have a small eye phenotype (Hill RE 1991) while homozygous mutants have no eyes and die shortly after birth due to nasal dysfunctions (Grindley JC 1995). Similarly, sine oculis homeobox homolog 3 (Six3), another TF, mRNA injections into embryos, results in lens formation in medaka fish (Oliver G 1996) as well as ectopic Pax6 expression (Loosli F 1999). Human mutations cause holoprosencephaly, a condition where the embryo's forebrain fails to fuse. Less severe phenotypes include microphthalmia and iris coloboma (Wallis DE 1999).

Retina and anterior neural fold homeobox gene RX, which is again a homeobox TF is expressed very early in eye development (E9). Mouse embryos lacking Rx do not have eyes (anlagen) and Pax6 fails to be expressed where appropriate in the eye primordium (Mathers PH 1997). Sex-determining region Y (SRY) of the SRY-related high mobility group (HMG) related box-gene 2 or successfully abbreviated as SOX2 is an important TF, with SOX2 heterozygous mutants lacking eyes (Fantes J 2003).

One of the first congenital disorders of the retina discovered was that of the microphthalmic (mi) mouse some 50 years ago. Since then, microphthalmia-associated transcription factor (MITF) has been characterised as a protein which, when its gene is affected by one of at least six mutations identified, will lead to a microphthalmic phenotype in humans with retinal pigment abnormalities (Hemesath et al. 1994; Steingrimsson et al. 1994; Hallsson et al. 2000). Another mutation is that of the recessive ocular retardation (orJ) mouse model, characterised by microphthalmia and blindness. Table 1.2 below indicates genes that have been identified to cause microphthalmia in mice and humans.

*Table 1.2 List of genes known to cause microphthalmia in the mouse or in humans (capitalised italics). Table includes syndromic and non-syndromic conditions.*

<b>Gene</b>	<b>Phenotype</b>	<b>Reference(s)</b>
Chx10, <i>CHX10</i>	RPC proliferation defect, microphthalmia	(Burmeister M 1996)
Pax6	Anophthalmia in homozygotes, microphthalmia in heterozygotes	(Hill RE 1991)
Rx, <i>RX</i>	No eye development	(Furukawa et al. 1997; Mathers PH 1997)
Pitx3	Microphthalmia in both eyes, aphakia	(Rieger et al. 2001)
Mitf	Microphthalmia. <i>Waardenburg syndrome in humans</i>	(Tachibana et al. 1994)
Cyclin D1	Impaired development of all layers of the retina	(Fantl V 1995)

Hes1	Premature and accelerated differentiation, resulting in microphthalmia with lens and corneal abnormalities in mice. No human mutations identified to date	(Tomita et al. 1996; Ohtsuka et al. 1999)
Lhx2	Failure of optic cup and lens placode formation leading to anophthalmia. No mutations identified in humans	(Porter et al. 1997)
Gas1	Ocular remnants ingress from the surface with hypocellular RPE, lens, cornea and NR and disorganised eyelid	(Lee et al. 1997)
Xt, XT	Variable ocular phenotypes	(Hui and Joyner 1993; Franz 1994)
Otx2, OTX2	Absence of forebrain and hindbrain in mice, microphthalmia and anophthalmia in humans	(Matsuo et al. 1995)
Pax2, PAX2	Defects in optic nerve development, renal-coloboma syndrome, associated with microphthalmia in some cases in heterozygotic humans. In homozygotic mutations coloboma, abnormal optic nerve development and lack of contralateral optic nerve fibre projections is observed in mice.	(Favor et al. 1996; Eccles and Schimmenti 1999)
Foxg1	Ventrally rotated ellipsoid eyes defective nasal retina	(Hatini et al. 1994)
Hesx1	Anophthalmia	(Dattani et al. 1998)
Msx1,2	Absence and over-expression of lead to microphthalmia	(Wu et al. 2003)
Bmp7	Anophthalmia	(Wawersik et al.



		1999)
SOX2	Anophthalmia	(Fantes J 2003)
SIX6 (SIX9)	Anophthalmia in both eyes	(Gallardo et al. 1999; Lopez-Rios et al. 1999)
SIX3	Microphthalmia in heterozygotes, and iris coloboma in humans. Does not affect mice. Forebrain including eyes is absent in homozygote mice.	(Oliver et al. 1995)
SHH	In humans, heterozygotic genotype causes holoprosencephaly which can involve anophthalmia, microphthalmia and coloboma, and colobomatous microphthalmia. No phenotype in mice, while homozygous mutation causes cyclopia in mice.	(Roessler et al. 1996)
BMP4	Microphthalmia, optic nerve hypoplasia in heterozygotic mice. Anophthalmia identified in humans. Embryonic lethal and failure of lens induction in homozygotic mice.	(Furuta and Hogan 1998) (Bakrania et al. 2008)
Cx43, CX43	Oculo-dento-digital syndrome in Heterozygotes, which is associated with microphthalmia, cataracts, and iris abnormalities in humans. One 3 bp homozygous duplication reported in an oculo-dento-digital syndrome patient. No eye phenotype in mice.	(White et al. 2001)

### 1.4.3 Chx10 transcription factor

Several homeodomain-containing TFs have been shown to play important roles in the specification and development of the mammalian retina. Homeodomain proteins contain a binding domain encoded by a 180 base pairs (bp) long homeobox sequence (Kissinger CR 1990). The structure consists of a 60 amino acid triple  $\alpha$  helix motif used for binding to deoxyribonucleic acid (DNA) target sequence. Mutations in some homeobox genes lead to anophthalmia or microphthalmia both in mice and humans (Percin et al. 2000).

Studies have shown that mutations in the *C. elegans* *ceh-10* homeo domain-containing homolog, *Chx10* gene (or its new name, *Vsx2*), encoding for a TF, is the cause of a microphthalmic phenotype (Burmeister M 1996). Mice lacking a functional *Chx10* protein express a phenotype of microphthalmic eyes characterised by diminutive retina, abnormal photoreceptor outer segments, iris abnormalities and truncated or absent optic nerve (Burmeister M 1996). In humans, two families with recessive mutations affecting the arginine residue at position 200, leading to a *Chx10* protein with diminished activity that degrades rapidly, showed a microphthalmic phenotype (Percin, Ploder et al. 2000).

*Chx10* is expressed in the presumptive neural retina of the invaginating optic vesicle, possibly due to signals deriving from the prospective lens ectoderm (Liu IS 1994). This homeobox gene is expressed in the proliferating RPC population throughout retinal development, hence is the earliest characterised specific marker for RPC. Its expression in the eye is observed at E9.5 when the surface ectoderm is still in contact with the optic vesicle (Burmeister M 1996; Chow RL 2001) and is restricted to the anterior region of the presumptive optic vesicle that will give rise to the neural retina (Liu IS 1994). After the last retinal mitotic event, its expression is narrowed to bipolar and a subpopulation of Müller cells in the INL (Burmeister M 1996; Rowan S 2004). *Chx10* is the earliest known marker

expressed in bipolar cells with a peak during bipolar cell differentiation and its expression persists during adult life. In humans the *CHX10* gene is located on chromosome 14q24.3 (Percin, Ploder et al. 2000). *Chx10* is essential for normal eye development and its absence causes abnormalities such as defective iris development, cataracts and microphthalmia (Burmeister M 1996; Ferda Percin et al. 2000).

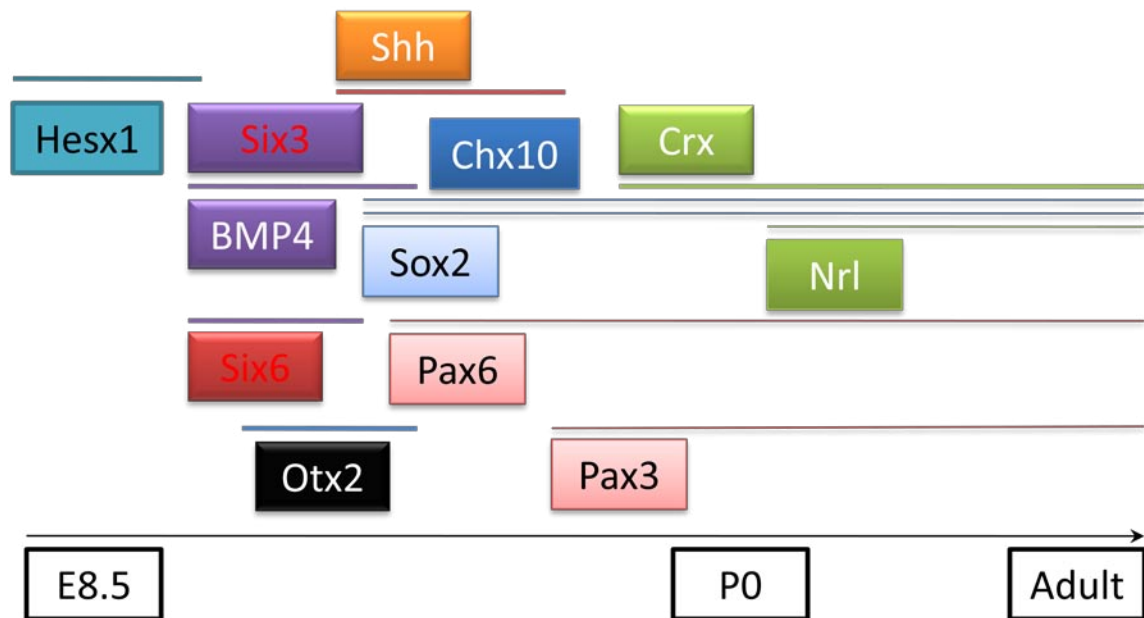


Figure 1.8 Transcription factors and retinogenesis in mice. Figure showing some transcription factors expressed in the eye field just before retinogenesis (E8.5) and after terminal mitosis and during adulthood. *Chx10* is expressed very early in retinogenesis (E9.5) and onwards.

#### 1.4.4 The *Chx10<sup>orJ/orJ</sup>* mouse model

The correlation between microphthalmia and *Chx10* was shown from a naturally occurring *Chx10* murine mutant, named *Chx10<sup>orJ/orJ</sup>* [orJ for ocular retardation,

(Truslove GM 1962)] deriving from a spontaneous null mutation leading to a premature stop codon and failure to produce Chx10 protein (Burmeister M 1996). This recessive mutation leads to a blind phenotype with five distinctive characteristics with the most obvious being their diminutive but laminated adult retinas (Theiler K 1976; Robb RM 1978; Burmeister M 1996; Rutherford AD 2004). CNR appears to undergo differentiation but a poorly laminated structure is created. Examination of the periphery revealed the progressive expansion of pigmented cells towards the neural retinal region at the expense of RPC (Rowan S 2004); new evidence suggested that the neural retina (NR) region transdifferentiates into RPE in *Chx10<sup>orJ/orJ</sup>* mice (Horsford DJ 2004). Additionally, there is a failure of retinal ganglion cell axons exiting the eye in the ventral fissure area and a total absence of bipolar cells (Burmeister M 1996).

I will attempt to explain the function of Chx10 protein in the RPC behavioural context, bipolar cell formation and transdifferentiation events between RPE and retinal cells. Chx10 TF function is based in a medley of events leading to normal RPE and NR differentiation. In addition, Chx10 is involved, as its knock-out model indicates, in RPC normal developmental patterns (Hatakeyama J 2004). Its abolition leads to failure of RPC proliferation and delayed differentiation with the consequence of an expanded pigmented epithelium in mice and with the absence of most of the NR in adult rodents (Dyer 2003; Rutherford AD 2004). Assumptions point towards an inverse condition of transdifferentiation of retinal cells to RPE (Rowan S 2004). To date, there has not been an exact establishment of where Chx10 TF stands in the signalling pathway, as its misexpression is not sufficient to direct rod bipolar cell fate on its own, or generate RPC while it seems to function as a repressor protein for other retinal cells types in development i.e. photoreceptors (Hatakeyama J 2001; Hatakeyama J 2004; Dorval KM 2006; Livne-Bar I 2006).

In Chx10<sup>orJ/orJ</sup> mutants, important cell cycle enhancers are downregulated, while MITF and other RPE markers are abnormally upregulated (Rowan S 2004). p27<sup>Kip1</sup>, a cell cycle enhancer is indirectly halted due to Chx10 absence, while *Mitf* gene expression is upregulated, due to direct inaction of Chx10 TF (Rutherford AD 2004). This possibly leads to an abnormal RPC proliferation. In addition, the absence of inhibitory signals towards RPE differentiation might lead to a default pathway of dedifferentiation of retinal cells to RPE, but again this needs more analysis. Recent data indicate that Math3 and Mash1 genes are responsible for bipolar cell fate specification while Chx10 might regulate layer specificity but cannot determine bipolar cell fate (Hatakeyama J 2001). Nonetheless, the saga of RPC proliferation and retinogenesis lies in a 4-dimensional complex that includes the number of intrinsic and extrinsic cues involved, their concentration, and the developmental window in which these cues are expressed and needed.

#### **1.4.5 Chx10 as a cell cycle regulator**

It is feasible to speculate that Chx10 regulates retinal cell proliferation, via the cell cycle. De-phosphorylation of negative cell cycle regulators such as retinoblastoma (Rb), p107 and p130 induces a halt in cell proliferation while loss of phosphorylated Rb (pRb) induces abnormal cell division status in humans and mice (Brinkert et al. 1998; Kastner et al. 1998).

The factors associating with retinoblastoma and pRb levels determine cell fate. Homeodomain factors such as Pax3 and Chx10 are involved in the interaction of pRb with the E2F-1 machinery. In that way, if Chx10 binds with the unphosphorylated form of Rb will act as cell cycle repressor, but if the phosphorylated form of Rb is bound then its role is switched to cell cycle enhancer (Dyer 2003).

Additionally, a study in the knockout mouse for *Cyclin D1*, a cell cycle component necessary for pRB phosphorylation by cyclin dependent kinase, which is expressed ubiquitously during embryogenesis, reveals that only a few tissues, including the retina, are sensitive to its absence. Nullizygous mice revealed a striking reduction in thickness and organisation of the retinal layers (Fantl et al., 1995). Comparison of cell numbers in the two nuclear layers confirmed that a reduction in thickness was most marked for the outer layer and accompanied by disorganisation of nuclear polarity. A reduction in thickness and organisation of the surface GCL was also observed (Fantl et al., 1995). A lack of Cyclin D1 seems to affect only proliferation, and it would therefore be interesting to see if cyclin D1 has any relationship with Chx10. Figure 1.8, indicates where the aforementioned proteins act on the cell cycle.

Image removed for copyright reasons

*Figure 1.8 the mammalian cell cycle. The point of action of proteins discussed are shown on graph (modified from [biomedcentral.com](http://biomedcentral.com))*

Another interesting cell cycle component, which might have some relationship with Chx10, is the cyclin-dependent kinase inhibitor, p27<sup>Kip1</sup>. *In vitro* analyses show that p27<sup>Kip1</sup> accumulation in retinal cells correlates with cell cycle withdrawal and differentiation, and when over-expressed, there is reduced RPC proliferation (Reh TA 1998). Results from another recent publication suggest

that p27<sup>Kip1</sup> is abnormally present in progenitors of the *Chx10<sup>orJ/orJ</sup>* mutant mouse, and that its deletion rescues the mutant phenotype (Green ES 2003). It was suggested that Chx10 influences p27<sup>Kip1</sup> at a post-transcriptional level, through a mechanism that is largely dependent on cyclin D1. Further, deletion of the *p27<sup>Kip1</sup>* gene restores normal development of cyclin D1 deficient mice (Geng Y 2001). Thus, these cell cycle regulators may play an important role in Chx10 regulation of neural retinal progenitor proliferation. Table **1.3** summarises how Chx10 is thought to interact with various cell cycle proteins.



*Table 1.3: Cell cycle components potentially affected by or under control of Chx10 in RPC populations*

Cell Cycle Component	Role in Cell Cycle	Phenotype deletion	Relationship with Chx10 and PRC?
pRb	Negative cell cycle regulator, binds E2F to cause exit of cell cycle	Retinoblastoma tumours uncontrolled proliferation	(Wiggan 1998) O
Cyclin D1	Activates cyclin dependent kinases cdk4 and cdk6, drives cell cycle progression	Severe retinopathy impaired development of all retinal layers	(Green 2003) ES
Cyclin D3	Interacts with cyclin dependent kinases cdk4 and cdk6 driving cell cycle progression	shortening of G <sub>1</sub> with no apparent effect, expressed postnatally	(Dyer and Cepko 2001)
P27 <sup>Kip1</sup>	Inhibits cyclin dependent kinases, required for timing of cell cycle withdrawal	Normal period of histogenesis of photoreceptors and Müller glia is extended	(Green 2003) ES

### **1.5 Cell transplantation for retinal disease**

Stem cell transplantation has been nominated as a potential therapeutic tool for repair of neuronal damage including retinal damage. The two cardinal properties making a stem cell the ideal cell type for this purpose is its ability to self-renew and to give rise to more than one different cell types. Bone marrow stem cells

have been studied more vigorously due to their easy accessibility and vast numbers as well as maintenance in *in vitro* culture (Yeager et al. 1986; Reiffers 1990). In addition to haematopoietic stem cells it was believed that only the epidermis of the skin, the digestive tract and muscles possess stem cells for tissue regeneration. However, recent research has identified stem-like cell populations in mammalian organs that lack regenerative capability such as the heart and CNS. With regards to the eye, the most exposed part of the CNS, several stem-like cells have been identified able to give rise to different ocular tissues such as in the near-corneal limbal region, the pars plicata and pars plana of the ciliary margin as well as the NR (Cotsarelis et al. 1989; Lindberg et al. 1993; Yang P 2002; Coles BL 2004; Mayer EJ 2005).

It has been widely known that fish and amphibians possess the ability to regenerate neural retina throughout life, a process that adult mammals lack (Meyer 1978; Raymond 1991). In recent years several studies have shown that transplantation of progenitors may hold the key for restoring neuronal function in the brain (Gage et al. 1995; Brustle et al. 1998; Zupanc et al. 2005) and retina (Young et al. 2000; Aramant and Seiler 2002). Here, I discuss on-going research for identifying the appropriate cell type for retinal transplantation as well as the criteria and conditions needed for *ex vivo* manipulation techniques.

### **1.5.1 The concept of retinal transplantation**

The mature retina is comprised of a photoreceptor, an interneuron and a ganglion cell layer. Degeneration of the photoreceptors (both rods and cones) causes a retinal disease known as *retinitis pigmentosa* (Milam and Jacobson 1990), while retinal ganglion cell degeneration gives rise to a disease named glaucoma (Takatsuji et al. 1988). These common retinal conditions affect those layers only and can lead to severe visual loss and eventually blindness. The two

important advantages for cell transplantation to the retina is the accessibility of the ocular structure followed by recent advancements in ophthalmologic surgical procedures (Humayun et al. 2000). Secondly, the eye is an immunologically privileged site to some extent since cells do not express Major Histocompatibility Complex (MHC) I peptides, essential for CD8<sup>+</sup> T-cell-mediated immune activation (Kaplan and Stevens 1975; Vessella et al. 1978). The hurdles of cell transplantation have been many. Although, the stem cell literature has been vast, delivering these cells to the tissue of interest has been a challenge. Even though some success has been achieved by transplanting RPE sheets in dystrophic rats, which showed increased photoreceptor survival (Binder et al. 2002; Arai et al. 2004), the major issues of survival, maturation and integration of grafted cells has not been solved with great success. Since only a very low percentage (1%) of grafted cells was able to survive in the mature mammalian retina (Sakaguchi et al. 2003) and in humans, direct photoreceptor transplantation did not enhance light perception (Kaplan et al. 1997), but in some cases grafted cells were able to mature and integrate (Mizumoto et al. 2001). Recent work showed that it is essential to improve *in vitro* conditions for progenitor/stem cell survival, expansion and differentiation in order to achieve visual enhancement in mammals (MacLaren, Pearson et al. 2006). This study showed that for effective rod integration into the P1 and adult retina donor cells need to be in a specific ontogenetic state, being post-mitotic but not fully differentiated. The importance of this finding is that the *in vitro* conditions need to be finely tuned in order to maintain the donor cell population in a retinal post-mitotic phase without pushing towards a fully differentiated state.

### **1.5.2 Stem cell sources for retinal transplantation**

Choosing the appropriate cell for retinal transplantation has been the focus of research for several years now. The two main areas of research are an

optimised *in vitro* culture system and post-transplantation performance. Initially, the appropriate candidate for effective retinal transplantation should be a cell able to undergo multiple cell divisions in culture in order to give rise to a large source of cells. It also needs to be plastic producing the appropriate cell type upon *in vitro* manipulation. Following transplantation into the appropriate retinal region, grafted cells should be able to survive, reach their final maturation stage and successfully integrate into the damaged neuronal network alleviating visual abnormalities. For those general properties, on-going research has been conducted on embryonic stem cells, adult non-neural as well as adult neural stem/progenitors.

### 1.5.2.1 Embryonic stem cells as transplants

Mammalian ES cells are multipotent with the ability to differentiate into CNS cells. Their high plasticity makes these cells a very attractive candidate for retinal transplantation, but ethical issues concerning their acquisition, has hindered efforts for retinal transplantation. In addition, ES cells can only be used in allogeneic transplantation which might trigger an immune response even in the immuno-privileged ocular region due to nonhuman sialic acid Neu5Gc (Martin et al. 2005; Cabrera et al. 2006). Human ES cells undergo cell division every 1-2 days *in vitro* and are highly active on both DNA and protein synthesis during cell division. Their antigen expression profile, when in an undifferentiated state, is characterised by SSEA3<sup>high</sup>SSEA4<sup>high</sup>Oct4<sup>pos</sup>Nanog<sup>pos</sup>Sox2<sup>pos</sup>TERT1<sup>pos</sup>DNMT3<sup>pos</sup>AP<sup>pos</sup>SSEA-1<sup>neg</sup>. Apparently, the ES cell field is not as advanced as the haematopoietic SC field and there are several reports highlighting differences as well as novel surface antigens for distinguishing undifferentiated ES cell populations (Sperger et al. 2003; Abeyta et al. 2004; Bhattacharya et al. 2004; Richards et al. 2004).

Embryonic stem cells derive from the inner cell mass of embryos (blastocyst stage). Several lines of work have showed mixed success for using ES cells in retinal transplantation (Hirano et al. 2003; Meyer JS 2004). ES cell lines are usually cultured in RA and/or FGF-2 inducing a neural cell fate (Zhao et al. 2002; Aoki H 2006; Zhao et al. 2006). Also, ES cells, in the absence of serum and the presence of LIF, can form primitive Nestin<sup>pos</sup>Sox1<sup>pos</sup>β-III-tubulin<sup>pos</sup> sphere structures, a characteristic of neural stem cells (Reynolds BA 1992; Reynolds BA 1992; Smukler SR 2006). Thus, it seems feasible to induce highly plastic ES to commit to the neural cell fate but with several limitations such as that only a fraction of ES cells acquire a neural cell fate (~0.2%) due to cell death and cross-contamination of non-neural precursors (Smukler SR 2006).

Following neural cell fate commitment, ES-derived neural precursors need to further acquire retinal stem-like cell characteristics. Researchers have attempted to obtain the later by culturing in substrates such as PLL and Laminin, both shown to promote retinal fate of neural precursors (Zhao, Liu et al. 2002; Zhao, Liu et al. 2006), while others have observed spontaneous differentiation to RPE cells in a high glucose-medium supplemented with LIF and bFGF (Lund et al. 2006; Aoki et al. 2007). Finally, EBs have also been recruited as a possible neural progenitor cell source ready for retinal transplantation (Mayer EJ 2005), with significant success using human, chick and mouse ES-derived cells (Zhao, Liu et al. 2002; Hara et al. 2004; Lund, Wang et al. 2006; Meyer et al. 2006; Aoki, Hara et al. 2007). It is clear that the proper epigenetic factors need to be “switched on” for obtaining the correct candidate cell for efficient retinal transplantation. The current *in vitro* culturing systems for ES cells are still in their infancy, with many questions and obstacles on how to obtain a homogeneous cell population and to control spontaneous cell differentiation and proliferation causing carcinoteratomas (Hardy et al. 1990; Arnhold et al. 2004).

### 1.5.2.2 Adult stem cells as transplants

An alternative source that has been revisited in the recent years is that of adult stem cells, since there are several ethical considerations of using human ES cells for treating retinal disease. One prominent source would be that of induced pluripotent stem cells (iPSC) that could be obtained from skin fibroblasts which would then be reprogrammed back to the embryonic stem cell stage by expression of Oct3/4, Sox2, c-Myc and Klf4 (Lowry et al. 2008). The advantage of using adult stem cells are numerous; first, they can be obtained from the patient, cultured *in vitro*, and then re-implanted where appropriate performing autologous transplantation with minimal risk of graft rejection. Second, ethical issues concerning the use of adult stem cells are not in place and unlikely to be raised in the future.

### 1.5.2.3 Adult non-neural stem cells as transplants

The stem cells of the bone-marrow are the best-studied stem cells to date, with a tremendous amount of literature. The two distinct populations residing at the bone marrow are the haematopoietic and mesenchymal stem cells. The former can give rise to any blood-cell type, while recent studies have shown that the latter, under certain conditions, can differentiate into cell types other than blood such as digestive tract-, cardiac- and skeletal muscle-specific cells as well as neurons and glia (Ferrari et al. 1998; Sanchez-Ramos et al. 2000; Woodbury et al. 2000; Krause et al. 2001; Orlic et al. 2001). Both of these cell populations are readily formed in the bone marrow throughout life. Mesenchymal stem cells can be obtained from the femurs and tibias in large quantities and their phenotype is characterised by CD34<sup>neg</sup>CD44<sup>neg</sup>CD45<sup>neg</sup>c-Kit<sup>neg</sup>MHC-class-I<sup>neg</sup>II<sup>neg</sup>Flk-1<sup>low</sup>Sca-1<sup>low</sup>Thy-1<sup>low</sup>CD13<sup>high</sup>SSEA-I<sup>high</sup> surface antigen expression as well as rapid proliferation (Jiang et al. 2002). These cells have been cultured in standard

medium conditions with or without LIF and then either directly used for retinal transplantation or modified in culture by chemical inducers such as  $\beta$ -mercaptoethanol or dimethylsulfoxide and butylated hydroxyanisole (BHA) (Woodbury, Schwarz et al. 2000), by supplementing with EGF and FGF-2 (Hermann et al. 2004), co-culturing with neuronal cells such as Nestin<sup>pos</sup> cerebellar granule neurons (Wislet-Gendebien et al. 2005) for inducing a neural stem-like cell fate. Mesenchymal stem cells isolated from these regions showed effective migration to the injured retina, with signs of proliferation and expression of several photoreceptor and glial markers (Tomita et al. 2002; Minamino et al. 2005). In addition, when these cells were injected into the sub-retinal space in the eyes of dystrophic animals there was a significant increase in photoreceptor survival (Kicic et al. 2003; Arnhold et al. 2007). Certainly, these studies have shown the potential benefit for the use of adult mesenchymal stem cells in retinal transplantation such as integration and differentiation, a theoretically infinite cell source and lower risk for neoplasias. On the other side of the spectrum, there are still considerations involving tumour formation and certainly cross-contamination by non-neural cells since marrow-derived stem cells do not show homogeneity in terms of becoming neural stem-like cells, under the current culture conditions. Clearly, there is a need for identifying the appropriate conditions *in vitro*, for obtaining a homogeneous neural stem-like cell population from marrow-derived stem cells.

#### **1.5.2.4 Adult neural stem cells as transplants**

Neural gliogenesis in the adult mammalian nervous system was first observed with the classic experiments from Joseph Altman in 1966, where he injected a DNA incorporation precursor, [ $H$ ]<sup>3</sup> Thymidine, into adult rats (Altman 1966). His experiments showed the existence of proliferating cells in the lateral ventricle with the ability to migrate to other parts of the brain. The possibility of identifying

NSC *in vitro* was demonstrated around three decades later by Brent Reynolds by culturing E14-derived striata in medium supplemented with EGF (Reynolds BA 1992). That gave rise to floating cell aggregates termed neurospheres with the ability to proliferate. In the following years, this “neurosphere” assay was used as a tool for discovering NSC from the adult brain that were able to self-renew and give rise to cells with neuronal, astrocytic and oligodendrocytic characteristics (Cameron et al. 1998; Gage FH 2000).

Adult NSC can be obtained and cultured by several CNS regions, but the most prominent source derives from the niches in the SVZ of the lateral ventricles as well as the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Gage FH 2000). These germinal centres have the appropriate microenvironment for stem cell maintenance (Doetsch 2003). Also, different brain regions give rise to different stem-like cells with different characteristics but high plasticity and adaptation when co-cultured with other parts of the brain or CNS, indicating that neurosphere-forming stem-like cells might be a mixture of true stem cells, progenitors and differentiated cells (A, B and C cells) (Hitoshi et al. 2002).

In the last decade, a considerable effort has been made to use mammalian CNS precursors in transplantation to damaged areas of the CNS. One of the areas that has drawn considerable attention is the developed retina. The obvious advantage for these cells was their designated neural cell fate which would ideally allow them to integrate to the retinal layers and differentiate into retinal neurons more effectively than embryonic or non-neural stem cells. Adult CNS precursors isolated from the mammalian SVZ and SGZ can be readily expanded in culture either as monolayers or sphere aggregates (Reynolds BA 1992; Svendsen CN et al. 1998). Protocols for obtaining CNS-stem cells *in vitro* have been established by mainly culturing cells in EGF- and FGF-2- rich medium (Ming and Song 2005; Pluchino et al. 2005). When in growth factor-rich culture



these cells readily express the proliferation marker Ki-67, the stem cell marker nestin, GFAP, microtubule-associated protein-2 (MAP-2) and radial glial marker RC2. These sphere aggregates can be induced to give rise to cells with neuronal characteristics ( $\beta$ -III<sup>POS</sup>) by culturing them in growth factor-free medium and in the presence of B27 supplement and low amounts of Fetal Calf Serum (FCS) in Poly\_L-Lysine (PLL) and Laminin.

The first attempts of transplanting adult CNS precursors to the retina came from cells obtained from the SGZ of adult transgene rats expressing Green Fluorescence Protein (GFP). Injection of neural precursors into the vitreous of dystrophic or ischemic and normal rat eyes showed that these cells were able to differentiate and integrate into the inner, outer and ganglion cell layers of the dystrophic or ischemic rats, enhancing host photoreceptor survival, but not into the normal retina (Nishida et al. 2000; Young, Ray et al. 2000; Kurimoto et al. 2001; Mizumoto et al. 2003; Grozdanic et al. 2006). This implied that normal adult retina is not permissive to new cell differentiation and integration probably due to the presence of inhibitory or absence of positive signals (Kurimoto, Shibuki et al. 2001). Also, it is possible that an intact developed retina possess physical barriers impermeable to cell migration, something that embryonic retina seems to lack, being more accessible to CNS-derived precursors upon treatment with RA (Akita et al. 2002). The use of neural stem cell lines both from mouse and human shed light into the problem of integration by showing that sub-retinal cell injections of CNS precursors could differentiate and integrate into non-traumatic retinas. In addition, integrated cells expressed both glial and neuronal cell markers (Nishida, Takahashi et al. 2000; Young, Ray et al. 2000; Kurimoto, Shibuki et al. 2001; Mizumoto, Mizumoto et al. 2003; Grozdanic, Ast et al. 2006). In essence, murine brain progenitors, when transplanted into eyes of Brazilian opossum (*Monodelphis domestica*) newborns showed increased morphological integration in comparison to adult hosts (Sakaguchi, Van Hoffelen et al. 2003; Sakaguchi et al. 2004).

These studies show that CNS precursors from the adult brain have the ability to differentiate and integrate to the damaged retina and RPE, expressing various retinal cell markers. Effectively, it is important to address the specification of these cells in culture, since it is important to find conditions that promote specific retinal cell types (photoreceptors, ganglion cells etc.). Their inherent progenitor/glial nature might cause a limitation factor in obtaining large numbers of retinal neurons needed for optimal retinal disease. One solution would be to co-culture adult CNS-precursors with medium derived from cultured RPC or growth factors such as TGF- $\beta$ 3 that promote a specific retinal fate (Dong et al. 2003). In that way it would be possible to dictate CNS progenitors to follow the retinal progenitor cell fate.

To date, transplant success of retinal stem cells has been limited. When RPC isolated from E17 rats were expanded *in vitro*, and injected in the sub-retinal space of P17 rats, they survived but showed a poor differentiation and integration outcome (Qiu et al. 2005). Upon isolation of human RPC from adult cadavers and culturing in neurosphere-formation conditions, and injection in the vitreous of an embryonic chick eye, indicated that these cells were able to survive for long periods but did not migrate and integrate successfully (Coles BL 2004).

## **1.6 Aim of the Thesis**

The aim of this thesis was to investigate the potential of the adult Chx10-deficient central neural retina of possessing neural progenitors, a phenomenon not apparent in the normal adult central neural retina.

Chx10 is a TF expressed in all retinal progenitors in the presumptive neural retina of the invaginating optic vesicle. After completion of retinogenesis, its expression is restricted to bipolar cells and a subpopulation of Müller glia. The orJ mouse model is a microphthalmic model which has a phenotype caused by point mutation in the *Chx10* gene creating a premature stop codon, leading to the translation of a non-functional Chx10 protein. The major phenotypic characteristic of the model is a diminutive retina and an expanded ciliary body, in an overall small eye phenotype. Our lab has previously reported a persistent dividing population residing in the CNR of the mature *Chx10<sup>orJ/orJ</sup>* eye (Dhomen NS 2006). In addition, it was reported recently that the CB is where a retinal stem cell niche exists in adult mammals, including humans (Ahmad I 2000; Tropepe V 2000; Coles BL 2004). Based on these, the following hypotheses were tested:

### Chapter 3

1. Based on the recently identified population, the population of dividing cells isolated from the adult *Chx10<sup>orJ/orJ</sup>* CNR is a retinal stem-like population.
2. The adult CNR *Chx10<sup>orJ/orJ</sup>* retinal stem-like cell population is distinct from the RSC identified in the CB with an altered proliferation capability and pigmentation profile *in vitro*.

### Chapter 4

1. The neurosphere-forming population in the adult *Chx10<sup>orJ/orJ</sup>* CNR possess Müller glia characteristics.
2. The dividing stem-like population found in the adult *Chx10<sup>orJ/orJ</sup>* CNR is a Müller/progenitor population.

3. The absence of a functional Chx10 protein leads to Müller glia to appear earlier in retinogenesis.

## Chapter 5

1. The differentiation potential of neurosphere-forming population from the adult  $Chx10^{orJ/orJ}$  is similar to wildtype CB one.

2. Neurosphere-forming cells from the adult  $Chx10^{orJ/orJ}$  show a higher differentiation potential for rod photoreceptor production in comparison to wildtype, *in vitro*.

3. The effect of different growth and differentiation factors alters the potential of wildtype and Chx10-deficient RPC *in vitro*.

4. Cells deriving from the  $Chx10^{orJ/orJ}$  are suitable for rod photoreceptor precursor retinal transplantation.

## Chapter 6

This chapter focused on the developmental origin of CE-derived neurosphere-forming retinal stem cells. Since the CE is part of the ciliary body in mammals, which includes cells of mesodermal, neural crest and neural ectodermal origin, I examined whether retinal neurospheres that are observed *in vitro*, originate from the embryonic anterior forebrain. *Hesx1*, a homeobox gene, is expressed at the forebrain region, including the presumptive optic vesicle until embryonic day 9.5, just before retinogenesis. For that reason a  $Hesx1^{Cre/+}Rosa26^{LacZ/+}$  knock-in mouse model was used to genetically label descendants of forebrain  $Hesx1^{pos}$  cells in the adult murine retina. The hypotheses tested in this chapter were:

1. Adult retinal neurospheres derive from Hesx1-expressing forebrain progenitors.
2. The adult retina is not only formed by Hesx1-expressing forebrain progenitors.

In all four results chapters the primary tool used was the neurosphere-forming assay for comparative analyses and assessing differences in proliferation rates. In **Chapter 3**, retinal cells were isolated from the wildtype and  $Chx10^{orJ/orJ}$  CE and CNR, and cultured in neurosphere-forming conditions. To explore the effect of CHX10 on the RPC population in cell culture conditions, comparative analysis was performed by assessing their protein expression profile by immunocytochemistry, neurosphere diameter, number and pigmentation, and gene expression analysis via Real-Time quantitative Polymerase Chain Reaction (Re-Ti PCR). In **Chapter 4**, the characterisation of the newly discovered  $Chx10^{orJ/orJ}$  CNR progenitors was extended towards a Müller characterisation in accordance to recent literature. This was achieved by using several Müller glia markers both in neurospheres and acutely dissociated retinal cells as well as in adult and embryonic retinal sections using a series immunocytochemistry and immunohistochemistry experiments. Also, Re-Ti PCR was used in the investigation of cell cycle proteins to verify the presence of a Müller/progenitor cell. Finally two photoreceptor degeneration mouse models were employed in order examine if neurospheres are formed due to reactive gliosis. In **Chapter 5**, the potential of retinal neurospheres from both wildtype and mutant CE and CNR was examined, for differentiating to retinal neurons and glia, while I focused my attempts on promoting a rod photoreceptor precursor fate by the use of different growth and differentiation factors. In addition, the  $Nrl.gfp^{+/+}$  and  $Chx10^{orJ/orJ}/Nrl.gfp$  transgenic mouse models were used for investigating the formation of rod photoreceptor precursors *in vitro*. Finally, several culture protocols were used for expanding both the wildtype and Chx10-deficient RPC population. In **Chapter 6**, by using the

*Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* and *Hesx1<sup>gfp/+</sup>* mouse models it was possible to visualise the developmental origin of CE-derived proliferating neurospheres and the adult retina and CB.

Hopefully, the results shown here will give a better understanding of the importance of Chx10 in retinal stem/progenitor proliferation and differentiation cascade. This thesis presents novel findings for the behaviour of Chx10-deficient retinal progenitors, *in vitro* and *in vivo*, indicating that for finely tuning RPC or RSC for either *in vivo* activation upon injury or cell-based therapies for degenerative diseases, Chx10 is an essential factor that needs to be considered for effective therapies.

## **Chapter 2 – Materials and Methods**

## **2.1 General solutions, media, and laboratory equipment**

### **2.1.1 General laboratory equipment**

Petri dishes, pipettes, eppendorfs and Falcon tubes were supplied by Costar, UK. Pipette tips were supplied by VWR, UK. Pipette tips and eppendorf tubes were autoclaved prior to use. Centrifugations were either performed in a bench-top Sigma 1-15 microfuge (Philip Harris Scientific Ltd., UK) or in a Heraus microfuge (Biofuge, Pico, UK)], as indicated in the thesis. Dissections were carried out under a Stemi SV11 Zeiss microscope (Zeiss, Germany).

### **2.1.2 General solutions and reagents**

Distilled water (dH<sub>2</sub>O) was purified using a MilliRo-15 Water Purification System (Millipore, USA), or further purified using a Milli-Q Plus Ultra Pure Water System (Millipore, USA), and autoclaved.

A 1:1000 dilution of diethylpyrocarbonate (DEPC, Sigma) was added to solutions stated to be DEPC-treated, left at 37° C overnight, and subsequently autoclaved.

Paraformaldehyde (PFA, Sigma-Aldrich, UK) was used at 4% w/v and dissolved in DEPC-treated 1X Phosphate buffered saline (PBS, Oxoid Ltd, UK), heated until ~60°C, aliquoted and stored at -20 °C until used.

For obtaining 5M NaCl, 292.2 g of NaCl were dissolved in 800 ml dH<sub>2</sub>O. The solution was adjusted to 1L and autoclaved.



For obtaining 1M MgCl<sub>2</sub>, 203.3g of MgCl<sub>2</sub>.6H<sub>2</sub>O was dissolved in 800 ml dH<sub>2</sub>O. The solution was adjusted to 1L and autoclaved.

## **2.2 Animals**

Mice were maintained in the animal facility at University College London. All experiments have been conducted in accordance with the Policies of the Use of Animals and Humans in Neuroscience Research, revised and approved by the Society of Neuroscience in January 1995. Animal strains used included the homozygous *Chx10<sup>orJ/orJ</sup>* (Burmeister M 1996; Dhomen NS 2006) bred on a SV/129 background. Age-matched wild-type SV/129 mice were used as a control upon experimentation and data comparison. Additional mouse strains used were the GFP-expressing mice/ C57BL/6 and CFP /C57BL/6-expressing mice (Hadjantonakis et al. 2002), the *rds* (retinal degeneration slow) mouse and the rhodopsin knockout (*rho<sup>-/-</sup>*) mouse (Humphries et al. 1997). The *Nrl.gfp<sup>+/+</sup>* transgenic mouse was obtained as a kind gift from A. Swaroop's lab (Akimoto M 2006). The *Chx10<sup>orJ/orJ</sup>/Nrl-gfp* backcross was created in collaboration with R. Ali's lab. The *Hesx1<sup>Cre/+</sup>Rosa26<sup>LacZ/+</sup>* and the *Hesx1<sup>GFP-dta/+</sup>* transgenic mice was a gift from JP Martinez-Barbera's lab (Andoniadou et al. 2007).

Timed matings were set up on the evening of a given day. Mice usually mate in the middle of the dark period (Hetherington 2000), the following morning was designated E0.5, if vaginal plugs were subsequently found. On required day of embryonic development, pregnant mice were sacrificed by cervical dislocation. Embryos were removed from the uterus for use in all experiments described, i.e. for fixing and producing sections, or further dissection. When required, eyes from the sacrificed mother were removed using bent forceps.

## **2.3 Retinal cryosections**

Eyeballs were obtained from adult and embryonic mice and fixed o/n in 4% PFA at 4°C. Samples were then washed 3X in 1X PBS and incubated o/n at 4°C in 20% sucrose for cryo-protection. Preparation for obtaining cryosections involved embedding eyeballs into O.C.T. (VWR International Ltd, UK) in foil-made chambers and snap-freezing them in 2-Methyl-butane on dry ice for 2-3 minutes. Samples were stored at -70°C.

### **2.3.1 Obtaining saggital retinal cryosections**

Adult and embryonic saggital cryosections of 12 µm were prepared on Superfrost plus™ glass slides (VWR International Ltd, UK) and used for immunohistochemical analysis to confirm antibody specificity.

### **2.3.2 Immunohistochemistry of saggital retinal cryosections**

Saggital ocular cryosections were washed in 1X PBS at room temperature (RT) for 5 minutes for dissolving O.C.T. Wax circles were drawn around the samples onto the glass coverslips and blocking solution [10% Bovine Serum Albumin (BSA, Sigma-Aldrich, UK)/ 1% Fetal Calf Serum (FCS, Invitrogen, UK) (adult) or 10% BSA/ 1% Goat serum (DakoCytomation, Denmark (embryonic))] was added for at least 1 hour at RT. After discarding blocking solution excess, appropriately-diluted primary antibodies (approximately 350 µl) were added in fresh blocking solution and incubated overnight (o/n) at 4°C. The next day, samples were washed 3X with 1X PBS and then the appropriate fluorescence-conjugated secondary antibody was added (approximately 350 µl) and incubated for 40-60 minutes at RT. Slides were then washed 2X with 1% PBS.

Initially, Hoechst nuclear dye (1 $\mu$ M, Sigma-Aldrich, UK) was incubated along with the secondary antibody for nuclear counterstaining and then a drop of Citifluor<sup>TM</sup> (BDH, UK) was added, but then switched to DAPI staining which was added along with the mounting substance (Vector Laboratories Inc, USA) before sealing with a glass coverslip.

### **2.3.3 Haematoxylin and Eosin staining**

Retinal cryosections obtained were dipped into dH<sub>2</sub>O for 2 minutes to dissolve O.C.T. They were then dipped into haematoxylin (Sigma-Aldrich, UK) for 5 minutes and then washed with dH<sub>2</sub>O for 1 minute to wash away excess dye, and then dipped into chamber with running dH<sub>2</sub>O for 5 minutes. Slides were then subjected to dehydration by dipping them into 1% HCL / 70% Ethanol for 2 seconds and back into running dH<sub>2</sub>O for 2 minutes. Eosin staining (1%, Raymond A Lamb, UK) was then applied for 10 seconds before dipping slides into dH<sub>2</sub>O for 2 minutes. Complete removal of water was achieved by subjecting slides to increasing concentrations of ethanol starting from 70% for 3 minutes, then 90% for 3 minutes, two rounds of 100% Ethanol (Hayman, UK) 3 minutes each before adding HistoClear (National Diagnostics, USA) for 3 minutes. For mounting, a streak of DPX (BDH, UK) was added while sealing with a glass coverslip. Slides were then left to dry o/n in a fume hood.

### **2.3.4 Staining retinal cryosections for $\beta$ -galactosidase activity**

Adult retinal cryosections were washed in 1X PBS for dissolving O.C.T. Samples were then washed in buffer L<sub>0</sub> (washing buffer) filtered through a 0.22  $\mu$ m syringe filter. LacZ staining solution (in buffer L) was added through a 0.22  $\mu$ m syringe filter and the enzymatic reaction allowed proceeding in the dark, o/n at 37°C.

## 2.3.5 X-Gal staining reagents and solutions

Table 2.1 Buffer L<sub>0</sub>

<i>Reagent</i>	<i>Supplier</i>	<i>[Final]</i>	<i>[Stock]</i>
<i>MgCl<sub>2</sub></i>	<i>Sigma-Aldrich, UK</i>	<i>2mM</i>	<i>1M</i>
<i>Sodium Phosphate (pH 7.2)</i>	<i>Sigma-Aldrich, UK</i>	<i>0.1M</i>	<i>1M</i>
<i>EGTA</i>	<i>Sigma-Aldrich, UK</i>	<i>5mM</i>	<i>100mM</i>

Table 2.2 Buffer L

<i>Reagent</i>	<i>Supplier</i>	<i>[Final]</i>	<i>[Stock]</i>
<i>MgCl<sub>2</sub></i>	<i>Sigma-Aldrich, UK</i>	<i>2mM</i>	<i>1M</i>
<i>Sodium Phosphate (pH 7.2)</i>	<i>Sigma-Aldrich, UK</i>	<i>0.1M</i>	<i>1M</i>
<i>EGTA</i>	<i>Sigma-Aldrich, UK</i>	<i>5mM</i>	<i>100mM</i>
<i>NP40</i>	<i>Fisons, UK</i>	<i>0.2%</i>	<i>10%</i>
<i>Na-Deoxycholate</i>	<i>Sigma-Aldrich, UK</i>	<i>0.1%</i>	<i>10%</i>

**Table 2.3 X-Gal staining solution**

<i>Reagent</i>	<i>Supplier</i>	<i>[Final]</i>	<i>[Stock]</i>
<i>Buffer L</i>	-	-	-
<i>K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O</i>	<i>Sigma-Aldrich, UK</i>	<i>10mM</i>	<i>0.1M</i>
<i>K<sub>3</sub>Fe(CN)<sub>6</sub></i>	<i>Sigma-Aldrich, UK</i>	<i>10mM</i>	<i>0.1M</i>
<i>X-Gal</i>	<i>Sigma-Aldrich, UK</i>	<i>1 mg/ml</i>	<i>50 mg/ml in DMF</i>

## **2.4 Dissection procedure**

Mice were sacrificed and eyes were removed and kept in oxygenated L-15 medium (Invitrogen, UK) upon dissection. Eyes were hemisected using forceps and 7 mm curved micro-scissors, with the distinctive non-pigmented CNR region carefully dissected from the overlying RPE of the posterior half of the eye. The anterior half, the overlying RPE, iris and cornea were removed, leaving the CE region attached to the lens. Any remaining neural retinal tissue was removed. The CE was then carefully dissected free from the lens capsule under a Zeiss (Stemi SV-6) dissection microscope. In *Chx10<sup>orJ/orJ</sup>*, CNR tissue amount obtained was considerably lower and attached to the posterior region of the lens. The CE was expanded towards the periphery of the neural retina but the central retina, even though small, was clearly distinguishable from the expanded CE.

Embryonic retinal regions were obtained by decapitating embryos and removal of skin when needed (in E18.5 but not in E13.5 mice) in oxygenated L-15 medium. The neuroblastic layer and presumptive CE were separated from the

presumptive RPE using forceps and 7 mm curved micro-scissors under a Zeiss (Stemi SV-6) dissection microscope

## **2.5 Culturing retinal cells**

### **2.5.1 Neurosphere-forming assay**

Cultures were initially setup using Dispase (2 units/ ml, MPBiochemicals, USA) for an enzymatically-based dissociation method, but later, it was observed that papain enzyme was a more appropriate reagent for dissociating adult retinal tissue. Components of the dissociation medium are reconstituted as described previously (Tropepe V 2000) with alterations. Tissue samples were dissociated in a solution containing 20 units/ml of papain enzyme diluted in Earls Balanced Saline Solution (EBSS, Worthington Biochemical, Lorne Laboratories, UK) along with DNase solution (1 µg/ µl) and the mixture was equilibrated with (20:1) O<sub>2</sub>:CO<sub>2</sub>. (Worthington Biochemical, Lorne Laboratories, UK). Cells were incubated for at least 20 minutes at 37°C followed by gentle trituration. Supernatant was transferred to a fresh tube and centrifuged for 5' at 2,000 rpm (223xg). Dissociated cells were pelleted and resuspended in a Ovomuroid solution (1X, Worthington Biochemical, Lorne Laboratories, UK), a papain enzyme inhibitor, containing DNase (1 µg/ µl, Worthington Biochemical, Lorne Laboratories, UK). Intact cells were separated from cell lysates by a one step discontinuous density gradient in Ovomuroid solution (stock) and centrifugation (2,000 rpm for 2 minutes, 223xg). Pelleted cells were resuspended in DMEM-F12 plus Glutamax™ (Invitrogen, UK) containing N2 supplement (1:100, Invitrogen, UK), Penicillin-Streptomycin solution (1:100, Invitrogen, UK), EGF (20 ng/ml, Peprtech EC, UK) and FGF-basic (10 ng/ ml, Peprtech EC, UK), named as Neurosphere Growth Medium (NGM). Cells were cultured either as 2

eyes/ well or 4 eyes/ well and as 20,000 viable cells per well, using the Trypan Blue exclusion assay (Sigma-Aldrich, UK). Fresh medium was added every other day without surpassing 750 µl in each well of the 24-well plate. Medium was exchanged every 6-7 days.

### **2.5.2 Dissociated adult CE and CNR cultures**

CNR and CE tissue samples were dissociated as described in the previous section. For neurosphere formation, cells were resuspended in NGM in untreated 24-well tissue culture plates. Alternatively, cells were seeded onto PLL-coated glass coverslips and treated with 4% PFA fixation agent for immunocytochemical analysis.

## **2.6 Immunocytochemical characterisation of adult retinal cells**

### **2.6.1 Preparation of glass coverslips**

Poly-L-Lysine and Poly-Ornithine (PO) glass 13 mm round coverslips (Deckglaser, Germany) were made by coating with 10 µg/ ml PLL (stock 1 mg/ml, Sigma-Aldrich, UK) and 15 µg/ ml PO (stock 2 mg/ml, Sigma-Aldrich, UK), respectively. Coverslips were dipped in pure ethanol and allowed to dry prior to coating. A hundred to two hundred µl of PLL or PO were added and incubated o/n at 37°C. Coverslips were then washed 4X with 1X PBS and allowed to dry for 30 minutes within a fume hood, preferentially in low light conditions. For well coating the same procedure was followed but with larger volumes applied around 300-350 µl.

Preparation of Laminin- and Fibronectin-coated coverslips and wells required the surface of interest to have initially been coated with either PLL or PO as described in the previous paragraph. After the first substrate has been applied and wells or glass coverslips have been allowed to dry, 100 ng/ ml of Laminin (1 mg/ml stock, Sigma-Aldrich, UK) or 5 µg/ml of Fibronectin (1 mg/ ml stock, Sigma-Aldrich, UK) were applied for 2 hours at RT within a fume hood. Excess was aspirated and wells/glass coverslips were washed 3X with 1X PBS and allowed to dry for 30 minutes at RT. Coated well-plates or glass coverslips were either used directly or stored at 4°C for up to a week.

### **2.6.2 Immunocytochemistry on acutely dissociated retinal tissue**

Acutely dissociated CE or CNR cells were re-suspended in 1X PBS and placed on PLL-coated glass slides and allowed to settle prior to fixation with 4% PFA for 5 m at RT. Cells were washed 3 x 2 m in PBS and pre-blocked in PBS containing normal goat serum (NGS) (10%), bovine serum albumin (BSA) (1%) and 0.05% Triton-X 100 for 1 hour (h) at RT before being incubated with primary antibody in blocking solution for 1 hour at RT. After rinsing 3 x 2 minutes with 1X PBS, cells were incubated with secondary antibody for 20 minutes at RT, rinsed (3 x 2 minutes) and counter-stained.

### **2.6.3 Immunocytochemistry on proliferating neurospheres**

Cells that produced spheres were cultured for 7 days *in vitro* culture (IVC) and seeded onto PLL-coated (10 µg/ ml) glass coverslips for immunolabelling. After addition of 200-400 µl PFA (4%) supernatant was aspirated and left for 10-15 minutes at RT. Coverslips were washed three times with 1X DePC-treated PBS; blocking solution containing FCS (10%) / PBS-diluted BSA (1%) / Triton X-100 (0.5%, BDH, UK) and incubated for at least 2 hours at RT. Primary antibody,



diluted in fresh blocking solution, was added and incubated for 1 hour on a slow-moving rack again at RT. Primary antibody was incubated overnight (o/n) at 4°C, where appropriate. After washing 3X in 1X PBS, the appropriate secondary fluorescent-conjugated antibody was incubated along with Hoechst nuclear dye for up to 1 hour at RT. Coverslips were sealed and a drop of Citifluor™ was added. Alternatively, DAPI was used instead of Hoechst and Citifluor™ as described earlier.

## **2.7 Characterisation of proliferating neurospheres**

### **2.7.1 Neurosphere number and diameter counts**

To assess size and diameter size, neurospheres were visualized using either an inverted Leica DMIL microscope (Leica, UK) or a Zeiss Axiovert™ 135 microscope (Zeiss, UK), each fitted with a camera and image capture system. Neurospheres were selected at random for analysis from the image set, which had previously been re-coded to ensure blind assessment.

At 7 days *in vitro*, neurospheres defined as free-floating spheres with a definite outer boundary, were counted and expressed as number of neurospheres/ eye and neurospheres/ 20,000 cells. Neurosphere diameter was measured twice, the second measurement taken perpendicular to the first, and an average of the two was calculated.

### **2.7.2 Pigmentation assessment using Volocity™ software**

Neurosphere pigmentation was assessed using the light microscope images captured for size analysis from a single microscope (Zeiss Axiovert 135), using

Volocity™ software 3.0 (Improvision, UK). Images were normalized to ensure the same background intensity. 100% black was given a pixel value of 2000, while white had a value of 0. Volocity™ software calculates the average pixel intensity of a given region. The regions were selected manually to encompass the whole neurosphere, as determined by drawing round the perimeter of the sphere.

### **2.7.3 BrdU *in vivo* neurosphere labelling**

Adult wildtype and Chx10<sup>orJ/orJ</sup> mice received intraperitoneal injections of the Thymidine analogue bromodeoxyuridine (BrdU, Sigma-Aldrich, UK) diluted at 10 mg / mL in PBS, at 100 µg / g body weight every day for 5 days. Tissue was prepared two days after the last injection. Eyes were processed either for sphere culture or fixed for sectioning. At 3 days in culture, CE and CNR spheres were transferred to PLL coated glass slides, fixed with 4% PFA in PBS for 30 m and processed for BrdU immunohistochemistry, as described above. Retinal sections were prepared by fixing the eyes in 4% PFA for 1 hour, before cryoprotecting in 20% sucrose for 1 h. Eyes were then embedded in Tissuetek (GMI Inc, USA) and snap frozen. Cryosections (20 µm) were fixed to PLL-coated slides (VWR International, UK) and processed for BrdU immunohistochemistry as previously described (MacLaren, Pearson et al. 2006).

### **2.7.4 Limiting Dilution Assay (LDA)**

Papain-dissociated retinal cells or neurosphere-derived cells obtained from one adult eye of wild-type or Chx10<sup>orJ/orJ</sup> CE and CNR were counted using the Trypan Blue Exclusion Assay (1:5 ratio, cell suspension: Trypan blue) and dissociated CE and CNR cells were serially diluted and plated into non-tissue

culture-treated 96-well plates (TPP, Germany) at dilutions of 5, 50, 500, 1500 and 2500 cells/well. Cells were cultured for 7 days in the presence of growth factors (EGF/FGF2) and wells where at least one neurosphere was observed were considered as positive hits. The data is represented as a plot on a semi-logarithmic scale, indicating the % of negative wells on the vertical axis versus the cell number per well on the horizontal axis. As previously described (Tropepe V 2000), a linear correlation indicates that a neurosphere derived from a single event. Based on the Poisson distribution equation  $F_0=e^{-x}$ ,  $F_0$  is the fraction of wells where no neurospheres were observed, while  $x$  is the number of neurosphere-forming units per well.  $F_0 = 0.37$ , (i.e.  $x=1$ ) corresponds to the dilution at which there is one neurosphere-forming cell per well ( $1/e$ ). Statistical and regression analysis was performed using GraphPad Prism™ 5.0 software.

### 2.7.5 Neurosphere clonal derivation

Confirmation of clonal derivation of neurospheres was determined by plating a mix of dissociated cells isolated from the CE of GFP-expressing mice (Okabe et al. 1997) and from expressing CFP-expressing mice (Hadjantonakis, Macmaster et al. 2002), (both purchased from Jackson Laboratories, USA), in NGM. Cells were plated at a range of densities from 10 to 50,000 cells per ml. After 7 days *in vitro*, the resulting spheres were seeded onto PLL-coated (10 µg/ml) glass coverslips and allowed to settle prior to fixation with 4% PFA for 20 minutes at RT. Neurospheres were processed for immunohistochemistry and visualised using confocal microscopy.

## **2.7.6 Neurosphere self-renewal potential**

### **2.7.6.1 Acute neurosphere dissociation**

To assess secondary neurosphere-forming potential, proliferating neurospheres that were cultured for 7 days *in vitro* in NGM, were digested using Trypsin/EDTA solution (Sigma-Aldrich, UK) and re-plated into untreated plates, in neurosphere-forming conditions (NGM without FCS) for 7 days *in vitro*.

Neurospheres were also passaged for assessing self-renewal potential using different solutions and/or cocktails of Papain/ DNase (20 units/ ml & 0.005% respectively, same technique as for dissociation of cells after retinal dissection), Trypsin (0.1-1 mg/ ml, Sigma-Aldrich, UK) and Accutase<sup>TM</sup> (0.5 mM, PAA, UK), in order to optimise sphere dissociation to single cell suspension. Sphere number was then counted at specified intervals. For investigating self-renewal, cells that produced spheres after 7 days *in vitro* in NGM were seeded onto 24 plastic-well plates in NGM. Spheres were cultured for further 16 days, replenishing mitogens every other day and exchanging with fresh mitogen-rich medium every week. For secondary sphere formation spheres were counted at 2-3 day intervals, visualised and counted under a Zeiss inverted microscope.

### **2.7.6.2 Neurosphere passaging**

Proliferating neurospheres cultured for 7 days *in vitro* in NGM, were subjected to monolayer forming conditions by supplementing 1% FCS in the NGM in

PLL/Fibronectin-coated wells, for 4 days prior to trypsinisation to reach single cell level. Cells were then replated in NGM and allowed to form secondary neurospheres. Secondary neurosphere number was counted 7 days after initial dissociation.

Passaged neurospheres also underwent immunocytochemical analysis. After addition of 200-400  $\mu$ l PFA (4%) supernatant was aspirated and left for 10-15 minutes at RT. Coverslips were washed three times with 1X PBS; blocking solution containing FCS (10%) / PBS-diluted BSA (1%) / Triton X-100 (0.1%, BDH, UK) and incubated for at least 2 hours at RT. Primary antibody, diluted in fresh blocking solution, was added and incubated for 1 hour on a slow-moving rack again at RT. Primary antibody was incubated o/n at 4°C, where appropriate. After washing 3X in 1X PBS, the appropriate secondary fluorescent-conjugated antibody was incubated along with Hoechst nuclear dye for up to 1 hour at RT. Coverslips were sealed with a drop of Citifluor™. Alternatively DAPI was used instead of Hoechst and Citifluor™ as described earlier.

### **2.7.7 BrdU pulse-labelling in neurospheres**

Incorporation of BrdU during S-phase was used as an assay for cell proliferation. Spheres derived from cells from the adult *Chx10<sup>orJ/orJ</sup>* mouse CE and CNR and wildtype mouse CE were cultured in the presence of mitogens, as described above, for 7 days. On day 7 *in vitro*, cells were pulse-labelled with BrdU (0.5  $\mu$ M) for 4 hours. Free-floating spheres were removed and transferred to PLL-coated coverslips, fixed in 4% PFA for 20 minutes and processed for BrdU immunohistochemistry. Briefly, cells were exposed to 2M HCl (BDH, UK) for 30 minutes at 30°C to denature cellular DNA. HCl was neutralized by application of 0.1M Na-borate (Sigma-Aldrich, UK) for 10 minutes, prior to

rinsing with PBS. Rat anti-BrdU (1:500; AbCam, Cambridge, UK), followed by Alexa-546 goat anti-rat (1:250; Molecular Probes, Invitrogen, UK) were used for BrdU staining with Hoechst 33342 to label all cell nuclei. The percentage of cells that were BrdU<sup>pos</sup> was determined by counting the number of BrdU-labelled and Hoechst-labelled cells in single confocal sections taken at 15 µm intervals through each sphere. This interval was sufficient to ensure a given nucleus appeared in only a single analyzed section.

### **2.7.8 PI exclusion assay**

Neurospheres cultured for 7 days *in vitro* in the presence of growth factors were incubated for 15 minutes in gassed DMEM-F12 medium with Hoechst (2 µM) nuclear dye and Propidium Iodide (0.5 mM; Sigma-Aldrich, UK). Neurospheres were then transferred onto a glass slide and fixed with 4% PFA for 10 minutes at room temperature. Cells stained with Hoechst dye and PI were apoptotic, while non-apoptotic cells show uptake of the Hoechst nuclear dye only (Crissman et al. 1979; Jones and Senft 1985; Suzuki et al. 2001).

### **2.7.9 Staining for β-galactosidase activity on neurospheres**

Culture medium was removed and neurospheres were either seeded onto PLL-coated glass coverslips or remained on the culture wells prior to fixation with 4% PFA for 10 minutes at RT. Wells and coverslips were washed 4X with 1X PBS and then washed in buffer L<sub>0</sub> filtered through a 0.22 µm syringe filter. LacZ staining solution was added through a 0.22 µm syringe filter and the enzymatic reaction allowed to proceed in the dark, o/n at 37°C.

### **2.7.10 X-Gal staining reagents and solutions**

Neurosphere-forming cells were treated with the same reagents as in section 2.3.5 but with a lower amount of NP40; 0.02% and Na-Deoxycholate; 0.01% used.

## **2.8 Expansion of adult retinal progenitors**

### **2.8.1 Expansion via GSK-3 $\beta$ inhibition**

Cultures were initially setup as described in Section 2.3.2. Pelleted cells were resuspended and allowed to form neurospheres for 7 days *in vitro* in NGM along with a cell-permeable inhibitor of glycogen kinase-3 (GSK-3 $\beta$ , Calbiochem, Germany) in concentrations ranging from 0 to 25  $\mu$ m. Neurosphere number, diameter and immunocytochemical analysis were performed in order to assess the effect of GSK-3 $\beta$  as described earlier.

### **2.8.2 Expansion via monolayer culture**

At 7 days *in vitro*, spheres were transferred to PO/Fibronectin-coated plates and cultured in the presence low serum (1% FCS) and growth factors, as described for the propagation of human (Coles BL 2004) and porcine CE cells (Macneil et al. 2007). Fresh growth factors were added every other day and cultures were split approximately every 7 days using trypsin/EDTA solution (Sigma-Aldrich, UK) and re-plated into fresh plates.

## **2.9 Differentiation potential of adult retinal progenitors**

### **2.9.1 Neurosphere differentiation assay**

Cells that produced spheres after 7 days *in vitro* in NGM were allowed to differentiate onto glass coverslips into wells containing mitogen-free medium, supplemented with 10% FCS plus additional factors and cultured for further 14-20 days IVC in a 24-well plate. Medium was exchanged every 3-4 days. Coverslips were then immunolabelled as mentioned before.

It was also attempted to differentiate spheres from Chx10<sup>orJ/orJ</sup> and wild-type retinal regions using different substrate combinations (PLL/Laminin, PO/Laminin & PO/Fibronectin) on plastic wells or glass coverslips and culturing in MFM for 4 days, followed by 14 days in 10% FCS (Lumelsky, Blondel et al. 2001; Balcells and Edelman 2002).

### **2.9.2 Immunocytochemistry on differentiated retinal cells**

After cells were allowed to differentiate, culture media was removed and 200 µl of PFA (4%) were added and left for 10-15 minutes at RT. Coverslips were washed three times with 1X DePC-treated PBS; blocking solution containing FCS (10%) / PBS-diluted BSA (1%) / Triton X-100 (0.1%, BDH, UK) and incubated for at least 2 hours at RT. Primary antibody, diluted in fresh blocking solution, was added and incubated for 1 hour on a slow-moving rack again at RT. Primary antibody was incubated o/n at 4°C, where appropriate. Secondary antibody was incubated as described earlier.



### 2.9.3 Puramatrix™ gel assay

Gel (BD Biosciences, UK) at 0.1 or 0.5% w/v (diluted in MQ H<sub>2</sub>O) was centrifuged for 2 minutes at 5,000 rpm before incubating in a bath sonicator for 30 minutes. For creating a cell adherent surface, 100 µl of the hydrogel was seeded onto the surface of glass coverslips to promote gelation and 100 µl of medium was added onto the glass coverslip. After 5-10 minutes the hydrogel should have assembled, the medium was changed every 30 minutes twice to equilibrate the growth environment to physiological pH of ~7.0. Cell suspension was carefully added on top of the hydrogel.

For 3-dimensional cell encapsulation, Puramatrix™ peptide hydrogel was centrifuged and sonicated as above, while the cell pellet was washed with 10% sucrose from any culture medium. Cell pellet was re-washed and re-suspended in fresh 10% sucrose. To achieve maximum encapsulation, sonicated hydrogel and cell/sucrose suspension were mixed in equal volumes creating a 0.5% w/v of hydrogel of the desired cell concentration and pipetted into 24 well-plates onto glass coverslips. Culture media was added slowly and changed 3 times 30 minutes each to achieve physiological pH.

## 2.10 Antibodies

### 2.10.1 Primary antibodies

**Table 2.4 Primary antibodies List**

Pax6		Glutamate synthetase	Vimentin	Nestin	Antibody name
1:300	1:100	1:20	1:20	1:20	Dilution
Covance	B&D Biosciences	AbCam	Developmental Studies	Developmental Studies	Manufacturer
Rabbit	mouse	mouse	mouse	mouse	Species Raised
pIgG	mIgG <sub>1</sub>	IgG <sub>1</sub>	mIgG <sub>1</sub>	mIgG <sub>1</sub>	Isotype
-	250 µg/ml				[Stock]
Retinal & Neural progenitors	Early Müller glia	Neural stem cells, RPC	Neural progenitors, RPC	Neural progenitors, RPC	Tissue Specificity
Nuclear Transcription factor	Mitochondrial marker	Intermediate Filament protein	Intermediate Filament protein	Intermediate Filament protein	Target
(Marquardt T 2001)	(Lavail and Reif-Lehrer 1971; Riepe and Norenburg 1977)	(Drager 1983; Kivela et al. 1986; Okada et al. 1990)	(Mayer et al. 2003; Walcott JC 2003; Xue et al. 2006)	(Mayer et al. 2003; Walcott JC 2003; Xue et al. 2006)	References

Doublecortin	$\beta$ -III tubulin	Brn-3b	Sox2	Chx10
1:100	1:1000	1:100	1:200	1:4000
Santa Cruz	Promega & Covance	Santa Cruz	AbCam	Kind gift from T. Jessell & R. McInnes
donkey	mouse	goat	rabbit	rabbit
200 $\mu$ g/ml	1 mg/ml	200 $\mu$ g/ml	0.3 mg/ml	
mIgG	mIgG <sub>1</sub>	plgG	plgG	
Horizontal cells	Early neurons	Retinal ganglion cells	ES cells, Neural stem cells	RPC, Müller glia & bipolar cells
Neuronal processes & cytoskeleton	Neuronal processes	Nuclear Antigen	Nuclear localisation	Nuclear Transcription factor
(Lee et al. 2003; Engelhardt M 2005)	(Vinores et al. 1995; Sharma and Netland 2007)	(Xiang et al. 1993)	(Graham et al. 2003; Ellis P 2004; Brazel et al. 2005; Episkopou V	(Burmeister M 1996; Rowan S 2004)

CRALBP	Phosducin	Recoverin	Rhodopsin	NeuN
1:1000	1:1000	1:100	1:50	1:100
Gift from J. Saari		Chemicon	Santa Cruz	Chemicon
rabbit	sheep	rabbit	rabbit	mouse
-	-	1 mg/ml	200 µg/ml	1 mg/ml
-	mIgG <sub>1</sub>	plgG	plgG	IgG <sub>1</sub>
Müller glia & RPE cells	Late Rod photoreceptors	Rod and cone photoreceptors & midget cone bipolars	Rod photoreceptors	Pan-neuronal except photoreceptors
cellular retinaldehyde-binding protein	cytoplasmic	Calcium-binding protein	G-protein	Nuclear Antigen
(Kennedy et al. 2003; Saari and Crabb 2005)	(Sokolov et al. 2004; Chen et al. 2005; Krispel et al. 2007)	(Lambrecht HG 1992; Wiechmann et al. 1994; Erickson et al. 1998; Strissel et al. 2005)	(Low 1987; Leonard et al. 1992; McNally et al. 1999; Terada et al. 2006)	(Kumar and Buckmaster 2007)

GFAP	Syntaxin	HNK-1/N-CAM	PKC
1:1000	1:100	1:100	1:100
Chemicon	Sigma-Aldrich	Sigma-Aldrich	Amersham
rabbit	mouse	mouse	mouse
pIgG	IgG <sub>1</sub>	IgM	IgG <sub>2a</sub>
	4.8 mg/ml	1.7 mg/ml	100 µg/ml
Reactive Müller glia & astrocytes	Amacrine Cells/INL	Amacrine & Horizontal cells	Bipolar cells
Intermediate Filament protein	Cell membrane protein	GABAergic neurons	subcellular localisation
(Dyer MA 2000)	(Inoue et al. 1992; Inoue and Akagawa 1993)	(Kosaka et al. 1989)	(Greferath et al. 1990; Zhang and Yeh 1991)

<b>Antibody name</b>	eGFP	Phospo-H3	p27 <sup>Kip1</sup>	RPE65
<b>Dilution</b>	1:200	1:1000	1:100	1:500
<b>Manufacturer</b>	Invitrogen	Upstate biotechnology	BD Pharmigen	Chemicon
<b>Species Raised</b>	rabbit	rabbit	mouse	mouse
<b>Isotype</b>	mIgG	mIgG	IgG	IgG <sub>1</sub>
<b>[Stock]</b>		-	-	100 µg
<b>Tissue Specificity</b>	Recognises GFP	Cell cycle protein	Cell cycle protein	RPE
<b>Target Protein</b>	GFP-expressing cells	RPC	Müller glia	RPE microsomal membranes
<b>References</b>	-	(Tapia et al. 2006)	(Dyer MA 2000; Levine et al. 2000)	(Hamel et al. 1993; Tsilou et al. 1997)

### **2.10.2 Secondary antibodies and controls**

The appropriate Cy3- or FITC- or Rhod- (Jackson ImmunoResearch, UK) or Alexa- (Molecular Probes, Invitrogen) tagged secondary antibodies were used. Cells were counter-stained with Hoechst 33342, DAPI, or PI. Negative controls omitted the primary antibody. Staining was visualized using either a Zeiss (LSM 510; Zeiss, UK) or Leica (SP2, Leica, UK) confocal microscope or an Axiophot 3 (Zeiss, Germany) fluorescence microscope. Dissociated wild-type adult retina was used as a positive control for testing antibody specificity.

An osteoblastic cell line (MC3T3) (Sudo et al. 1983) was used as a negative control for immunocytochemistry, along with several immunoglobulin isotypes (all from Sigma-Aldrich, UK) on retinal neurospheres.

### **2.11 Confocal Microscopy**

Neurospheres or retinal sections were mounted under glass coverslips and imaged using a confocal microscope (LSM510, Zeiss or Leica SP2, Leica). The fluorescence of Hoechst 33342, Alexa-488 and Alexa-546 were excited with the 350 nm line of the UV laser, the 488 nm line of the Argon laser and the 543 nm line of the HeNe laser, respectively. Images show projections of multiple single confocal sections taken at approximately 5-10  $\mu\text{m}$  steps, unless otherwise stated.

### **2.12 Viral Transfection**

Dissociated cells obtained from 4-10 dissected eyes of wildtype and Cxh10<sup>orJ/orJ</sup> animals were cultured for 7 days *in vitro* to obtain neurospheres as previously

described. Neurospheres were then seeded on pre-coated Poly-Ornithine & Laminin 24-well plates and cultured for further 3 days with 1% FCS/ 20 ng/ml EGF / 10 ng/ml FGF-2/ 10 ng/ml NT3/10 ng/ml Heparin. A Chx10-lentiviral construct (MOI=50) was used to transfect spheres along with a control lentiviral construct [Multiples Of Infection (MOI) of 50] carrying a Red Fluorescence Protein (RFP) reporter gene. Spheres were cultured for further 3 days, and viewed under fluorescence microscope to visualise RFP emission.

## **2.13 RNA extraction**

### **2.13.1 RNA extraction using TRI Reagent™**

For RNA cell purification from neurospheres, TRI Reagent (Sigma-Aldrich, UK) was used. Table below shows the reagents used for RNA extraction.

**Table 2.5 TRI REAGENT™ RNA extraction reagents**

Reagent	Supplier	[Stock]
TRI Reagent™	Sigma-Aldrich, UK	N/A
Chloroform	Sigma-Aldrich, UK	100%
2-Isopropanol	Sigma-Aldrich, UK	100%
Ethanol	Hayman, UK	75%

In theory, 1 ml of TRI REAGENT™ can isolate RNA from 5-10 x 10<sup>6</sup> cells or 10 cm<sup>2</sup> of culture dish surface for cells grown in monolayer. For cell suspension, cells are isolated by centrifugation and lysed in 1 ml of TRI REAGENT™ by



repeated pipeting. Alternatively, for monolayers cells can be lysed directly on the culture dish.

Samples were allowed to stand for 5 minutes at RT before adding 0.2 ml of chloroform and shaking vigorously for 15 seconds. Lysates were then left for 10 minutes at RT and then centrifuged at 12,000 g (8,000 rpm) for 15 minutes at 4°C. Centrifugation separated the mixture into 3 phases: a red phase containing protein, an interphase containing DNA and an upper aqueous phase (colourless) containing RNA.

Upper aqueous phase was transferred to a new tube containing 0.5 ml isopropanol and mixed gently. Sample was left at -20°C overnight for better RNA precipitation. The next day, sample was centrifuged at 12,000 g (maximum speed on microfuge) for 10 minutes at 4°C, to allow RNA to form a tight pellet. Supernatant was discarded and pellet was washed with 1 ml (75%) ethanol before vortexing and centrifuging at 7,500 g (6,000 rpm) for 5 minutes at 4°C. Supernatant was removed carefully and RNA-containing pellet was left to dry in upright position for 15 minutes before adding the appropriate amount of DepC-treated distilled H<sub>2</sub>O.

### **2.13.2 RNA extraction using Genra Purescript™ extraction kit**

For  $2 \times 10^5$  to  $5 \times 10^5$  cells, it was decided to use an RNA extraction kit that did not contain Trizol™, since it has been shown that in very low RNA yields this reagent can cause degradation. The expected yield of RNA using the below extraction method was between 1-5 µg. Table below shows all the reagents used for the RNA extraction.

**Table 2.6 Genra Purescript™ RNA extraction reagents**

Component	Supplier
Cell Lysis Solution	Genra systems, USA
Protein-DNA precipitation solution	Genra systems, USA
RNA Hydration Solution	Genra systems, USA
2-Isopropanol	Hayman, UK
Ethanol (75%)	Hayman, UK

Culture medium was added to 1.5 ml microfuge tube on ice and centrifuged for 5-10 seconds at 13,000 rpm (maximum speed). Supernatant was removed leaving 10-20  $\mu$ l of residual fluid vortexing vigorously to resuspend cells. Cell Lysis Solution™ was added to resuspended cells at RT, to avoid SDS precipitation, and pipeting up and down no more than 3 times to lyse the cells. Alternatively, adherent cells were lysed directly in the culture dish by withdrawing culture medium and adding 150  $\mu$ l Cell Lysis Solution™. Extensive pipeting could shear DNA causing it to precipitate less efficiently at the next step. Immediately, 50  $\mu$ l of Protein-DNA Precipitation Solution™ was added to the cell lysates inverting the tube(s) gently 10 times and placed into ice for 5 minutes. Cell lysates were centrifuged for 3 minutes at 13,000 rpm. Precipitated proteins and DNA formed a white pellet (if white pellet is not tight repeat centrifugation). Supernatant was poured into a fresh 1.5 ml tube containing 150  $\mu$ l 2-propanol (at RT in order to keep residual proteins in solution and not precipitate) and 1  $\mu$ l blue pellet co-precipitant dye (Novagen, USA). Tubes were inverted 50 times and centrifuged for 3 minutes at maximum speed before discarding supernatant and drained briefly on clean absorbent paper. RNA pellet was then washed in 150  $\mu$ l 70% Ethanol (also at RT in order to remove salt

more efficiently) and centrifuged for 1 minute at maximum speed. Supernatant was discarded and tube(s) left to drain for 15 minutes in upright position, making sure that RNA is not over-dried or it will be very difficult to re-hydrate it. RNA samples were rehydrated in appropriate volume of RNA Hydration Solution™ (10–25 µl) on ice for at least 30 minutes. Samples were then vortexed and pulse-spinned before storing them at -80°C.

### **2.13.3 Buffer preparation for RNA gel electrophoresis**

For a 500 mL stock solution of 0.5 M ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich, UK), 93.05 g of EDTA disodium salt (FW = 372.2) was dissolved in 400 mL deionised water. The pH was adjusted with NaOH (Sigma-Aldrich, UK) to 7.5, and the volume adjusted to a final volume of 500 mL. For preparation of stock TAE (50X), 242 g of Tris base (FW = 121.14) were dissolved in approximately 750 mL deionised water. Carefully, 57.1 ml of glacial acid were added along with 100 mL of 0.5 M EDTA (pH >7.8) and solution's volume adjusted to 1 L.

The working solution of 1x TAE buffer was made by simply diluting the stock solution by 50X in deionised water. Final solute concentrations were 40 mM Tris acetate and 1 mM EDTA. Stock buffer were stored at room temperature but working buffer needs its pH adjusted above 8.0. RNA ladder used indicated RNA fragment lengths from 0.24-9.5 kb (Gibco, UK)

### **2.13.4 RNA gel electrophoresis**

RNase-free agarose (Sigma-Aldrich, UK) was made up to 1% by adding 0.5 gr of agarose in 50 ml 1x TAE buffer. The solution was boiled and then 5 µl Ethidium Bromide (EtBr, Sigma-Aldrich, UK) was added. Solution was added

into a clean rack and allowed to solidify. Rack was loaded with 300 µl of TAE (pH >8.0).

Sample preparation involved 1 µl of RNA, 2 µl purple loading buffer (5X stock) and 7 µl formamide (RNA-protective). Gel was run at 90 Volts/ 100 Amperes for at least 20 minutes at 4°C. After run was complete, additional 5 µl of EtBr was added and viewed under a UV light.

## **2.14 Reverse Transcription**

RNA Samples were thawed from -80°C and kept on ice at all times. Table 2.7 shows the reagents used for the 1<sup>st</sup> step of the reaction. Samples in the RT-solution I were incubated in a PCR machine for 10 minutes at 72°C. Then they were placed on ice for 5 minutes before proceeding to the next step.

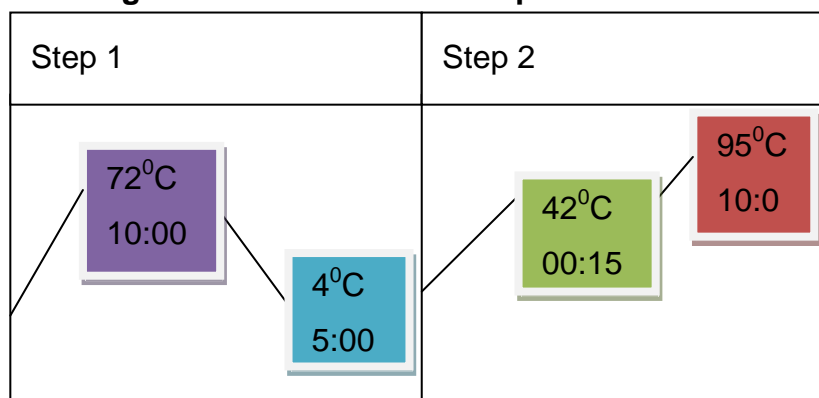
**Table 2.7 RT-Solution I**

Reagent	Supplier	Volume/reaction	Final concentration
pN <sub>6</sub> (10pM)	Roche, UK	1 µl	1 pM
DepC-treated H <sub>2</sub> O	-	1 µl	-
RNA	-	8 µl	<1 µg
Total	-	10 µl	-

Table 2.8 shows the 2<sup>nd</sup> RT-solution added to the 1<sup>st</sup> RT-Solution mix. Samples were incubated on a PCR machine for 1 hour at 42°C and then 10 minutes at 95°C. Samples were stored in -20°C (Figure 2.1). **Table 2.8 RT-Solution II**

Reagent	Supplier	Volume/reaction	[Final]
PCR buffer (5X)	Promega, UK	4 µl	1X
dNTPs (10mM)	Promega, UK	2 µl	1 mM
RNAsin	Promega, UK	1 µl	1 unit/ml
MMLV	Promega, UK	1 µl	1 unit/ml
RT-Solution I	-	10 µl	-
DepC-treated H <sub>2</sub> O	-	2 µl	-
Total		20 µl	-

**Figure 2.1 Reverse Transcription Reaction**



### 2.15 Genomic DNA isolation

Tailsnips were obtained from at least 4 week-old mice digested in Lysis buffer (Table 2.9) containing Proteinase K (0.1 mg/ml Sigma-Aldrich, UK) o/n at 55°C. The next day lysates were placed for 5 minutes onto a horizontal shaker at low speed at RT. Then 100 µl NaCl were added and samples were additionally replaced on the horizontal shaker for a further 5 minutes. Samples were then

centrifuged at 13,000 rpm for 10 minutes and supernatant was collected in new eppendorf tubes containing 250  $\mu$ l of 2-Isopropanol. Tubes were inverted gently 3-5 times and then centrifuged at 13,000 rpm for 10 minutes. Supernatant was discarded and DNA pellet was washed with 70% Ethanol prior to centrifugation at 13,000 rpm for 2 minutes. After discarding supernatant DNA pellets were allowed to dry for 10-15 minutes before resuspending them to 100  $\mu$ l DepC-treated milli-Q (MQ) H<sub>2</sub>O. Samples were stored in -20°C.

**Table 2.9 Lysis buffer**

<i>Reagent</i>	<i>Supplier</i>	<i>[Final]</i>	<i>[Stock]</i>
<i>Tris pH 8.0</i>	<i>Sigma-Aldrich, UK</i>	<i>50 mM</i>	<i>0.5M</i>
<i>NaCl</i>	<i>Sigma-Aldrich, UK</i>	<i>100 mM</i>	<i>5M</i>
<i>EDTA</i>	<i>Sigma-Aldrich, UK</i>	<i>100 mM</i>	<i>0.5M</i>
<i>SDS</i>	<i>BDH, UK</i>	<i>1%</i>	<i>10%</i>

## **2.16 Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

### **2.16.1 TaqMan™ qPCR**

cDNA samples were used as templates for quantitative real-time polymerase chain reaction (Re-Ti-PCR) on a 7500 Fast Real-Time PCR™ System, using TaqMan™ (Applied Biosystems, UK) gene expression assay kits according to the manufacturer's protocol (Tables 2.10 and 2.11). Quantitative Real Time PCR (RT PCR) stages were: (i) denaturation stage, 1 cycle at 50°C for 2 minutes and

95°C for 10 minutes (ii) amplification stage, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute (Figure 2.2). Data collection was set at the amplification stage in each cycle. At least four independent cultures of each genotype and region, each using neurospheres from four eyes, were analysed in duplicate TaqMan™ assays. *Gapdh* and 18S rRNA was used as endogenous controls and both showed similar expression between cultures; *Gapdh* data is presented. Relative quantities of the target mRNAs were normalized against the endogenous control by subtracting CT (threshold cycle) values for *Gapdh* from CT values for all gene expression assays; relative expression values were calculated as  $2^{-\Delta CT}$ . The Re-Ti PCR values obtained from this analysis were represented as Log values for comparison purposes.

**Table 2.10 Primer Pairs for Real-Time TaqMan™ qPCR**

Primer Pair	Part No/Assay ID	[Stock]	Company	Amplification length
(GAPDH) Glyceraldehyde-3-phosphate dehydrogenase	4352932E	20X	Applied Biosystems	-
Eukaryotic 18S ribosomal RNA	4352930E	20X	Applied Biosystems	-
Recoverin	Mm00501325_m1	20X	Applied Biosystems	80

(Nrl) Neural Retinal Leucine Zipper	Mm00476550_m1	20X	Applied Biosystems	61
(Crx) cone-rod homeobox	Mm00483994_m1	20X	Applied Biosystems	74
Sox2	Mm00488369_s1	20X	Applied Biosystems	68
(Gap43) growth associated protein 43	Mm00500404_m1	20X	Applied Biosystems	64
$\beta$ -III tubulin	Mm00727586_s1	20X	Applied Biosystems	64
Dct	Mm00494456_m1	20X	Applied Biosystems	72
Mitf	Mm00434954_m1	20X	Applied Biosystems	78

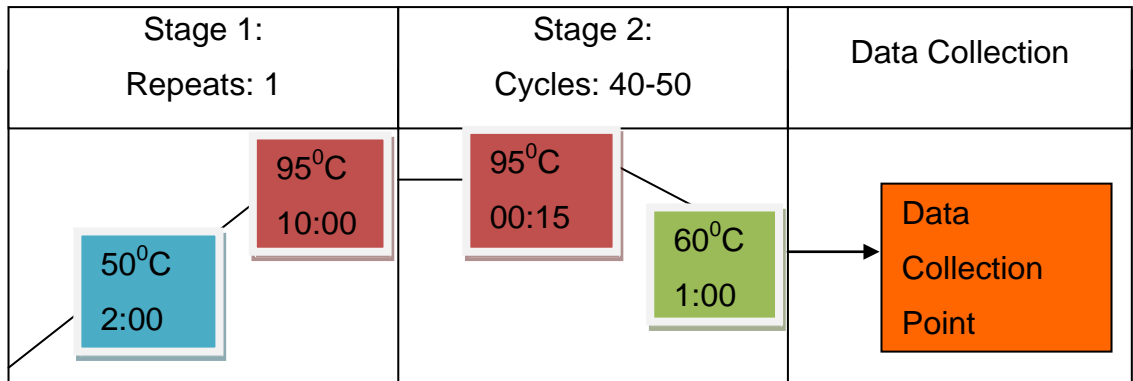


GFAP	Mm01253034_m1	20X	Applied Biosystems	64
Arrestin 3	Mm01227032_m1	20X	Applied Biosystems	69
Silver	Mm00498996_m1	20X	Applied Biosystems	65
ApoE	Mm00437573_m1	20X	Applied Biosystems	66
Cyclin D1	Mm00432359_m1	20X	Applied Biosystems	71
Cyclin D3	Mm03053314_g1	20X	Applied Biosystems	172
p27 <sup>Kip1</sup>	Mm00438168_m1	20X	Applied Biosystems	81

(PTEN) Phosphatase and Tensin homolog	Mm00477210_m1	20X	Applied Biosystems	84
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**Table 2.11 Re-Ti PCR Master Mix using TaqMan™**

Reagent	Volume/reaction	Final concentration
Master Mix (2X). It contains AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTP mix with dUTP [10mM each], 25mM MgCl in Mg <sup>+2</sup> -free buffer solution.	10 µl	1X
Primer pair (20X)	1 µl	1X
cDNA	1-5 µl	variable
DepC-treated H <sub>2</sub> O	4-8 µl	-
Total	20 µl	-

**Figure 2.2 Quantitative Real-Time PCR using TaqMan™**

### 2.16.2 SYBR™ Green qPCR

Genomic DNA samples were used as templates for quantitative real-time polymerase chain reaction (Re-Ti-PCR) on a 7500 Fast Real-Time PCR™ System, using SYBR™ Green (Qiagen, UK) according to the manufacturer's protocol for genotyping analysis (detection of *Nrl.gfp* transgene in genomic tail-snip DNA, Tables 2.12 and 2.13). Quantitative Real Time PCR (RT PCR) stages were: (i) denaturation stage, 1 cycle at 95°C for 15 minutes, (ii) amplification stage, 40 cycles of 94°C for 15 seconds, then at 55°C for 30 seconds and 72°C for 30 seconds (Figure 2.3). Data collection was set at the amplification stage in each cycle. *Gapdh* was used as an endogenous control. Relative quantities of the target alleles were normalized against the endogenous control by subtracting CT (threshold cycle) values for *Gapdh* from CT values for copy number analysis, and then subjected to normalisation by comparing with standard samples expressing the alleles of interest with either 3 or 6 copies of GFP. Relative expression values were calculated as  $2^{-\Delta CT}$  and fold differences were calculated using the  $2^{-\Delta\Delta CT}$ . The Re-Ti PCR values obtained from this analysis were represented in as Log values for comparison purposes. The same method was used for gene expression assays performed for *Gfp* mRNA from cDNA samples.

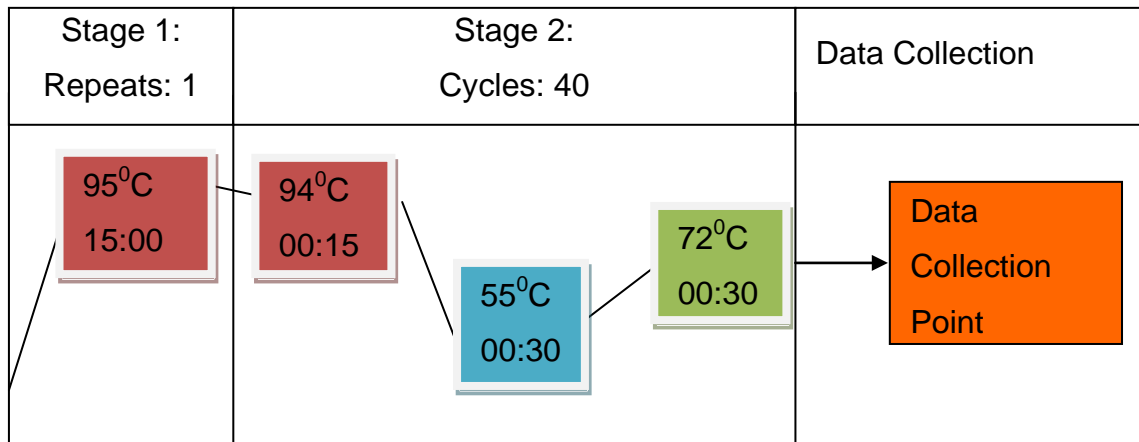
**Table 2.12 Primer Pairs for Real-Time SYBR™ Green qPCR**

Primer Pair	Part No/Assay ID	[Stock]	Company	Amplification length
GAPDH	Forward: 5'- ATGACATCAAGAAGG TGGTG-3', Rev: 5'- CATACCAGGAAATGA GCTTG-3'	10X	Qiagen, UK	102
eGFP	eGFP_1_SQ	10X	Qiagen, UK	85

**Table 2.13 Re-Ti PCR Master Mix using SYBR™ Green**

Reagent	Volume/reaction	Final concentration
Master Mix (2X). It contains SYBR™, dNTP mix with dUTP [10mM each], 25mM MgCl in Mg <sup>+2</sup> -free buffer solution.	10 µl	1X
Primer pair (20X)	1 µl	1X
gDNA	1 µl	variable
DepC-treated H <sub>2</sub> O	8 µl	-
<b>Total</b>	<b>20 µl</b>	<b>-</b>

**Figure 2.3 Quantitative Real-Time PCR using SYBR™ Green**



## **2.17 Statistical analysis**

### **2.17.1 Statistical analysis software**

Statistical packages used were GraphPad™ Prism 4.0 and Microsoft (XP, 2003) Excel for obtaining Mean values, Standard Deviation (SD), Standard Error of Mean (SEM) and graphical representation. For testing statistical tools SPSS 12.0.1 software was used and one-way Analysis of Variance (one-way ANOVA) was selected as the most appropriate one for these experiments over Student's t-test. Dunnett's correction was used as an Ad-Hoc. The reason is that by comparing means from three different sources as a whole, this overcomes the possible error of performing multiple t-tests between each group of means. In this way, when t-tests would give many p values between groups, one-way ANOVA will pool all the means into one group and give one p value. The use of the post-hoc analysis gave individual p values between samples. Where a non-parametric distribution was prominent, a Mann-Whitney test was used for obtaining statistical significance and p values.

### **2.17.2 Independent samples T-Test**

A t-test is any statistical hypothesis test in which the test statistic has a distribution arising from the mean value of a normally distributed small sample size population if the null hypothesis stands (meaning that there is no statistical significance, unless there is a high degree of confidence, more than 95%). Therefore, if the statistical approach fails to reject the null hypothesis then the latter stands and no statistical significance is stated. The statistical significance will arise from the comparison between two sample means.

When the same variance is assumed, the  $t$  statistic to test whether the means are different can be calculated as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_{\bar{X}_1 - \bar{X}_2}} \text{ where } s_{\bar{X}_1 - \bar{X}_2} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}$$

Where  $s^2$  is the unbiased estimator of the variance of the two samples,  $n$  = number of participants, 1 = group one, 2 = group two.  $n - 1$  is the number of degrees of freedom for either group, and the total sample size minus 2 is the total number of degrees of freedom, which is used in significance testing. The degrees of freedom used in significance testing are  $n_1 + n_2 - 2$  (Press W.H. 1999).

### 2.17.3 One-Way Analysis of Variance (ANOVA)

For the analysis of differences between more than two groups of specimens, which were differentiated from each other by only one factor, the One-Way ANOVA test was employed. This test is based on the differences in means between various groups. An example of a One-Way ANOVA table produced by SPSS is illustrated in Table 2.14 and the mathematical formulae explained.

**Table 2.14 Example of a One-Way ANOVA table produced by SPSS**

	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
Between Groups	$\frac{\sum (\sum \text{observed values in a treatment group})^2 - (\sum \text{observed values})^2 / n. \text{ observations}}{n. \text{ groups}}$	$n. \text{ groups} - 1$	$\frac{\text{sum of squares}_{\text{between groups}}}{df_{\text{between groups}}}$	$\frac{\text{mean square}_{\text{between groups}}}{\text{mean square}_{\text{within groups}}}$	P-value for difference between groups
Within Groups	$\frac{\sum (\text{observed value})^2 - \sum (\sum \text{observed values in a treatment group})^2}{n. \text{ observations} - n. \text{ groups}}$	$n. \text{ observations} - n. \text{ groups}$	$\frac{\text{sum of squares}_{\text{within groups}}}{df_{\text{within groups}}}$		
Total	$\frac{\sum (\text{observed value})^2 - (\sum \text{observed values})^2 / n. \text{ observations}}{n. \text{ observations} - 1}$	$n. \text{ observations} - 1$			

Key: n, number of; df, degrees of freedom; Sig, significance.

### 2.17.4 Mann-Whitney U test

The Mann-Whitney U is a non-parametric test (i.e. a normal distribution of values within a population is not assumed). It requires the two samples to be independent and the observations to be ordinal. In this thesis, I used this test when at least one outlier was found in one of the samples being examined and thus the ANOVA (with post-hoc) and T-test were not able to give a valid p value.



**Chapter 3 – Isolation of Retinal Progenitors from the Adult  
orJ Central Neural Retina**

### **3.1 Introduction**

The adult mammalian eye possesses a retinal stem cell niche in the CE of the CB but its function *in vivo* is still unclear (Ahmad I 2000; Tropepe V 2000). By culturing *in vitro* with specific growth factors, a small percentage of cells from the CE form multicellular spheres that quickly increase in size owing to cell division. Conditions for neurosphere formation were established in 1992 by culturing brain-derived stem cells and were found to also apply for proliferation of progenitor populations (Reynolds BA 1992; Reynolds BA 1992). This rare cell population in the CE, appears mitotically quiescent *in vivo* and proliferates extensively upon isolation and mitogen administration, whilst gaining differentiation capability when no mitogen is present and substantial amount of serum is added (Ahmad I 1999).

In lower vertebrates the CMZ at the periphery of the retina, is a retinal stem cell niche and produces new retinal neurons throughout life. The analogous region in mammals is the CB, in respect to adult stem/progenitor cell residing territory. To date, no reports indicate an equivalent peripheral retinal region resembling the CMZ in mammals. Recent literature tends to describe two different cell populations; RSC and RPC, and of those (Cepko CL 1996; Reh TA 1998; Ahmad I 2000; Tropepe V 2000; James J 2004; Zaghloul NA 2005), the latter is purported to derive from asymmetric proliferation of the former (Zhong W 2003). However, there is limited evidence of either specific cell markers or assays that permit the distinction between these two cell types (Bhattacharya S 2003; Das AV 2005).

RSC appear to persist during adulthood in the CE and when stimulated by growth factors *in vitro*, proliferate and show properties of multipotentiality and self-renewal (Ahmad I 2000; Tropepe V 2000). Although retinal stem or

progenitor cells have not been isolated from the adult mouse CNR (Tropepe V 2000; Coles BL 2006), our laboratory recently showed that a population of dividing cells persist *in vivo* in the CNR of the  $Chx10^{orJ/orJ}$  mouse (Dhomen NS 2006). Here, I sought to determine if the dividing cells in the  $Chx10^{orJ/orJ}$  CNR are a population of retinal progenitor cells by comparing their behaviour to the previously characterised CE RSC (Ahmad I 2000; Tropepe V 2000).

## **3.2 Results**

### **3.2.1 Embryonic retina can give rise to neurospheres *in vitro***

The potential of the embryonic mammalian wildtype and  $\text{Chx10}^{\text{orJ/orJ}}$  retinas to give rise to neurospheres *in vitro* was investigated at two embryonic stages, a developmentally early retinal stage (E11.5, Figure 3.1) and a late one (E18.5, Figure 3.2). At E11.5, wildtype and  $\text{Chx10}^{\text{orJ/orJ}}$  retinas resemble each other phenotypically with a similar eye size and pigmentation (Compare Figures 3.1b and 3.1d). At E18.5, lack of Chx10 has produced a severe phenotype with a thinner retina close to the lens vesicle (compare Figures 3.2b and 3.2d). The eye is considerably smaller at this stage than wildtype.

Cells that were enzymatically dissociated from the wildtype E11.5 and E18.5 retinas and cultured for 7 days in FGF-2/EGF/N2-enriched medium (NGM) consistently gave rise to spheroid structures with a diameter of up to 30  $\mu\text{m}$  with the majority of them showing an adherent morphology (Figures 3.1a and 3.2a, respectively). Upon immunolabelling with an antibody against the neuronal stem/progenitor marker nestin, few positive cells were identified at late retinal developmental stages in the wildtype retina (E18.5, Figure 3.2a). Similarly, some retinal cells from E11.5 and E18.5  $\text{Chx10}$ -deficient retinas gave rise to spheroids upon similar *in vitro* culture conditions (Figures 3.1c and 3.2c, respectively). Interestingly, cells isolated from the  $\text{Chx10}^{\text{orJ/orJ}}$  central and peripheral retina/presumptive CE had a neurosphere-like phenotype, although were still adherent (Figure 3.2c). Mutant spheres obtained from E18.5 retinas had a clearly demarcated outline compared to the more loosely aggregated cell clusters observed from the wildtype E18.5 cultures. Finally, immunolabelling with  $\alpha$ -Nestin showed an increased expression of this protein in comparison to wildtype E18.5-derived retinal spheroids.

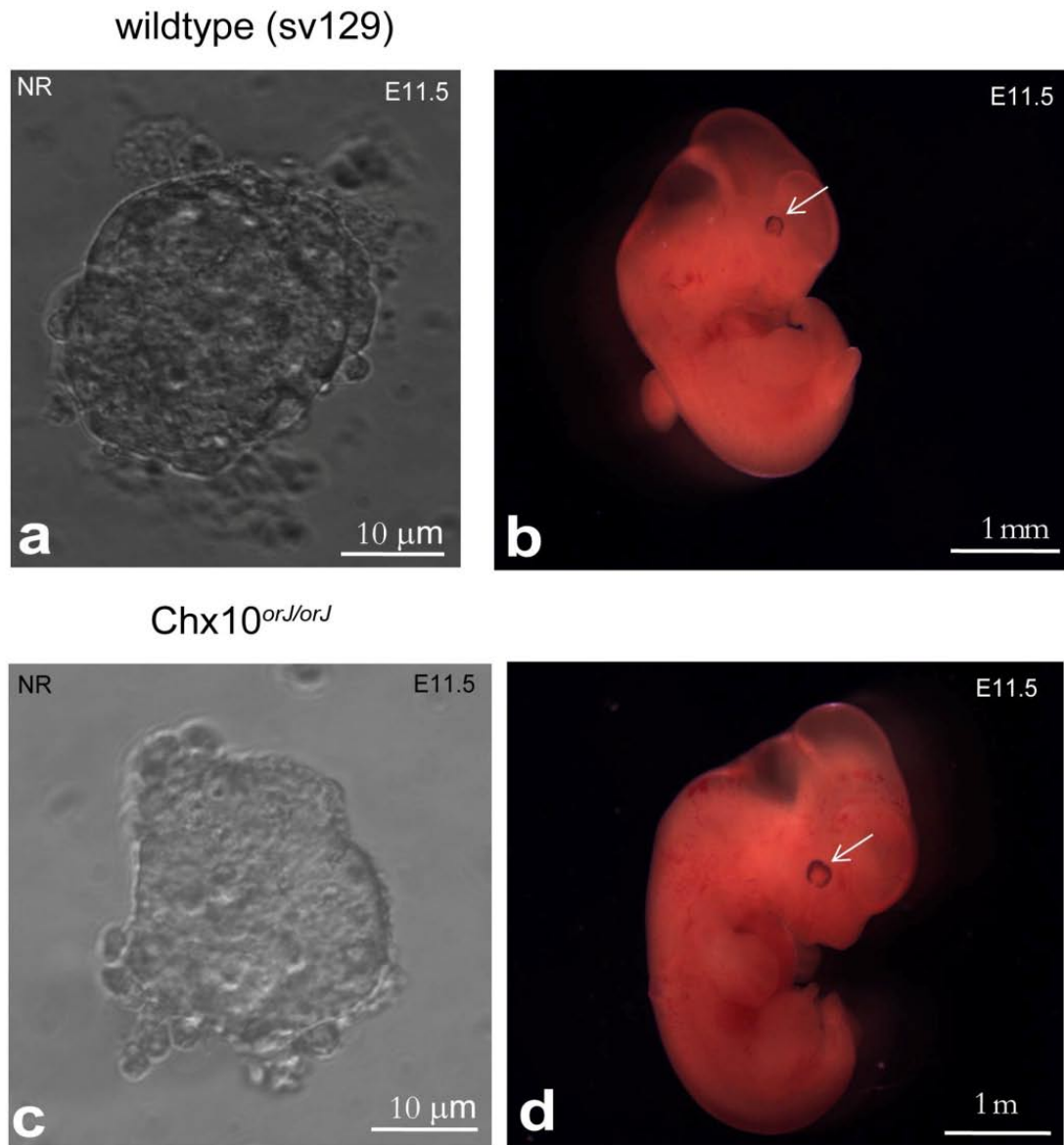


Figure 3.1 Neurosphere potential in the developing wildtype and mutant murine retina

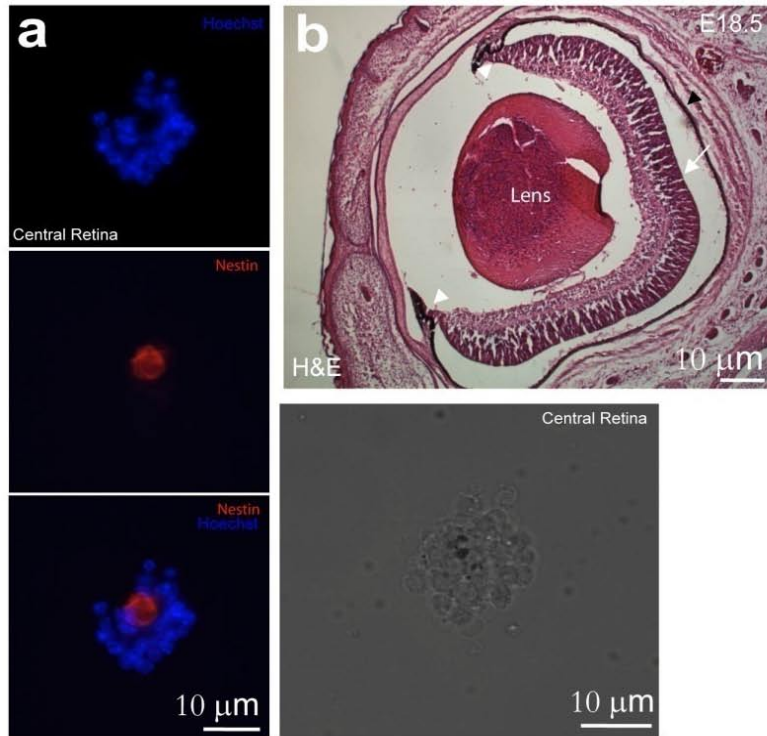
(a) Cells isolated from the presumptive neural retina and cultured in Neurosphere Growth Medium (NGM) giving rise to small adherent spheroid structures.

(b) Neurospheres from E11.5 wildtype (sv129) mouse. Photograph of a wildtype embryo.

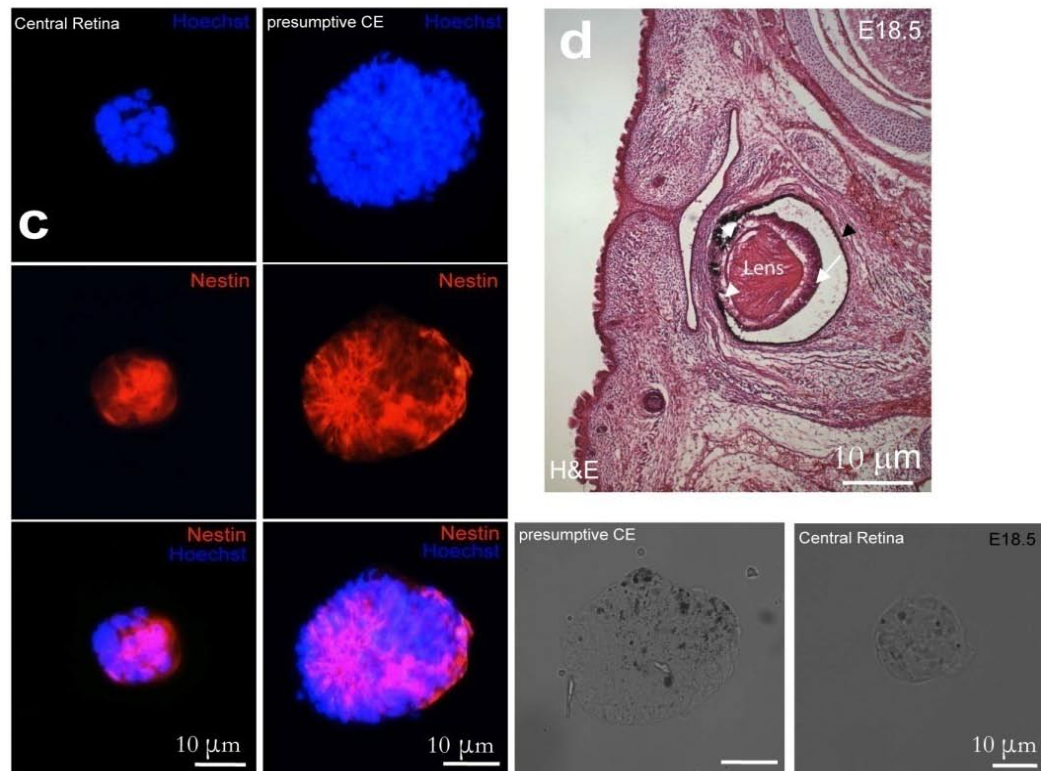
(c) Cells isolated from the presumptive neural retina and cultured in NGM giving rise to small adherent spheroid structures.

(d) Neurospheres from E11.5  $Chx10^{orJ/orJ}$  mouse. Photograph of a  $Chx10^{orJ/orJ}$  embryo. At this developmental stage there is no phenotypic alteration in the eye in comparison with E11.5 wildtype.

wildtype (sv129)



*Chx10<sup>or/J</sup>orJ*



**Figure 3.2. Neurosphere potential in the developing Wildtype and  $Chx10^{orJ/orJ}$  murine retina**

(a) Neurospheres from E18.5 wildtype (sv129) mouse. Photomicrograph of a Haematoxylin and Eosin staining of a wildtype saggital eye cryosection. The RPE (black arrowheads), presumptive CE (bold white arrowheads) and the retinal neuroblastic layer (white arrows) are visible.

(b) Cells isolated from the presumptive neural retina and cultured in NGM giving rise to small neurosphere-like structures which contain cells positive for the neural stem/progenitor marker Nestin.

(c) Neurospheres from E18.5  $Chx10^{orJ/orJ}$  mouse. Microphotograph of a Haematoxylin and Eosin staining of a  $Chx10^{orJ/orJ}$  embryo. The RPE (black arrowheads), presumptive CE (bold white arrowheads) and the retinal neuroblastic layer (white arrows). Neural retina is visible with a poor lamination in comparison to E18.5 wildtype eye. The eye as a whole is less than half in size and deeper within the skull than wildtype.

(d) When cells isolated from the presumptive neural retina and cultured in NGM giving rise to small neurosphere-like structures which contain cells positive for the neural stem/progenitor marker Nestin.

N=2 independent experiments.

### 3.2.2 Cells from the adult $\text{Chx10}^{\text{orJ/orJ}}$ central neural retina can form neurospheres *in vitro*

The adult murine eye is similar to the adult human eye, although the lens structure in the latter is proportionally smaller to the eye than the former. As Figure 3.3a indicates schematically, the neural retina is a thin structure posterior to the lens with the RPE adjacent and posterior to the former. The bilayered CE structure within the CB, resides in between, adjacent to the Iris and peripheral neural retina. Eyes dissected out from the adult wildtype and  $\text{Chx10}$ -deficient mice show considerable phenotypic differences with the latter being smaller in size, hypomorphic, lacking an optic nerve. A hemisection indicates a hypocellular retina and an expanded CB, characteristic of the  $\text{Chx10}^{\text{orJ/orJ}}$  eye (Figure 3.3b).

As a consequence of the developmental defect due to the lack of functional  $\text{Chx10}$  protein, the mutant murine adult eye is considerably smaller in size and fails to open the eyelids (Figure 3.4d). I investigated the possibility that adult  $\text{Chx10}$ -deficient retinal cells from the CNR can give rise to neurospheres *in vitro*, using wildtype CE- and CNR-derived as well as  $\text{Chx10}^{\text{orJ/orJ}}$  CE cells as controls (Figure 3.4b). By dissociating and culturing cells from these two regions and phenotypes under similar conditions, a fraction of cells derived from the  $\text{Chx10}^{\text{orJ/orJ}}$  CNR were shown to have the ability to form neurospheres *in vitro* while wildtype CNR cells never gave rise to a neurosphere *in vitro* (Figure 3.4b-e). Neurospheres could also be readily obtained from wildtype and  $\text{Chx10}^{\text{orJ/orJ}}$  CE cultures. Using an antibody against  $\text{Chx10}$  protein it was confirmed that the latter, in the adult, is only expressed in the NR of the wildtype and not in the CE of both mutant and wildtype, nor in the mutant retina (Figure 3.4b-e).



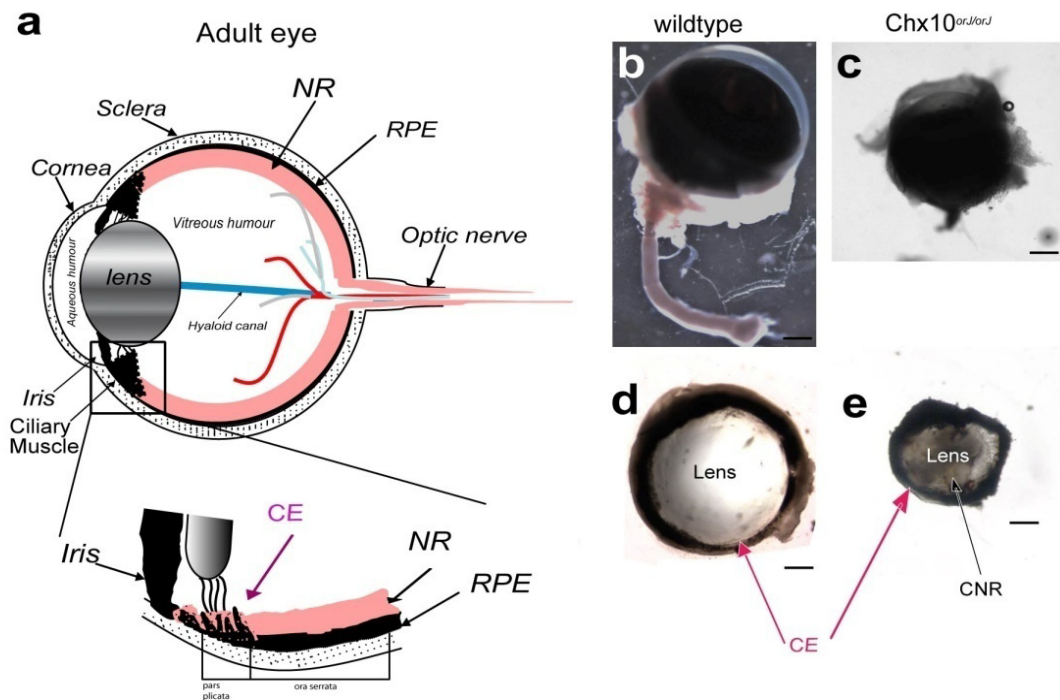


Figure 3.3 Murine adult wildtype and  $Chx10^{orJ/orJ}$  retina

(a) Schematic representation of a sagittal section of the adult murine eye. The adult eye is comprised by non-neuronal tissues such as the Cornea, Sclera and the Lens, while of neuroectodermal origin but non-neuronal are the iris and the CB as well as the RPE. Neuronal tissues are the retina along with the optic nerve. Magnification in the region where the RPE, peripheral retina and iris meet and next to the CB. Zonule of Zinn (Ciliary zonule) connects the CB with the lens. Ora serrata is the edge (serrate) of the retina located next to the CB. The Ciliary Epithelium is considered analogous to the CMZ region found in teleost and is comprised of an interleaved pigmented and non-pigmented layer, extensions of RPE and neural retinal tissue, respectively. The pigmented CE region adjacent to the iris tissue is called the pars plicata while the posterior region connected to the neural retina is called pars plana in humans, while mice have a miniature analogous region.

(b-e) Adult wildtype and  $Chx10^{orJ/orJ}$  whole and dissected eyes. The wildtype adult eye (b) dissected with surrounding mesenchyme removed is twice in size in comparison to the mouse  $Chx10^{orJ/orJ}$  adult eye (c) which has a malformed optic nerve. A posterior view of the anterior segment of the eye obtained by equatorial incision posterior to the ciliary muscle plane of dissection shown by red arrow, it can be clearly seen the CE in wildtype (d). In mutant eyes, the incision is performed more towards the centre of the ocular region since lens is located more centrally (e). It can be noticed the expanded CE region and the diminished retina which is attached to the back of the lens in the mutant.

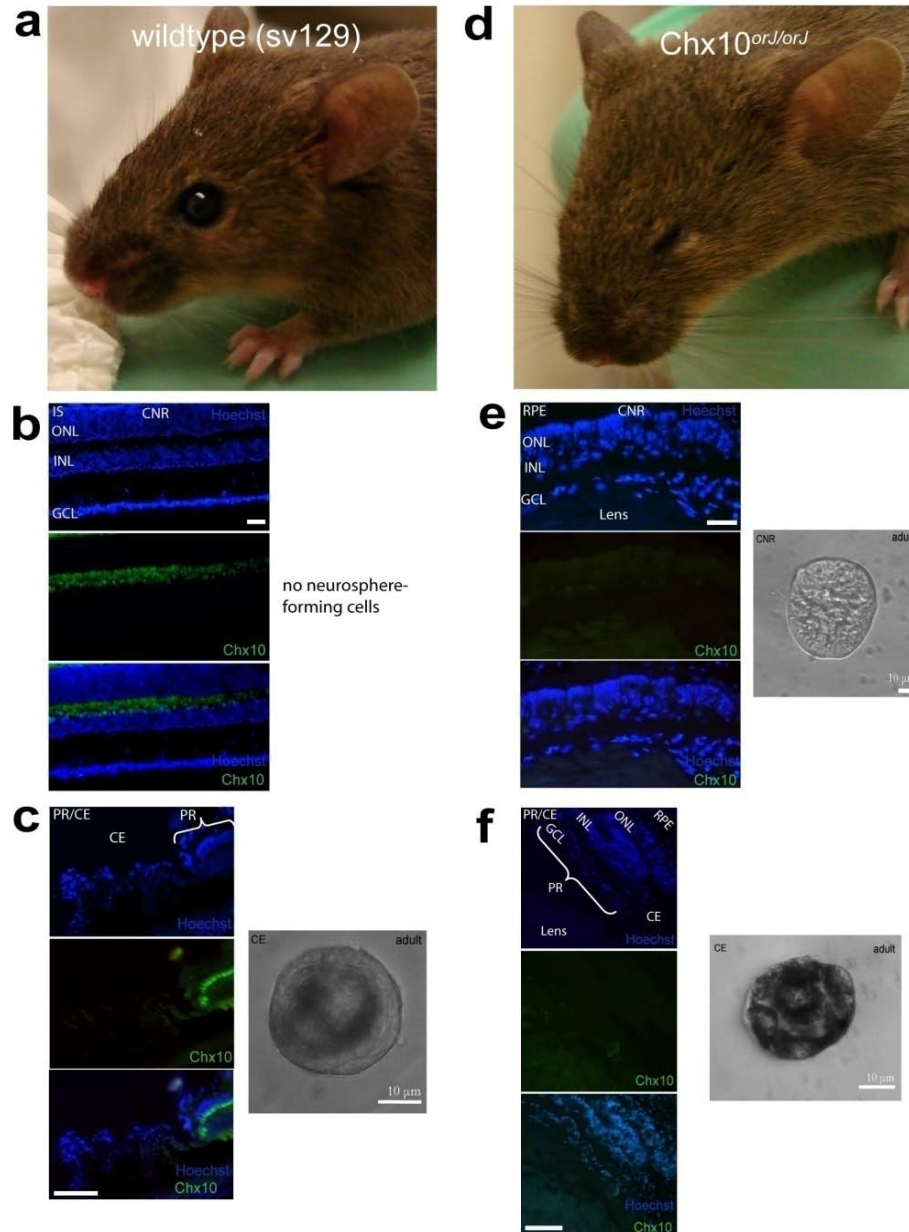


Figure 3.4 Neurospheres are formed from the adult *Chx10<sup>orJ/orJ</sup>* but not from the wildtype CNR

(a) The wildtype (SV129) adult mouse. Photograph of an adult mouse with normal eye.

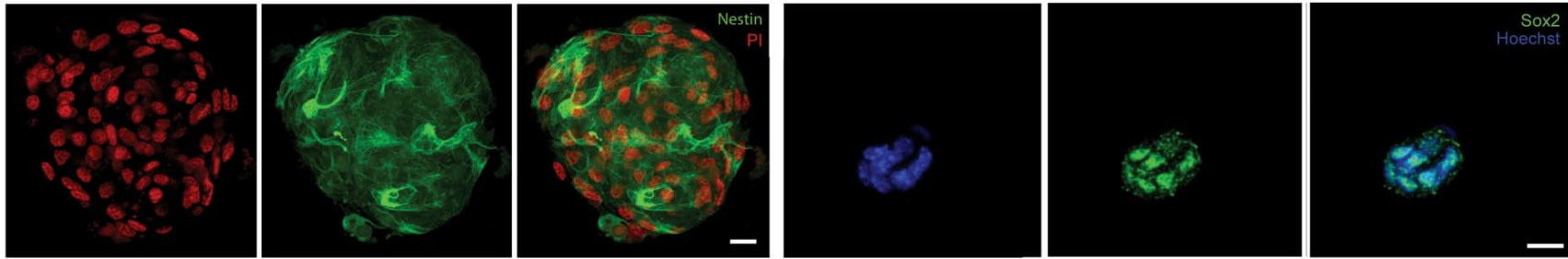
(b-c) Immunolabelling with  $\alpha$ -Chx10 shows the presence of the protein only in the wildtype central and peripheral retina (b) but not in the ciliary epithelium (c). Cells isolated from the wildtype CNR do not give rise to neurospheres, while cells isolated from the wildtype CE form neurospheres when cultured in NGM.

(d) The *Chx10<sup>orJ/orJ</sup>* adult mouse. Photograph of a microphthalmic adult mouse. The mouse is blind and the eyelids are closed over the microphthalmia.

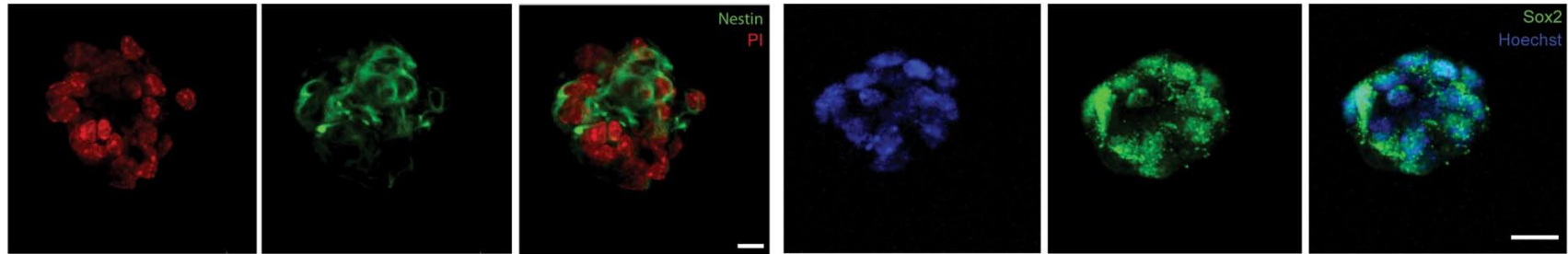
(e-f) Immunolabelling with  $\alpha$ -Chx10 shows the presence of the protein only in the wildtype central and peripheral retina (e) but not in the CE (f). Cells isolated from the *Chx10*-deficient CNR and CE give rise to neurospheres, when cultured in NGM.

To further characterise the newly-discovered neurosphere-forming cells, indirect immunocytochemistry was performed with the neural stem/progenitor markers Nestin and Sox2 neurospheres from wildtype CE and Chx10<sup>orJ/orJ</sup> CE and CNR grown for 7 days *in vitro* (Figure 3.5). Wildtype CE-derived neurospheres showed a strong expression of both these markers as well as the Chx10-deficient CE ones (Figure 3.5a, b). The expression of these neural progenitor markers was also strong in the Chx10-deficient CNR derived neurospheres (Figure 3.5c). Similarly, the expression of Pax6, a retinal-specific stem/progenitor marker was strong on wildtype and Chx10<sup>orJ/orJ</sup> CE as well as Chx10<sup>orJ/orJ</sup> CNR derived neurospheres (Figure 3.6a, b and c, left column). The expression of Chx10 protein was, as expected, restricted to wildtype CE-derived neurospheres with no expression observed in the Chx10<sup>orJ/orJ</sup> CE- and CNR-derived neurospheres (Figure 3.6a, b and c, middle column). Finally, to assess the differentiated state of the neurosphere-forming cells by day 7 *in vitro*, I immunolabelled with anti- $\beta$ -III tubulin, a marker of early-born neural cells/retinal ganglion cells. Rarely, cells were found positive for this marker on all different neurosphere cell sources (Figure 3.6a, b and c, right column). Immunostaining experiments were performed on at least 4 different neurosphere preparations. Data shown is representative examples of each.

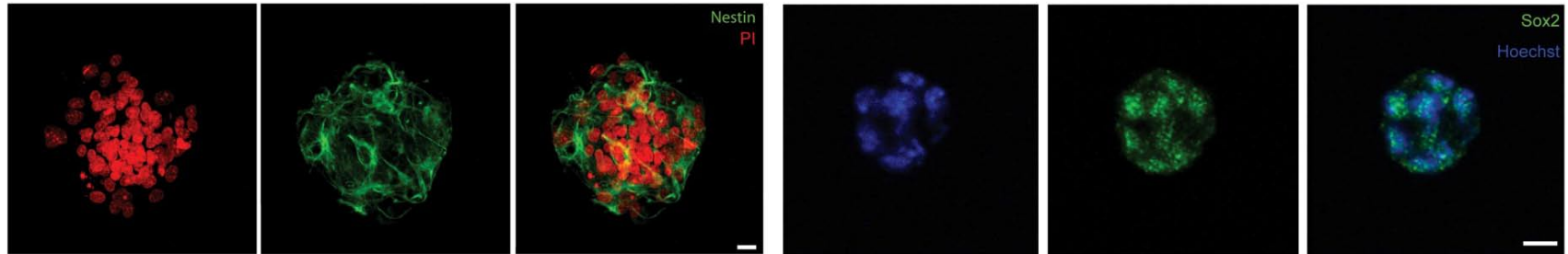
(a) adult wildtype CE



(b) adult *Chx10<sup>orJ/orJ</sup>* CE



(c) adult *Chx10<sup>orJ/orJ</sup>* CNR



**Figure3.5. Neurospheres from Chx10-deficient adult central neural retina express neural stem/progenitor markers.**

**(a)** Cells isolated from the CE were allowed to form neurospheres for 7 days *in vitro* in NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for neural stem/progenitor markers Nestin and Sox2.

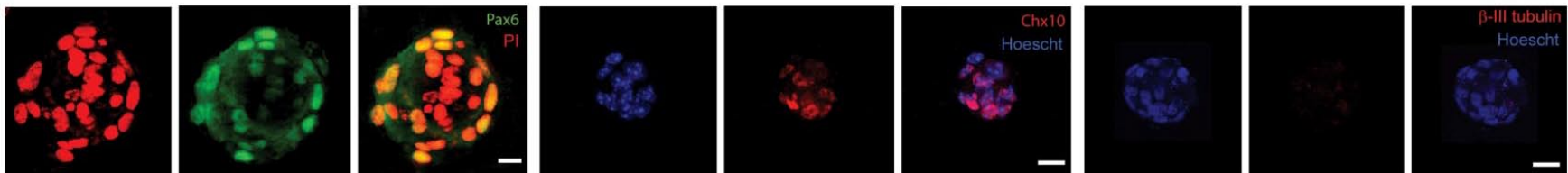
**(b)** Chx10<sup>orJ/orJ</sup> adult CE neurosphere-forming cells are positive for neural stem/progenitor markers. Cells isolated from the CE were allowed to form neurospheres for 7 days *in vitro* in NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for neural stem/progenitor markers Nestin and Sox2.

**(c)** Chx10<sup>orJ/orJ</sup> adult CNR neurosphere-forming cells are positive for neural stem/progenitor markers. Cells isolated from the CNR were allowed to form neurospheres for 7 days *in vitro* in NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for neural stem/progenitor markers Nestin and Sox2. Scale bars; 10  $\mu$ m.

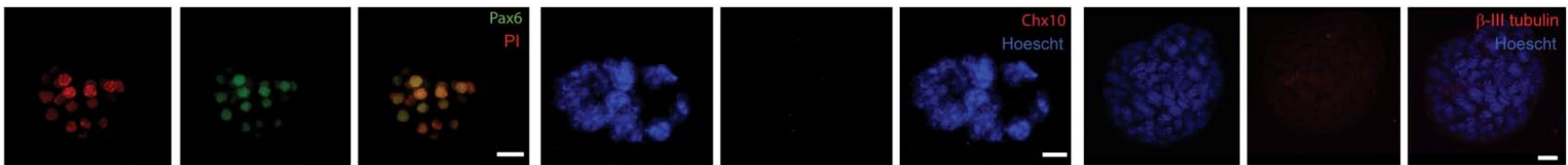
N=4-5 independent experiments.



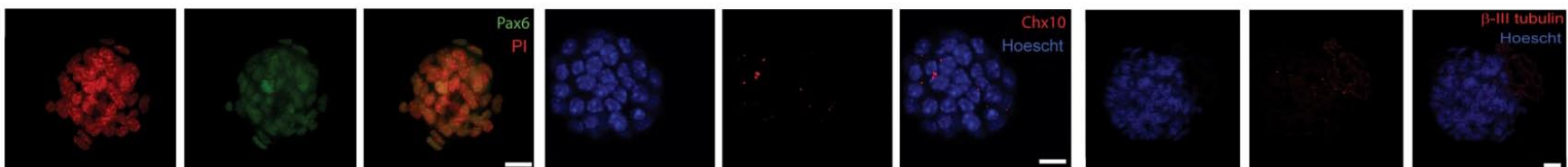
(a) adult wildtype CE



(b) adult Chx10<sup>orJ/orJ</sup> CE



(c) adult Chx10<sup>orJ/orJ</sup> CNR



**Figure 3.6. Neurospheres from Chx10-deficient adult central neural retina express retinal stem/progenitor markers and negative for early neuronal markers.**

**(a)** Cells isolated from the CE were allowed to form neurospheres for 7 days *in vitro* in NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for retinal stem/progenitor markers Chx10 and Pax6 but negative or low expression of  $\beta$ -III tubulin, a protein expressed in early neuronal/retinal ganglion cells.

**(b)**  $Chx10^{orJ/orJ}$  adult CE neurosphere-forming cells are positive for retinal stem/progenitor markers. Cells isolated from the CE were allowed to form neurospheres for 7 days *in vitro* in NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for retinal stem/progenitor marker Pax6 but negative for Chx10 as expected, and low  $\beta$ -III tubulin expression, a protein expressed in early neuronal/retinal ganglion cells.

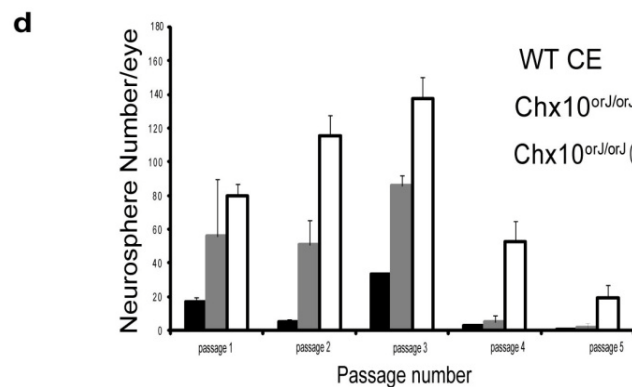
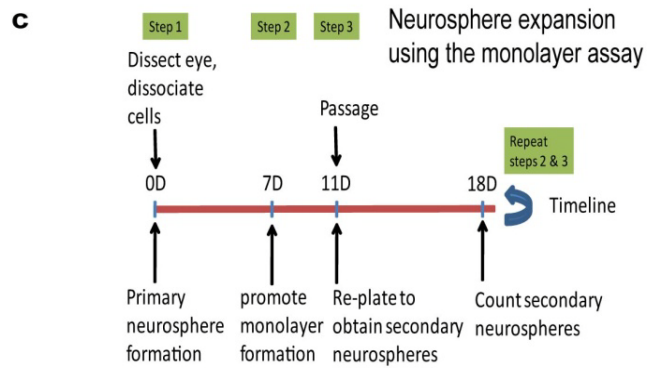
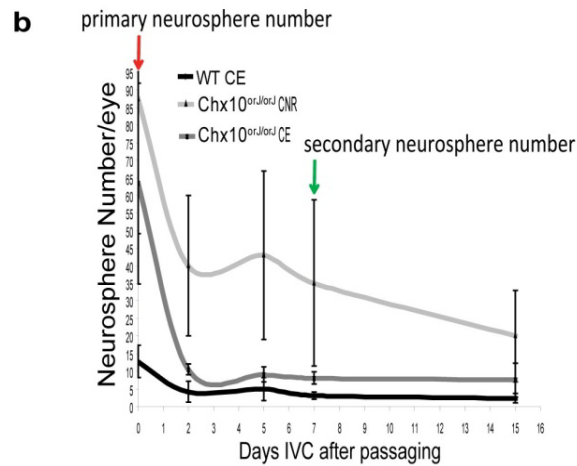
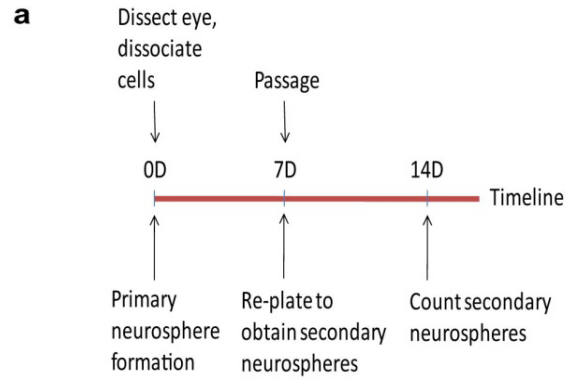
**(c)**  $Chx10^{orJ/orJ}$  adult CNR neurosphere-forming cells are positive for retinal stem/progenitor markers. Cells isolated from the CNR were allowed to form neurospheres for 7 days *in vitro* in NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for retinal stem/progenitor marker Pax6 but negative for Chx10 as expected, and low  $\beta$ -III tubulin expression, a protein expressed in early neuronal/retinal ganglion cells. Scale bars; 10  $\mu$ m.

N=4-5 independent experiments.

### 3.2.3 Cells from the adult Chx10<sup>orJ/orJ</sup> central neural retina behave as progenitor/stem cells *in vitro*

Following the observation of the newly discovered neurosphere population and its strong expression of neural and retinal stem/progenitor proteins, the next step was to assess if neurosphere-forming cells were behaving as progenitors in the culture system. The self-renewal potential was investigated by using two different *passaging* techniques; initially, cells were *passaged* directly after primary neurosphere formation with trypsin (Figure 3.7a), but neurospheres from both regions and genotypes were difficult to *passage* and the secondary neurosphere formation outcome was poor (Figure 3.7b). Also, obtaining tertiary neurosphere formation was difficult. The second method included an addition in the timeline, between neurosphere formation and *passaging*, of a 4-day monolayer formation step in low serum conditions (1% FCS), in the presence of growth factors (Figure 3.7c). This additional step promoted the formation of an increased number of neurospheres, but only transiently, which after the fourth *passage* started to diminish (Figure 3.7d). Interestingly, neurosphere-forming cells deriving from the Chx10-deficient central neural retina consistently showed a higher secondary neurosphere formation and a better response to neurosphere formation after prolonged *passages*.



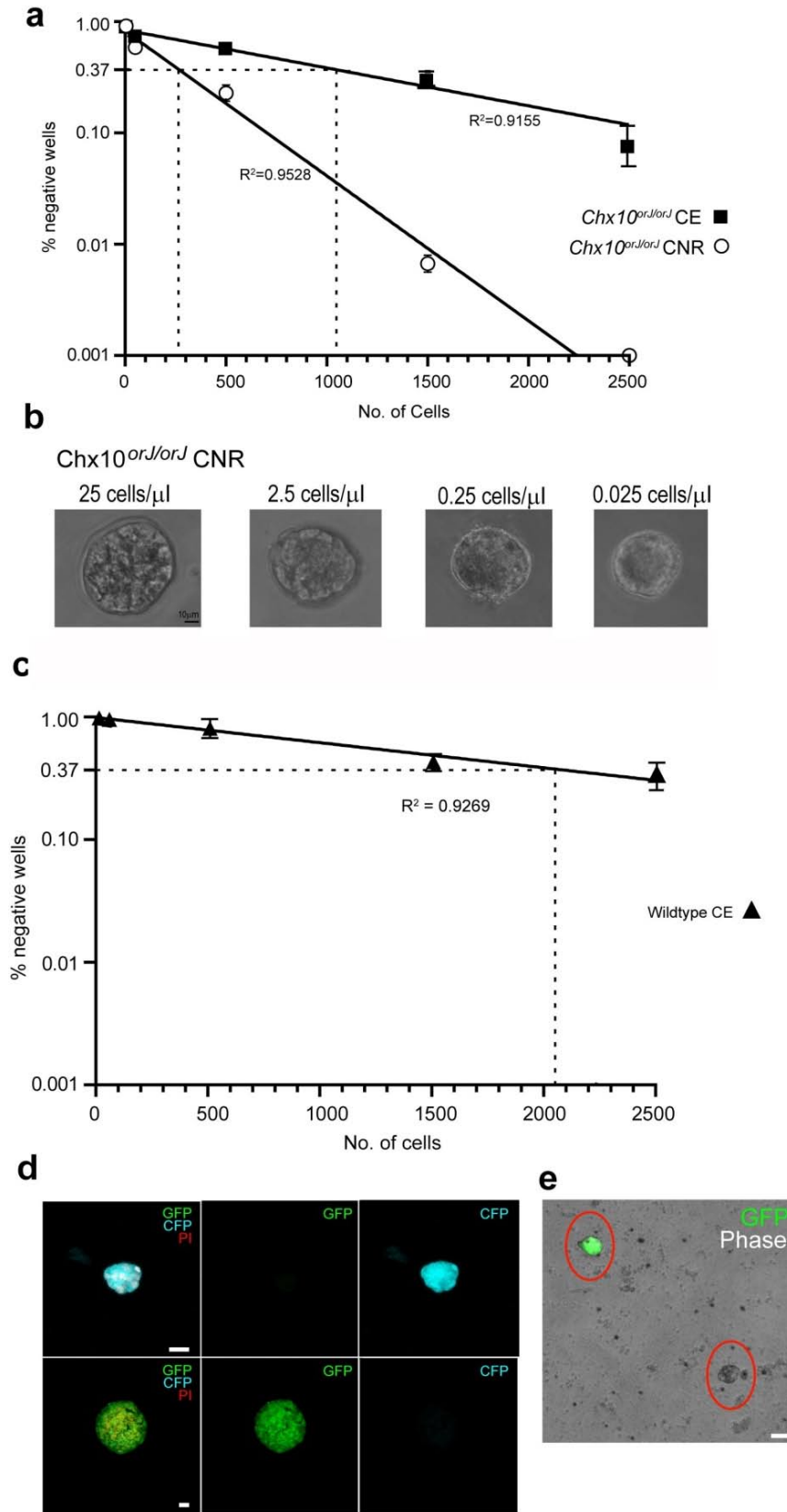


**Figure 3.7 Comparison of self-renewal capability and stem cell frequency between wildtype CE and  $Chx10^{orJ/orJ}$  CE and CNR tissues.**

**(a)** Timeline of neurosphere self-renewal assay from primary neurosphere formation assay. Adult wildtype and  $Chx10$ -deficient CE and CNR regions were dissected out and cells were enzymatically dissociated. Cells were allowed to form primary neurospheres for 7 days in NGM before *passaging* to reach single cell level. Cells were then replated in NGM and allowed to form secondary neurospheres for further 7 days. Secondary neurosphere number was counted 14 days after initial dissociation. **(b)** Neurosphere-forming cells from the  $Chx10^{orJ/orJ}$  CNR have a better self-renewal capability after one passage than wildtype and  $Chx10^{orJ/orJ}$  CE ones. Neurospheres formed from 7 days IVC in NGM of cells isolated from wildtype and  $Chx10^{orJ/orJ}$  CE (black line and dark grey line, respectively) and  $Chx10^{orJ/orJ}$  CNR (light grey line) were *passaged* down to single cell level allowed to form secondary spheres for 14 days in NGM; N=3, error bars; SEM. Neurosphere number was counted 7 days after passaging (green arrow), to compare with primary neurosphere formation (red arrow). **(c)** Timeline of neurosphere self-renewal assay from monolayer cultures. Adult wildtype and  $Chx10$ -deficient CE and CNR regions were dissected out and cells were enzymatically dissociated. Dissociated cells were allowed to form primary neurospheres for 7 days in NGM. An additional step was added (Step 2) with the addition of low FCS (1%) to promote a better passaging yield before trypsinising to reach single cell level. Cells were then replated in NGM and allowed to form secondary neurospheres. Secondary neurosphere number was counted 14 days after initial dissociation. **(d)** Neurospheres from the  $Chx10^{orJ/orJ}$  CNR and CE show a better self-renewal capability after serial *passaging* in comparison to wildtype ones. Neurospheres formed from 7 days IVC in NGM of cells isolated from WT and  $Chx10^{orJ/orJ}$  CE (black bar and dark grey bar, respectively) and  $Chx10^{orJ/orJ}$  CNR (white bar) were plated for 4 days in low-serum levels (1% FCS) and then passaged down to single cell. They

were then allowed to form secondary spheres for further 7 days; N=4-5, error bars; SEM.

Adult progenitor cells from the central retina lacking *Chx10* showed a higher self-renewal potential in comparison to their wildtype and *Chx10<sup>orJ/orJ</sup>* counterparts. Next, a limiting dilution assay (LDA), commonly used in haematopoietic stem cell biology, and recently used in adult CE RSC (Tropepe V 2000), was performed. The concept of this is to assess if the neurosphere formation is a cell- or a non-cell autonomous condition and the frequency of stem cells (neurosphere-forming cells) within a tissue. These experiments confirmed a higher number of neurosphere-forming cells present in the *Chx10<sup>orJ/orJ</sup>* CNR compared with either the *Chx10<sup>orJ/orJ</sup>* or wildtype CE; 1 per 280 cells (0.35%) plated for CNR; 1 per 1,050 cells (0.1%) for *Chx10<sup>orJ/orJ</sup>* CE (Figure **3.8a**); and 1 per 2,050 cells (0.05%) for wildtype CE (Figure **3.8c**). It was rarely possible to obtain neurospheres from cells plated at a density of 1 cell per well, even in the *Chx10<sup>orJ/orJ</sup>* CNR derived cultures. Neurospheres of similar size were obtained at densities as low as 5 cells per well for *Chx10<sup>orJ/orJ</sup>* CNR, leading to the assumption that neurosphere formation is a cell autonomous condition (Figure **3.8b**).



**Figure 3.8. More neurosphere-forming cells reside in the *Chx10*-deficient retina CNR**

**(a)** A limiting dilution assay revealed a linear relation between the number of cells plated and the formation of neurospheres deriving from *Chx10*<sup>*orJ/orJ*</sup> CE (black squares) and CNR (white circles) (N=3, error bars; SEM). The frequency of neurosphere hit was 1 per 280 cells and 1 per 1,050 cells for CNR and CE, respectively.

**(b)**, Dissociated cells deriving from the *Chx10*<sup>*orJ/orJ*</sup> central neural retina were plated in different cell concentrations for 7 days in NGM. No significant alteration was observed in neurosphere diameter between different primary cell concentrations.

**(c)** A limiting dilution assay revealed a linear relation between the number of cells plated and the formation of neurospheres deriving from wildtype CE (N=3, error bars; SEM). The frequency of neurosphere hit was 1 per 2,050 cells for wildtype CE cells.

**(d)** Neurospheres do not result from cell aggregation. Mixed cultures of cyan fluorescent protein (CFP) and green fluorescent protein (GFP) cells result in neurospheres that comprise either of only GFP-positive cells or of CFP-positive cells. Nuclei are counter-stained with propidium iodide (PI). Scale bar 10  $\mu$ m.

**(e)** Neurospheres do not result from cell aggregation. Mixed cultures of sv129 wildtype and GFP) cells result in neurospheres that comprise either of only GFP-positive cells or non-fluorescent cells. Scale bar 20  $\mu$ m.

Plating cells at low density is reported to result in clonal neurospheres that are derived from the proliferation of a single parent cell, rather than cell aggregation (Tropepe V 2000). To confirm that the neurospheres observed in our set-up were generated clonally, rather than by aggregation, we examined neurospheres generated from mixtures of dissociated CE cells GFP-expressing mice and from mice expressing CFP or wildtype sv129. When plated at densities ranging from 10 to 50 cells per  $\mu\text{L}$ , neurospheres were only ever observed to contain either GFP-positive or CFP-positive or non-fluorescent cells, with no evidence of cell mixing within a given neurosphere (Figure 3.8d, e). Together with the LDA data, this indicates that the neurospheres observed here are highly unlikely to arise by cell aggregation.

### **3.2.4 Cells from the adult $\text{Chx10}^{\text{orJ/orJ}}$ central neural retina are larger and more numerous than ciliary epithelium-derived neurospheres *in vitro***

Neurosphere size and cell number within a sphere, provide measures of proliferation. To determine whether the lack of Chx10 led to a change in the number of proliferative cells in the adult eye, the numbers of neurospheres arising from the CNR and CE of wildtype and  $\text{Chx10}^{\text{orJ/orJ}}$  mice were quantified. Initially, the number of neurospheres per CNR or CE obtained per eye (Figure 3.9a) was measured. Although there was a significant increase between the number of  $\text{Chx10}^{\text{orJ/orJ}}$  CNR compared to wildtype CE, there was no significant difference between wildtype and  $\text{Chx10}^{\text{orJ/orJ}}$  CE or  $\text{Chx10}^{\text{orJ/orJ}}$  CE and CNR neurosphere numbers per eye, contradicting an earlier report (Coles BL 2006). Since cell plating density was higher than that which allows neurospheres to arise clonally in these experiments and different volumes of tissue were present in each of the regions of the two genotypes, cells were plated at a standard density of 20,000 cells per well. When plated at a threshold clonal cell density of 20,000 cells per well, the absence of the Chx10 led to a marked increase in the

number of neurospheres generated from both the CE (7-fold) and the CNR (15-fold), compared with wildtype CE (Fig. **3.9b**), with the CNR providing the richest source of neurospheres at seven days in culture. These data show that the microphthalmic *Chx10<sup>orJ/orJ</sup>* eye harbours a large population of cells that can proliferate to form neurospheres *in vitro*.

Neurospheres deriving from the *Chx10<sup>orJ/orJ</sup>* CNR were found to have the largest diameter in comparison to their CE counterparts (Figure **3.10a**) when plated at 20,000 cells per well. Neurospheres lacking *Chx10* from the CE and CNR were 50% and 60% larger in diameter than wildtype CE, respectively (mean neurosphere diameters; wildtype CE; 49  $\mu\text{m}$ , *Chx10<sup>orJ/orJ</sup>* CE; 70  $\mu\text{m}$ , *Chx10<sup>orJ/orJ</sup>* CNR; 84  $\mu\text{m}$ ). Interestingly, upon analysing the diameter distribution, the majority of wildtype CE-derived neurospheres fell within a 20-40  $\mu\text{m}$  dispersion, while *Chx10<sup>orJ/orJ</sup>* CE and CNR within 70-90  $\mu\text{m}$  and >100  $\mu\text{m}$ , respectively (Figure **3.10b**).

The above observations led us to design experimental procedures to assess the possibility of the adult *Chx10*-deficient retinal cells possessing a higher proliferation potential *in vitro*. By analysing each neurosphere under a confocal microscope it was found that *Chx10<sup>orJ/orJ</sup>* CE- and CNR-derived neurospheres contained approximately 30% and 40% more cells than their wildtype CE counterparts, respectively (Figure **3.11a**).



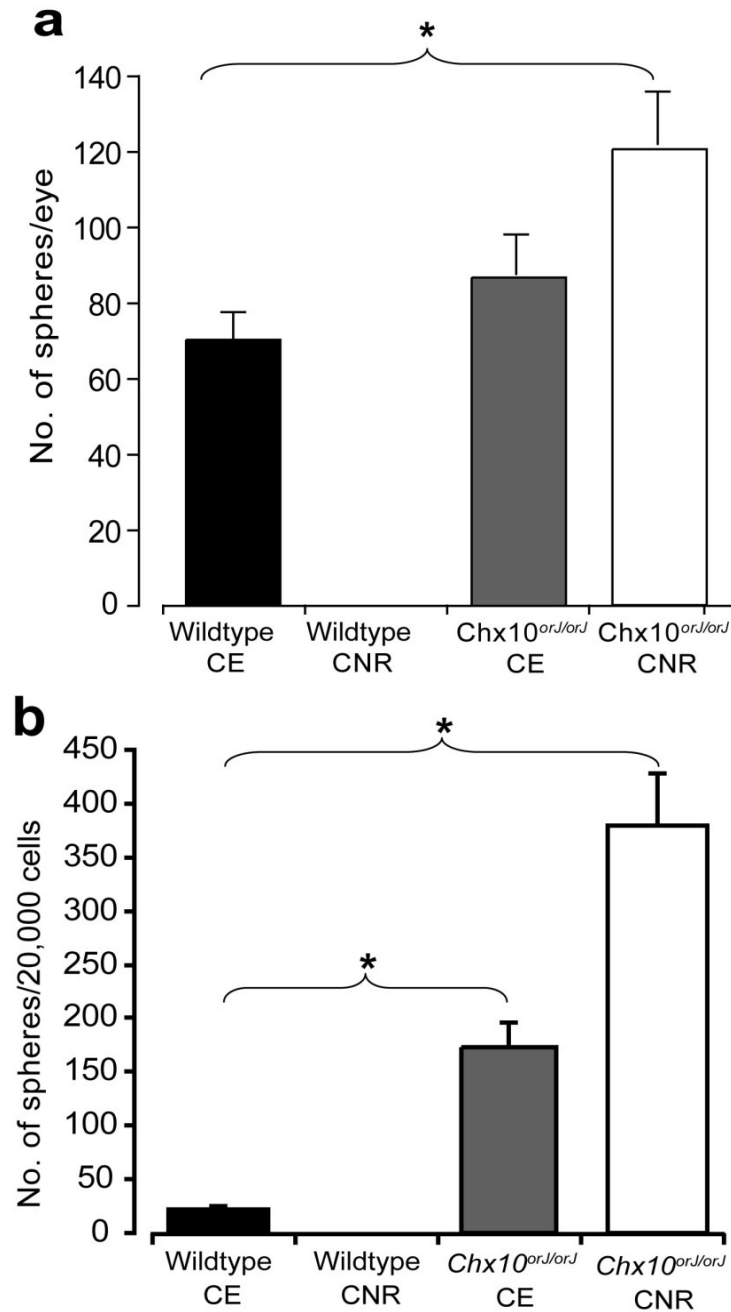


Figure 3.9 Cells from *Chx10<sup>orJ/orJ</sup>* CNR give rise to more neurospheres *in vitro* in comparison to WT and *Chx10<sup>orJ/orJ</sup>* CE cells.

Cells were enzymatically dissociated from WT and *Chx10<sup>orJ/orJ</sup>* CE and *Chx10<sup>orJ/orJ</sup>* CNR and allowed to form spheres for 7 days in NGM.

(a) Histogram showing the average number of primary neurospheres derived per eye. N=13 eyes for each region; one-way ANOVA \*P<0.01. Error bars; SEM.

(b) Histogram showing the average number of primary neurospheres derived per 20,000 cells. N=17 eyes for each region and for wildtype CNR region N=15; one-way ANOVA \*P<0.01. Error bars; SEM.

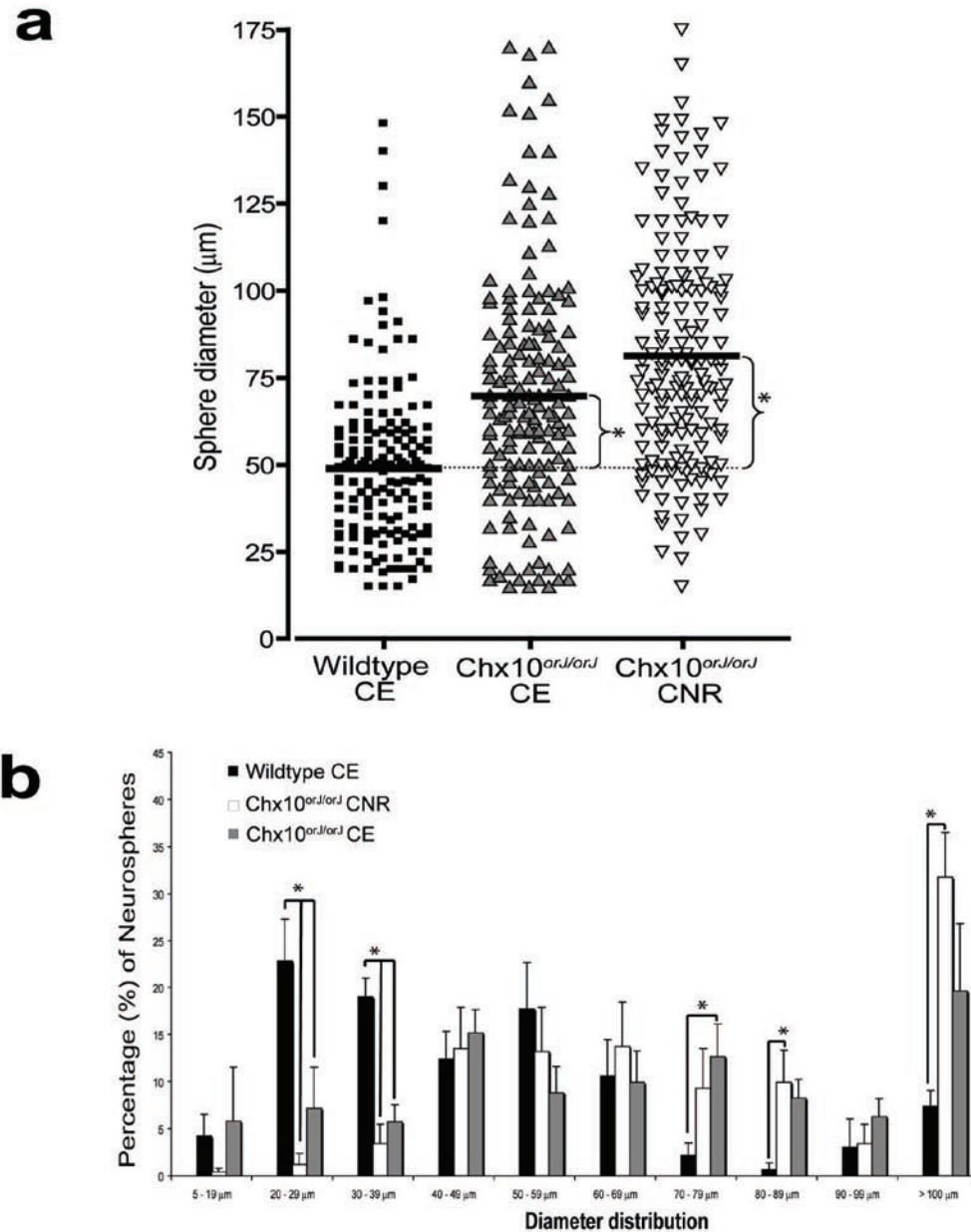


Figure 3.10 Cells from Chx10<sup>orJ/orJ</sup> produce larger neurospheres in vitro in comparison to WT CE.

(a) Graph showing diameter of neurosphere derived from wildtype CE (black squares; N=10 eyes, n=132 neurospheres), Chx10<sup>orJ/orJ</sup> CNR (white triangles; N=10, n=154) and Chx10<sup>orJ/orJ</sup> CE (grey triangles; N=10, n=154).

(b) Histogram showing average diameter distribution of neurospheres formed from cells isolated from wildtype (black) and Chx10<sup>orJ/orJ</sup> CE (light grey) and Chx10<sup>orJ/orJ</sup> CNR (white) regions. N=5 independent cultures, n=20-30 neurospheres measured per culture.

One-way ANOVA, Post-Hoc; Dunnet's T3 \*P<0.01.

Pulse-labelling with the proliferative marker BrdU at seven days in culture demonstrated that CNR and CE *Chx10<sup>orJ/orJ</sup>*-derived neurospheres had a higher percentage of BrdU-labelled cells compared with wildtype CE spheres (Figures **3.11b** and **3.12a**). A PI dye exclusion assay was performed on neurospheres at seven days in culture to assess whether different rates of cell death affect the neurosphere size and cell number. Negligible levels of PI staining of dying or dead cells were observed in all neurospheres indicating death rates were not a significant factor. Some rare cells were found being co-labelled with PI and Hoechst, usually found in the periphery (Figure **3.12b**). Most of the PI shown in the Figure 3.11d is non-specific staining, that probably has incorporated at RNA transcripts outside the cell nucleus.

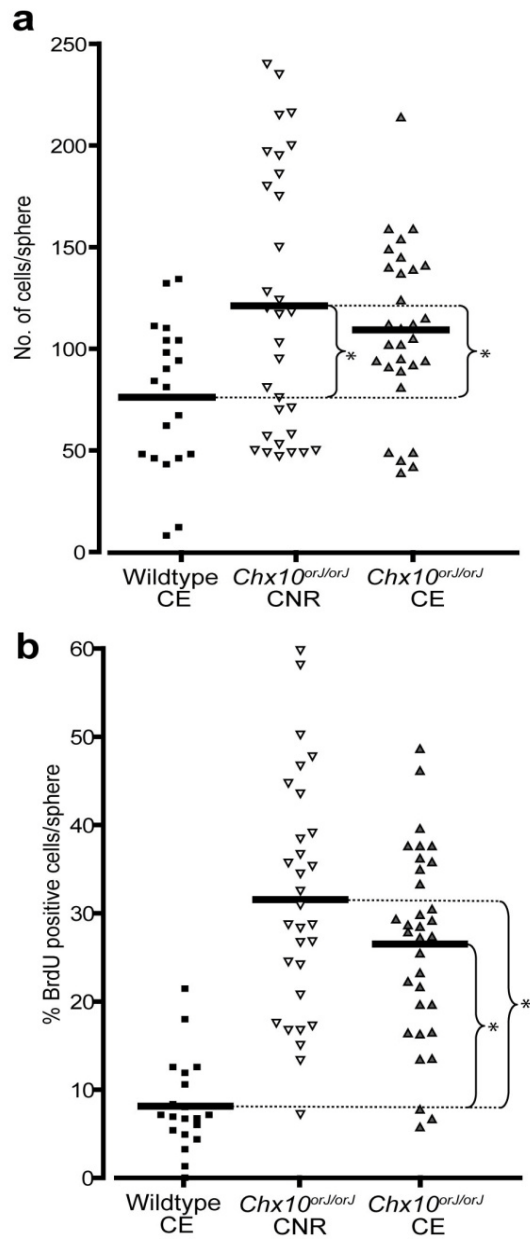


Figure 3.11 Neurospheres from *Chx10<sup>orJ/orJ</sup>* contain more BrdU<sup>pos</sup> cells in vitro in comparison to wildtype ones.

(a) Graph showing cell number per neurosphere derived from wildtype CE (black squares; n=22), *Chx10<sup>orJ/orJ</sup>* CE (grey triangles; n=33) and *Chx10<sup>orJ/orJ</sup>* CNR (white triangles; n=30).

(b) Graph showing the percentage of cells per neurosphere that were BrdU<sup>pos</sup> following a 4 h pulse in vitro from wildtype CE (black squares, n=22), *Chx10<sup>orJ/orJ</sup>* CNR (white triangles, n=30) and *Chx10<sup>orJ/orJ</sup>* CE (grey triangles, n=33).

One-way ANOVA \*P<0.005

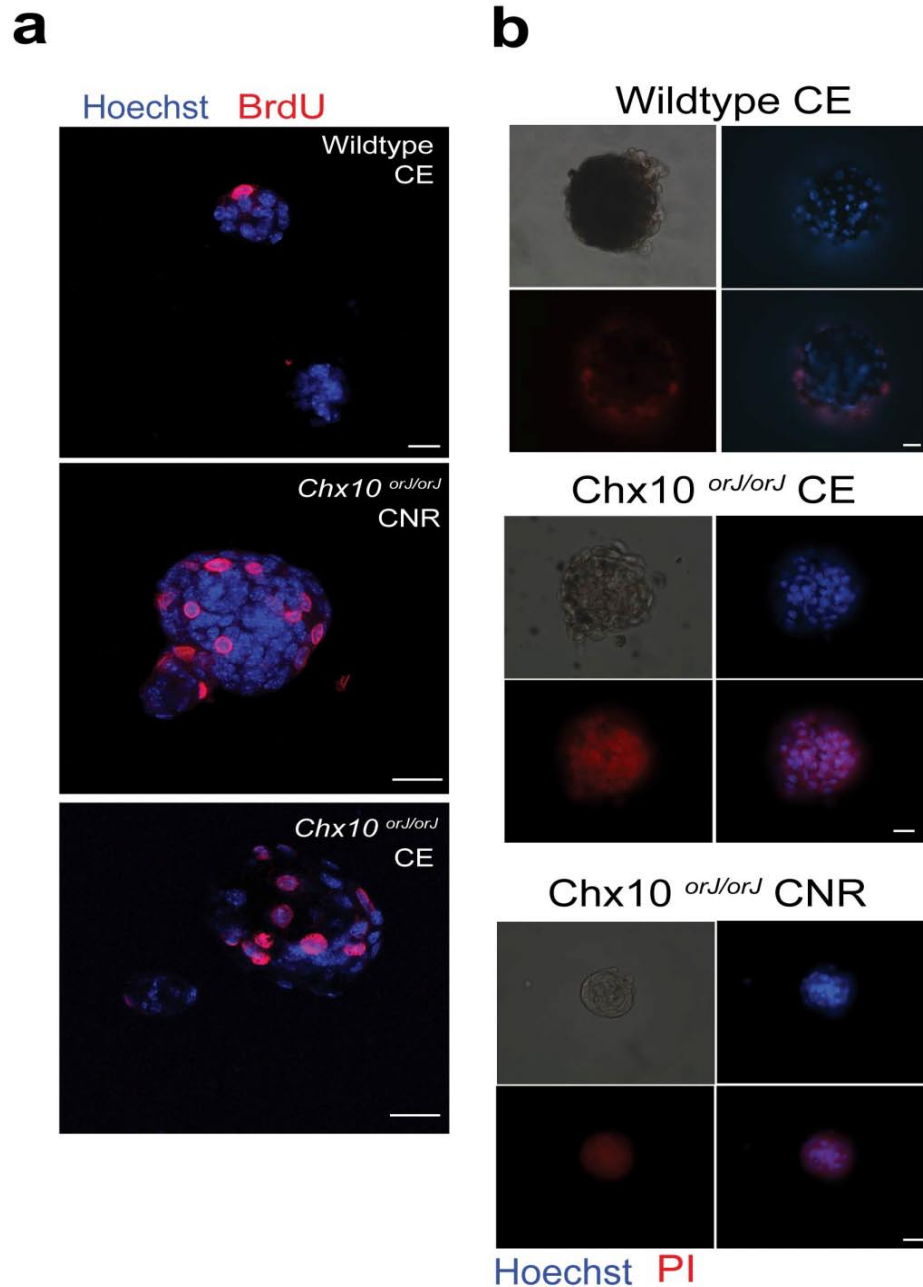


Figure 3.12 Neurospheres from wildtype CE are not smaller than *Chx10<sup>orJ/orJ</sup>* due to increased apoptosis.

(a) Examples of confocal sections through neurospheres from wildtype CE (top), *Chx10<sup>orJ/orJ</sup>* CNR (middle) and *Chx10<sup>orJ/orJ</sup>* CE (bottom) following a 4 h pulse of BrdU (red). Nuclei are labelled with Hoechst 33342 (blue).

(b) No alteration in apoptotic/necrotic cell level between *Chx10*-deficient and wildtype derived neurospheres. Neurospheres cultured for 7 days in NGM were subjected to PI exclusion assay prior to fixation, to assess double-labelled (PI-red, Hoechst-blue) cells. Only rarely cells were found positive for both nuclear dyes in the periphery of neurospheres (N=2). Scale bar: 10  $\mu$ m

### 3.2.5 Neurospheres derived from the $Chx10^{orJ/orJ}$ CNR are de-pigmented

In addition to my comparative analysis, it was observed that neurospheres deriving from the  $Chx10^{orJ/orJ}$  CNR consistently had very little or lacked pigmentation compared with wildtype and  $Chx10^{orJ/orJ}$  CE-derived neurospheres (Fig. **3.13a**). To quantify this difference, I employed the Volocity 3.0.1™ software, which is able to measure the black-white level of an area of interest and gives a measuring scale of black & white ratio (Black 2,000 & white 0). We found that  $Chx10^{orJ/orJ}$  CNR-derived neurospheres scored at very low Black-White ratio levels in comparison to their wildtype and  $Chx10^{orJ/orJ}$  CE counterparts (Figure **3.13b**). This difference in pigmentation was also assessed by quantitative real time-PCR. It was found that Dct (Dopachrome tautomerase) and Silver, two genes involved in pigment biosynthesis, were expressed only at low levels in  $Chx10^{orJ/orJ}$  CNR-derived neurospheres compared to those derived from the mutant or wildtype CE (Fig. **3.14a, b** Dct; 14-fold and 4-fold decrease, respectively; Silver; 5-fold and 6-fold decrease, respectively). Interestingly, Mitf gene expression levels were similar but considerably higher than in the wildtype retina (Figure **3.14c**).

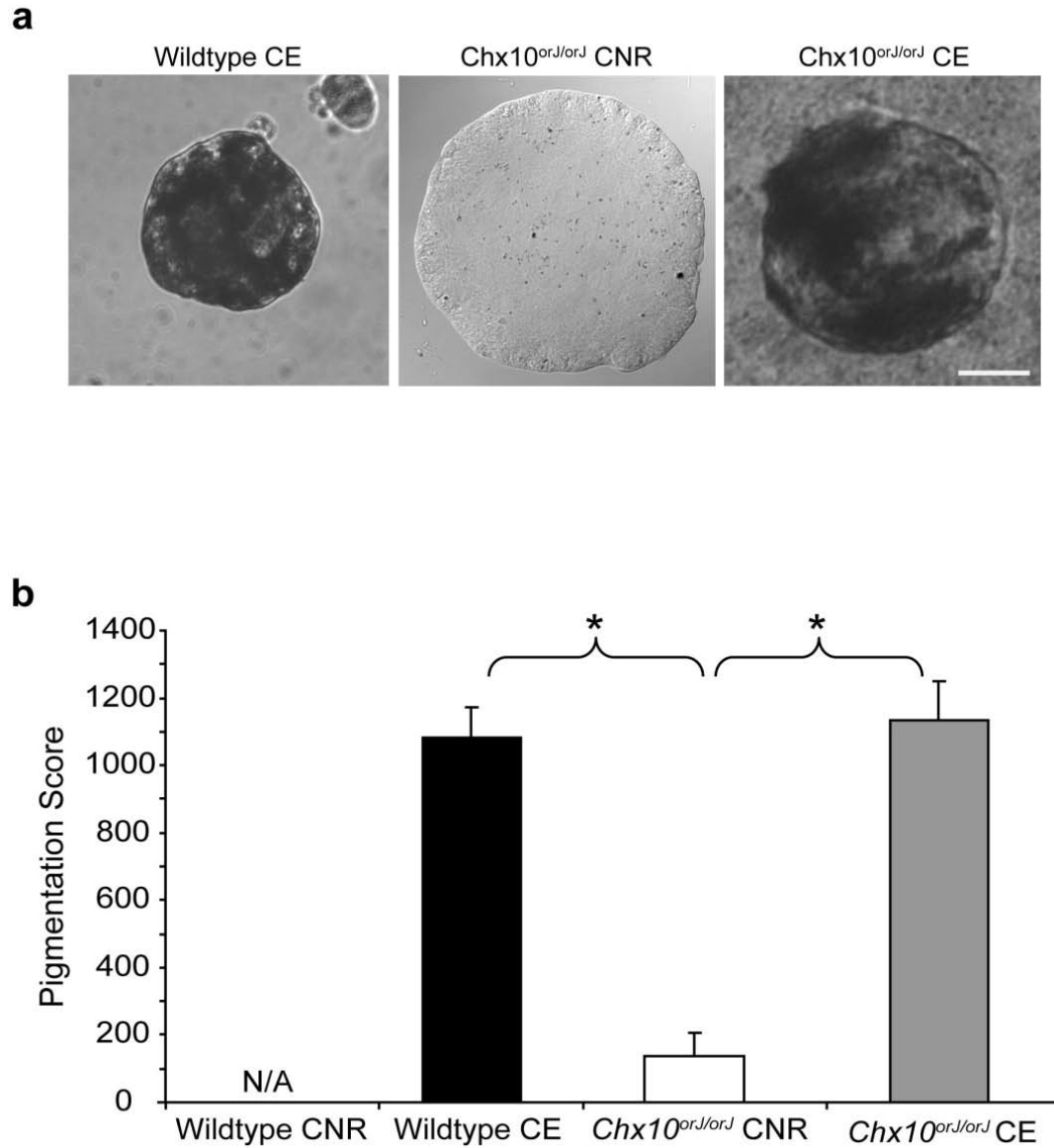


Figure 3.13 Pigmentation levels in Chx10-deficient and wildtype neurospheres. Neurospheres derived from the CNR of Chx10<sup>orJ/orJ</sup> mice virtually lack pigmentation, compared with those derived from wildtype and Chx10<sup>orJ/orJ</sup> CE.

(a) Light microscope images showing typical neurospheres derived from each genotype and region. Scale bar 10 mm.

(b) Histogram showing average pigmentation levels of neurospheres cultured for 7 days in vitro, as determined using Volocity software 3.0™ analysis. N=5 eyes; n=10 neurospheres for each region, one-way ANOVA test \*P<0.001.

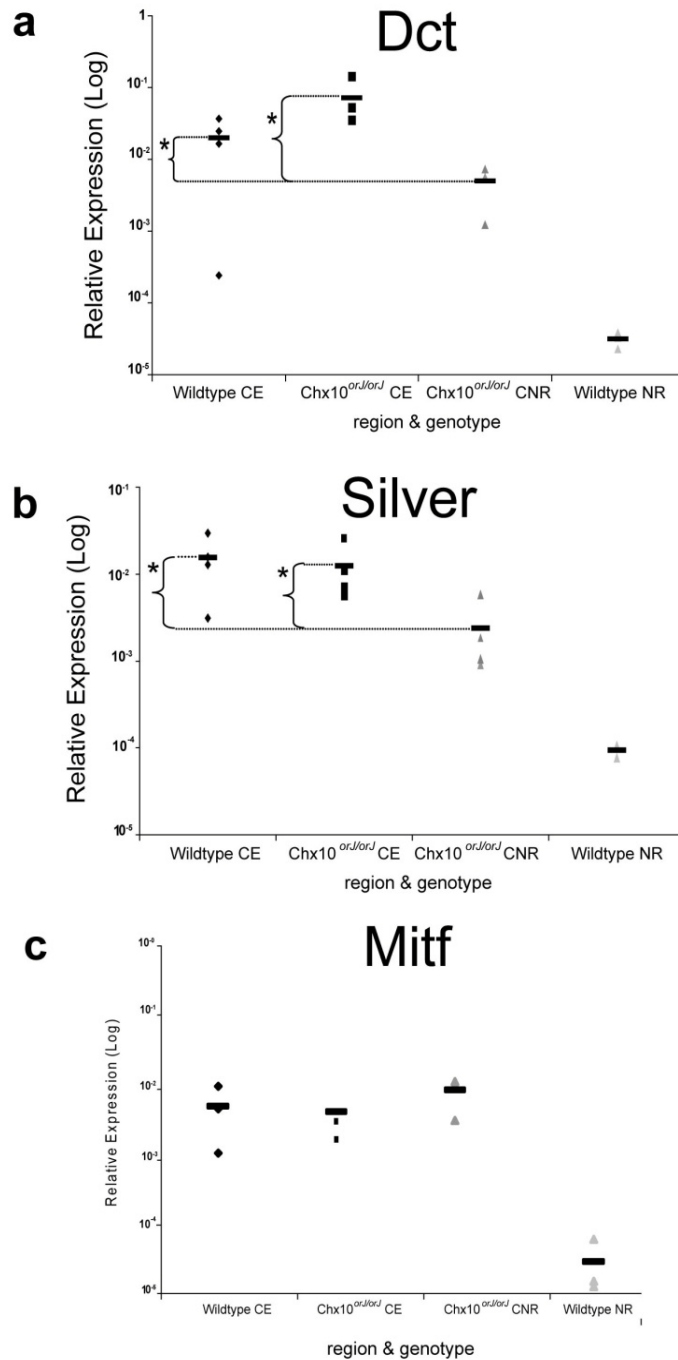


Figure 3.14. Pigment gene expression levels in Chx10-deficient and wildtype neurospheres.

Quantitative Real-Time PCR analysis of expression of Dct (a), Silver (b) and Mitf (c) in neurospheres derived from Chx10-deficient CE and CNR, and wildtype CE (cultured for 7 days in vitro as proliferating neurospheres), and adult neural retina for comparison. Graphs show relative expression levels from four independent experiments normalized to Gapdh (N=4 eyes from each genotype per experiment); Mann-Whitney test \* P<0.05.



### **3.3 Discussion**

RPC express *Chx10* throughout retinal development (Liu IS 1994; Burmeister M 1996). After terminal division, expression is restricted to bipolar cells (Liu IS 1994; Burmeister M 1996; Ferda Percin, Ploder et al. 2000) and a recently described subpopulation of Müller glia (Rowan S 2004). Mutations in both the human and mouse *Chx10* genes lead to microphthalmia (Burmeister M 1996; Ferda Percin, Ploder et al. 2000; Bar-Yosef et al. 2004). The naturally-occurring *Chx10* mouse mutant, *ocular retardation* ( $Chx10^{orJ/orJ}$ ) (Burmeister M 1996), is characterised by a severely underdeveloped neural retina and expanded CE, absence of bipolar cells and disrupted photoreceptor differentiation (Robb RM 1978; Silver and Robb 1979; Burmeister M 1996; Bone-Larson C 2000; Rowan S 2004; Rutherford AD 2004). In cultures of cells derived from the adult CE, notably more neurospheres arise from the CE of the  $Chx10^{orJ/orJ}$  mice than from wildtype mice (Tropepe V 2000). Similarly, our laboratory has previously shown that there are increased numbers of CE cells proliferating *in vivo* in the mutant compared to the wildtype (Dhomen NS 2006).

#### **3.3.1 Summary of findings**

The purpose of this study was to investigate the potential of the adult  $Chx10^{orJ/orJ}$  neural retina to give rise to neural progenitors *in vitro*. Previous studies, described earlier showed the persistence of a BrdU<sup>pos</sup> cell population within the adult  $Chx10^{orJ/orJ}$  retina.

From this study the following conclusions can be drawn;

- i. Early and late embryonic  $Chx10^{orJ/orJ}$  retinal cells showed similar ability to form neurosphere-like structures *in vitro* as wildtype.

- ii. Cells from the adult *Chx10<sup>orJ/orJ</sup>* CNR have the ability to form neurospheres *in vitro* and express neural and retinal stem/progenitor cell markers. Neurospheres are never formed from adult wildtype CNR.
- iii. Retinal stem/progenitor cells isolated from the retina of *Chx10<sup>orJ/orJ</sup>* mice are different from wildtype CE ones, lacking pigmentation and with a higher proliferation potential.
- iv. These data suggest that loss of a single TF, Chx10, alters the retinal stem cell potential of the mature retina in mammals.

### **3.3.2 Retinal progenitor/stem cells from the embryonic retina form neurospheres *in vitro* in the absence of Chx10**

The ability to form neurospheres, whereby a stem cell proliferates to form a free-floating sphere containing stem and progenitor cells, is well documented and is thought to provide an indication of the number of endogenous stem cells in a given tissue (Tropepe V 2000; Coles BL 2004; Parati EA et al. 2004; Coles BL 2006). The embryonic retina consists of a pool of retinal progenitors that will undergo a finite number of cell divisions, giving rise to a fully mature retina after postnatal day 11 in mice. My results show that retinal cells from early (E11.5) and late (E18.5) developing retina, when cultured in the presence of NGM show a limited ability for neurosphere formation and phenotypically an adherent morphology in culture rather than a free-floating neurosphere state. This was observed for both wildtype and Chx10-deficient retinal embryonic cultures. More Nestin<sup>pos</sup> cells were observed within the neurospheres deriving from the mutant E18.5 developing retina. Although lack of Chx10 causes a decrease in the RPC number from early retinal development *in vivo*, this does not seem to affect the proliferation of them *in vitro*. Previous work from our lab has shown that during embryonic stages, cell number in the metaphase stage of mitosis (H3-labelling) remains constant and lower in *Chx10<sup>orJ/orJ</sup>* retina from E11.5 through to E18.5 in

wildtype (Dhomen NS 2006). The mutant mitotic labelling index is similar to wildtype until E13.5, and then significantly decreases on later time points (E15.5 & E18.5), but only in wildtype. The *in vitro* data presented in this chapter show that the potential of embryonic retinal progenitors lacking Chx10 to form spheroid structures is similar to wildtype ones and that by E18.5 the Chx10-deficient cells give rise to most well-defined neurospheres *in vitro*.

### 3.3.3 Cells from the adult $Chx10^{orJ/orJ}$ mammalian retina form neurospheres

Mutations in several TFs, as well as cell cycle proteins, result in major alterations in eye size, often due to effects on the expansion of the progenitor pool during early development (Fantl V 1995; Kobayashi M 2001). *Chx10*, a paired-like homeobox TF gene, is one of the earliest markers of the presumptive neural retina expressed in the invaginating optic vesicle (Liu IS 1994).

The CNR of the mature  $Chx10^{orJ/orJ}$  eye is notably smaller than that seen in the wildtype eye, while the CE is expanded. However, our lab has previously reported a persistent dividing (BrdU<sup>pos</sup>) population of cells in the CNR of the  $Chx10^{orJ/orJ}$  mutant eye that occasionally differentiated to express markers of retinal neurons *in vivo* (Dhomen NS 2006). To determine whether these dividing cells have proliferative properties and whether lack of Chx10 causes the activation, or maintenance, of a dormant stem-like potential in the mature CNR, the ability of the  $Chx10^{orJ/orJ}$  eye to form neurospheres, compared with that of the RSCs isolated from the wildtype CE was examined (Ahmad I 2000; Tropepe V 2000). Here, I describe a population of cells with neural progenitor properties that gives rise to neurospheres deriving from CNR cells of the adult  $Chx10^{orJ/orJ}$  mouse. The CNR and CE of  $Chx10^{orJ/orJ}$  and wildtype mice were dissociated and cultured using conditions that have previously been described to promote CE-derived neurosphere formation (Ahmad I 2000; Tropepe V 2000; Macneil,

Pearson et al. 2007). Both the adult wildtype and  $Chx10^{orJ/orJ}$  CE gave rise to neurospheres while the wildtype CNR did not. This is in contrast to previous reports that concluded neurospheres could not be formed from the adult  $Chx10^{orJ/orJ}$  CNR (Tropepe V 2000; Coles BL 2006). I assessed the properties of the CNR cells by comparing them to the previously identified CE RSCs (Ahmad I 2000; Tropepe V 2000; Das AV 2005). The  $Chx10^{orJ/orJ}$  CNR cells fulfil several criteria of being a stem-like population. Like a rare population of CE cells they: (i) form neurospheres *in vitro*, (ii) express neural and retinal progenitor cell markers and (iii) can be expanded *in vitro*. Another cardinal feature of stem cells, multipotentiality, is discussed in Chapter 5 of this Thesis.

### 3.3.4 $Chx10$ as a retinal stem/ progenitor cell regulator

The neurosphere formation assay provides also a measure of the number of neurosphere-generating cells within a given population (Tropepe V 2000; Coles BL 2004; Parati EA, Pozzi et al. 2004; Coles BL 2006). Upon passaging neurospheres from both  $Chx10$ -deficient and wildtype neurospheres from both CE and CNR, a better secondary neurosphere formation was noticed in the mutant CNR, indicating the possibility that the mutant CNR tissue contains a higher number of transiently dividing progenitor or stem-like cells than the mutant or wildtype CE. In addition, we found that the  $Chx10^{orJ/orJ}$  CNR region contained significantly more neurosphere-generating cells than the wildtype CE excluding increased neurosphere fusion events between wildtype CE-derived neurospheres by co-culturing *Gfp*- with *Cfp*- and wildtype CE cells. Limiting dilution assays also confirmed the increased neurosphere-forming capacity of the  $Chx10^{orJ/orJ}$  central neural retina. Both CFP/GFP and GFP/Sv129 neurosphere aggregation assays and the limiting dilution assays support the notion that the CE and CNR neurospheres arise via clonal expansion, although the value of the neurosphere assay system for determination of stem cell

frequency is currently a subject of debate in the field (Louis et al. 2008). Nevertheless these data conclusively demonstrate that the absence of Chx10 leads to the maintenance of a significant number of progenitor cells within the adult CNR.

Lack of Chx10 led to larger diameter neurospheres containing more cells in the S-phase of the cell cycle from both the CE and CNR. This set of results indicate that a population of proliferative cells exists in the adult *Chx10<sup>orJ/orJ</sup>* CNR and that these and their daughter cells proliferate readily *in vitro* in the absence of Chx10 more potently than adult wildtype CE cells. Interestingly, this contrasts the proliferation defect observed in retinal progenitor cells of the developing *Chx10<sup>orJ/orJ</sup>* CNR *in vivo* (Burmeister M 1996; Bone-Larson C 2000). However, the properties of the progenitor cells from the CNR of the *Chx10<sup>orJ/orJ</sup>* mouse appear to be distinct from those derived from the wildtype CE in that they are de-pigmented with significantly lower gene expression levels of key pigment genes such as *Silver* and *Dct*; interestingly *Chx10<sup>orJ/orJ</sup>* CNR *Mitf* gene expression levels were similar to wildtype and mutant CE, although *Mitf* has also been reported to be implicated in promoting cell proliferation, acting as a cell cycle enhancer (Bismuth K 2005; Loercher AE 2005). A recent study has demonstrated the importance of Chx10 for maintaining retinal identity (Rowan S 2004); these researchers elegantly showed that on the *Chx10<sup>orJ/orJ</sup>* mouse, peripheral parts of the neural retina become pigmented, trans-differentiating possibly into RPE-like cells. This neo-pigmentation event was not observed in our mutant CNR neurosphere cultures after 7 or 14 even days *in vitro* in the presence of growth factors, although the possibility cannot be excluded that the primary neurosphere-forming cell was a de-differentiated cell that lost its pigmentation after several mitotic rounds.

### 3.3.5 Chx10 may act as a cell switch from symmetric to asymmetric division

These findings suggest that the lack of the TF, Chx10, leads to the presence of a distinct population of progenitor cells in the adult mammalian CNR. From both the *in vitro* and *in vivo* data, I propose, different models in which retinal progenitors lacking Chx10 behave differently from their wildtype counterparts. As Diagram **3.3.1a** indicates, in the Chx10-expressing presumptive NR of the optic vesicle, retinal stem cells will initially undergo a number of symmetric cell divisions to expand their number and then proceed onto asymmetric cell divisions population of progenitors that in turn will divide further to eventually give rise to RPC of the optic cup. The latter will not be post-mitotic but will be destined to become one of the 6 retinal cell types or a Müller glial cell. Lack of Chx10 that shows a hypocellular developing and adult retina is directly linked to the reduced retinal stem/progenitor cell proliferation (Dhomen NS 2006). Since neurospheres can be produced from the *Chx10<sup>orJ/orJ</sup>* CNR in combination with previous data from our lab showing BrdU<sup>pos</sup> cells residing in the adult *Chx10<sup>orJ/orJ</sup>* CNR, I propose that lack of Chx10 may affect retinal progenitors in three ways; during retinal development, lack of Chx10 (Diagram **3.3.1b**) does not allow a number of retinal stem cells in the periphery to divide. These cells may undergo apoptosis or reside in the retina in a quiescent state during optic cup specification. The remaining central retinal stem cell population increases its number of cell divisions to compensate for the absence of a “threshold” of retinal stem cells. This shift in the increased frequency of symmetric cell divisions leads to less asymmetric divisions and delays maturation (Rutherford AD 2004). Thus, these retinal stem/progenitors lose their specific developmental window and fail to differentiate.

Another possible scenario is that lack of Chx10 induces all retinal stem cells to divide symmetrically more times since they have lost one of the signals that will

induce them to proceed from a less primitive cell type towards a precursor state. That is Chx10 might be involved in the switching from stem cell amplification to an RPC phase, leading to lack of NR specification (Diagram **3.3.1c**).

A third possibility is for Chx10 not affecting the early retinal progenitor proliferation expansion (E11.5 to E13.5) but rather, lack of it to alter the late retinal developmental progenitor pool, perhaps after E13.5, arresting them in a more primitive (with increased symmetric divisions at the expense of asymmetric cell divisions) or even a quiescent state of cells residing in the S-phase of the cell cycle (Diagram **3.3.1d**). The last two scenarios might lead to a poorly differentiated retina with an increased number of quiescent retinal progenitors and possibly stem cells along with a number of post-mitotic retinal cells. A recent study from Rod Bremner's lab (Livne-Bar I 2006) showed that Chx10 is essential only for early retinal proliferation and then due to changes in the regulatory elements of *Chx10* targets may shift to affect late and postnatal RPC proliferation and/or maturation. In that context, scenario b would not apply since *Chx10* will only affect progenitors later in development (E15.5 and onwards). Although it is thought to modulate the level of various cell cycle proteins, including Cyclin D1 and p27<sup>Kip1</sup> (Green ES 2003), direct regulation of these factors by *Chx10* has not been proven. *Chx10* has a number of documented roles with respect to RPC proliferation and differentiation during development (Konyukhov and Sazhina 1971; Burmeister M 1996; Rowan S 2004; Livne-Bar I 2006). Proliferation is vastly reduced in the peripheral *Chx10*<sup>orJ/orJ</sup> retina at E11.5 (Burmeister M 1996; Bone-Larson C 2000),

Here, I show that *Chx10* is not essential for proliferation of adult-derived progenitor cells *in vitro*, in contrast to its effect on RPC proliferation during development (Burmeister M 1996; Bone-Larson C 2000). Interestingly, recent work suggests that *Chx10* is not required to maintain the proliferation of RPC at late stages of retinal development (Livne-Bar I 2006), and our laboratory has

previously showed that the *Chx10<sup>orJ/orJ</sup>* retina has an abnormally high mitotic index in late prenatal development (Dhomen NS 2006).



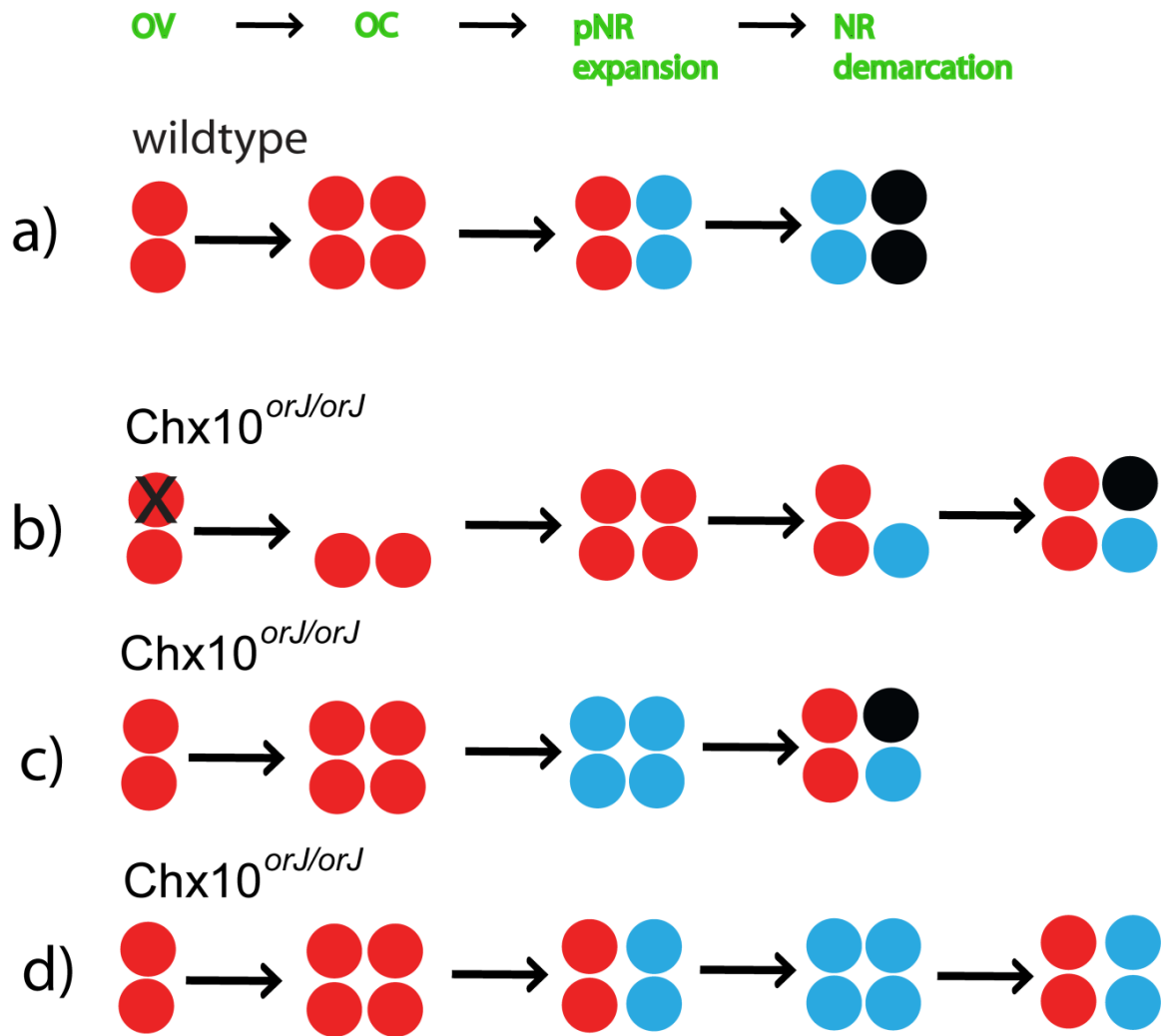


Diagram 3.3.1 Lack of *Chx10* alters the symmetric-asymmetric division status of retinal progenitors (key- red; RSC, light blue; RPC, black; post-mitotic).OV; optic vesicle, OC; optic cup, pNR; presumptive neural retina, NR; neural retina.

All possible scenarios mentioned above are consistent with the results obtained from the neurosphere-forming assay; apart from the presence of neurospheres deriving from the adult CNR, a phenomenon that is not observed in wildtype CNR cultures, they show an increased *in vitro* proliferation potential than adult CE-derived neurospheres. In addition, self-renewal capacity of the mutant

neurospheres and the frequency of neurosphere formation extracted from the LDA, also lead to the conclusion for the presence of more retinal stem-like and/or progenitor cells in the *Chx10*-deficient CNR. All of the above-mentioned models reconcile that *Chx10* may act to promote the developmental progression of progenitor cells. Without *Chx10*, a greater proportion of cells may remain in a more immature state during development leading to a reduced RPC number, and consequently, an underdeveloped neural retina. Absence of *Chx10* might then be predicted to lead eventually to an increased number of persisting plastic immature cells, but would not affect the rate of proliferation *per se*. It has been also proposed that loss of RPC proliferation causes an expansion in the number of RSCs in the mutant CE (Coles BL 2006).

In this chapter, it was shown that the *Chx10*-deficient adult neural retina possess a population of cells with stem-like properties. How is it possible for the adult mutant eye to have a persisting CNR stem-like cell but fails to grow during development? Findings presented here indicate that there is a population of cells within the adult mutant retina that has either acquired or reserved the ability to proliferate *in vitro*. This population appears to rise from cells different than those found in the wildtype and *Chx10<sup>orJ/orJ</sup>* CE in pigmentation and proliferation potential with similar expression of neural and retinal stem/progenitor markers. Tuning in with recent literature, a plausible candidate is an abnormal Müller glial population that due to lack of *Chx10* might behave abnormally in the retina. In higher vertebrates upon neuronal damage Müller glia undergo mitosis (Dyer MA 2000), but with no observed de-differentiation. This discrepancy raises interesting subjects for investigation concerning the extrinsic and intrinsic cues that might be involved in adult retinal stem cell regulation (Levine EM 2004). In the next chapter, I am attempting to characterise the adult murine central neural retinal *Chx10<sup>orJ/orJ</sup>* cells that are able to proliferate and give rise to neurospheres *in vitro*.

## **Chapter 4 – Müller Glia as Endogenous Retinal Progenitors**

## **4.1 Introduction**

Lower vertebrates display the capacity to generate new retinal neurons in response to injury (Hollyfield JG 1971; Reh TA 1998). New neurons arise mainly from the CMZ located around the anterior margin of the retina, which maintains a population of retinal stem cells throughout life. Regenerative potential in the central retina is more limited. A population of rod precursor cells exists in teleost fish that can regenerate all retinal neuronal types after damage [for review, see (Raymond 1991)]. Similarly, recent work showed that in zebrafish, the Müller glial cells function as multipotent retinal stem cells that respond to loss of photoreceptors by specifically regenerating the missing neurons (Bernardos, Barthel et al. 2007). Conversely, acute damage of the postnatal chick retina induced Müller glial cells to undergo limited cell division and rarely to express markers characteristic of retinal neurons (Reh TA 1998; Dyer MA 2000; Fischer and Reh 2001). Müller glia cells residing in the inner nuclear layer comprise 90% of the glial population in the retina (non-neural cell population) (Piccolino M 1989), while the rest are astrocytic populations found in the nerve fibre layer adjacent to the RGC layer making unspecialised vascular contacts (Ogden 1978). Although the primary role of Müller glia was thought to be for retaining retinal architecture and orientation scaffolding as well as circuitry, a recent study shows that in mammals (Ogden 1983), upon injury these cells re-enter the cell cycle and perhaps play a role in photoreceptor degeneration (Wan et al. 2008), similarly to posthatch chick and lower vertebrate retina. Additionally, two papers have indicated that Müller glia acquire potentially neural stem cell characteristics upon injury in mammals and then differentiate to retinal cell types (Ooto S 2004; Das et al. 2006). These data were based on the fact that BrdU<sup>POS</sup> cells were found to co-express retinal cell types and in the appropriate microenvironment form new neurons.

Chx10 was found to be expressed in Müller glia by using a bacterial artificial chromosome (BAC) vector carrying the Chx10 promoter with a GFP and alkaline phosphatase reporter construct (GFPCre-IRES-AP) (Rowan S 2004; Rowan S 2004). In the microphthalmic early postnatal and late embryonic retina, the Müller glia cell population has been reported to be directly affected by the lack of Chx10, producing an abnormal progenitor/Müller glia cell population (Rowan S 2004). This population was found to express both retinal stem/progenitor (Pax6) as well as Müller glia cell markers [i.e. glutamine synthetase (GS)] in early postnatal stages.

In this chapter, I investigated the possibility that the newly-discovered CNR-derived progenitor population has characteristics of Müller glia cells and may arise from Müller cells in the adult, or the abnormal/arrested development of early or late stage RPC.

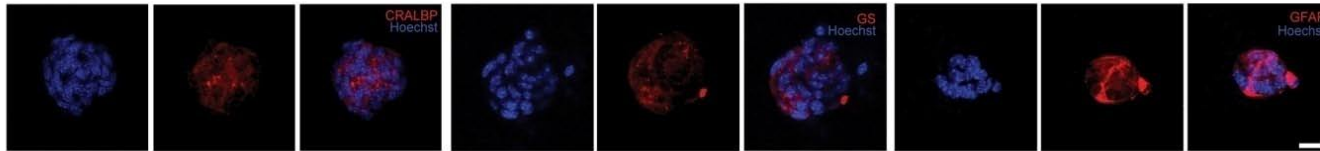
## **4.2 Results**

### **4.2.1 Neurospheres from the adult Chx10<sup>orJ/orJ</sup> central neural retina express Müller glia markers**

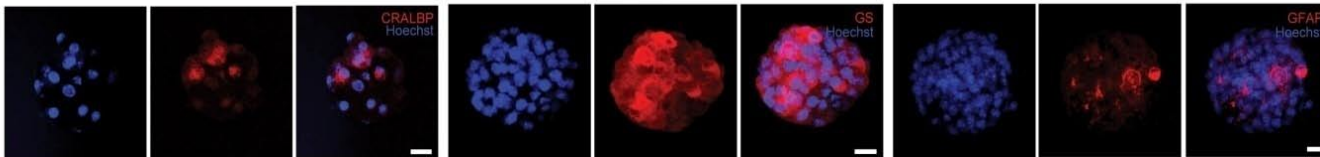
Neurospheres from the Chx10-deficient CNR were previously shown to express neural and retinal stem/progenitor markers (Nestin, Pax6, Sox2, Chapter 3, figures 3.5 & 3.6). To further characterise the neurosphere-forming cells, neurospheres deriving from wildtype CE and Chx10<sup>orJ/orJ</sup> CE and CNR were immunolabelled with Müller glia markers CRALBP, GS and GFAP after culturing for 7 days *in vitro* (Figure 4.1). Wildtype CE-derived neurospheres expressed all of these markers (Figure 4.1a). Similarly, the expression of those three markers was also detected in Chx10<sup>orJ/orJ</sup> CE- and CNR- derived neurospheres (Figure 4.1b, c). Interestingly, GS and GFAP immunolabelling expression was stronger in Chx10<sup>orJ/orJ</sup> CNR-derived neurospheres.

Neurospheres cultured for seven days deriving from the Chx10-deficient CE and CNR and wildtype CE were also co immune-labelled with GS and GFAP (Figure 4.1d). Double GS/GFAP-immunolabelled cells were observed frequently and with a strong expression in both wildtype and mutant CE and CNR neurospheres suggesting the presence of Müller cell markers in cells that also label with retinal progenitor markers (Chapter 3).

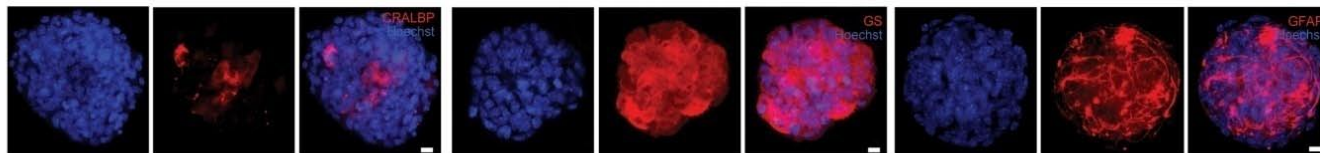
a) adult wildtype CE



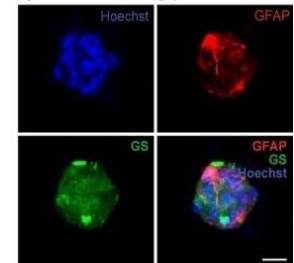
b) adult *Chx10<sup>orJ/orJ</sup>* CE



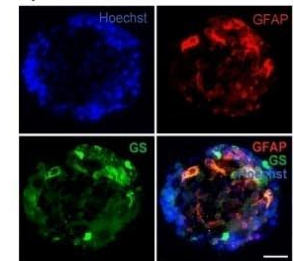
c) adult *Chx10<sup>orJ/orJ</sup>* CNR



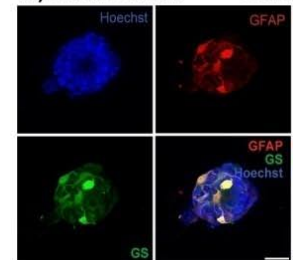
d) i) adult wildtype CE



ii) adult *Chx10<sup>orJ/orJ</sup>* CE



iii) adult *Chx10<sup>orJ/orJ</sup>* CNR



**Figure 4.1 Neurospheres from Chx10-deficient adult central neural retina express Müller glial/progenitor markers.** Cells isolated from the CE and CNR were allowed to form neurospheres for 7 days *in vitro* in the presence of NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for Müller glial/progenitor markers CRALBP, Glutamine Synthetase (GS) and GFAP. **(a)** Wildtype adult CE neurosphere-forming cells are positive for Müller glial/progenitor markers. **(b)** Similarly, Chx10<sup>orJ/orJ</sup> adult CE neurosphere-forming cells are positive for retinal stem/progenitor markers. **(c)** Also, Chx10<sup>orJ/orJ</sup> adult CNR neurosphere-forming cells are positive for retinal stem/progenitor markers. Scale bars; 10 µm. N=3-4 independent experiments. **(d)** Adult wildtype CE **(i)**, Chx10<sup>orJ/orJ</sup> CE **(ii)** and Chx10<sup>orJ/orJ</sup> CNR **(iii)** neurospheres were co-immunolabelled with anti-GFAP (red) and anti-GS (green). N=2-5 independent experiments.



Since immunolabelling for GFAP and GS was consistently stronger in neurospheres derived from the  $Chx10^{orJ/orJ}$  CNR, I sought to examine these differences in levels of Müller gene expression by quantitative Re-Ti PCR. Gene expression levels of GFAP along with Apo-lipoprotein E, another Müller glial cell marker, as well as Sox2, a neural stem/progenitor marker (Figure **4.2**), were consistent with the raised level of protein expression of Müller markers, in neurospheres derived from the  $Chx10^{orJ/orJ}$  CNR, compared to those derived from either the mutant or the wildtype CE. *GFAP* mRNA levels in  $Chx10^{orJ/orJ}$  CNR were increased 12-fold and 52-fold compared with  $Chx10^{orJ/orJ}$  CE and wildtype CE, respectively (Fig.**4.2a**), while for *Apo-lipoprotein E (ApoE)* mRNA  $Chx10^{orJ/orJ}$  CNR versus  $Chx10^{orJ/orJ}$  CE and versus wildtype CE there was a 102-fold and 138-fold increase, respectively (Fig.**4.2b**). In addition, Sox2 mRNA levels were higher in the mutant with the highest from mutant CNR-derived neurospheres in comparison to wildtype and  $Chx10^{orJ/orJ}$  CE ( $Chx10^{orJ/orJ}$  CNR versus  $Chx10^{orJ/orJ}$  CE and versus wildtype CE there was a 1.7-fold and 4.5-fold increase, respectively and  $Chx10^{orJ/orJ}$  CE versus wildtype CE there was a 2.5-fold increase, Figure **4.2c**). At least 4 independent neurosphere cultures were tested in these experiments.

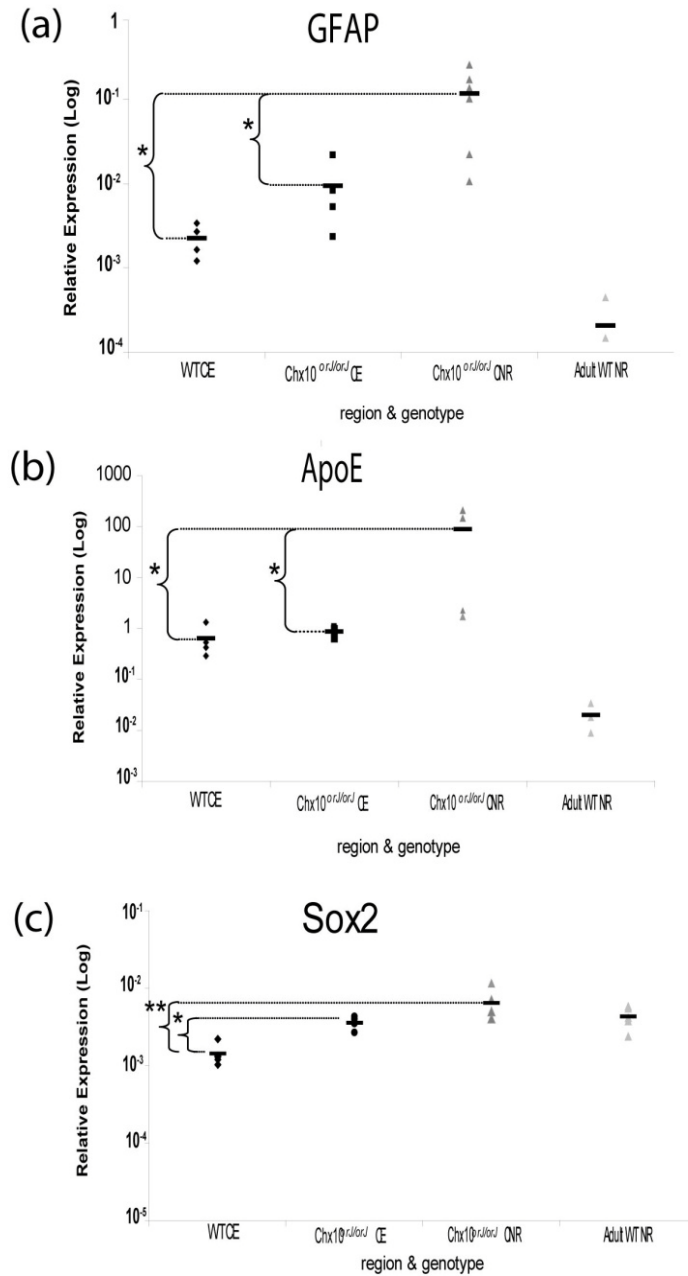


Figure 4.2 Neurospheres from Chx10-deficient adult central neural retina express high levels of Müller glial/progenitor genes in comparison to WT and Chx10<sup>orl/orl</sup> CE neurospheres.

Quantitative Real-Time PCR analysis for expression of GFAP (a), Apo-lipoprotein E (ApoE) (b) and Sox2 (c) in neurospheres derived from Chx10<sup>orl/orl</sup> CE and CNR, and wildtype CE (cultured for 7 days in vitro in NGM), and adult neural retina for comparison. Graphs show relative expression levels from four independent experiments normalized to Gapdh (N=4 eyes from each genotype per experiment); Mann-Whitney test \*\* P<0.05, \* P<0.01.

The above data indicate that while the majority of cells within neurospheres from each region expressed markers consistent with a retinal progenitor identity (see figure 3.5 & 3.6), the CNR neurospheres were distinctive in that a large number also displayed Müller glial cell characteristics. It has been shown that GFAP<sup>pos</sup> glial populations are found in neural tissues undergoing reactive gliosis, probably an after-effect of an injury response (Kivela, Tarkkanen et al. 1986; Okada, Matsumura et al. 1990; Sarthy and Egal 1995). I considered the possibility that the microenvironment of the mature *Chx10<sup>orJ/orJ</sup>* retina mimics that found in the injured retina and that the resulting reactive gliosis may lead to increased levels of activated Müller cells, which possibly proliferate *in vitro* to form neurospheres. If this was the case, it should be possible to establish neurospheres from other degenerating retinæ undergoing reactive gliosis (Dithmar et al. 1997). To examine this possibility, cultures were setup from two mouse models of retinal degeneration, the *rds* (retinal degeneration slow) (Reuter and Sanyal 1984) and the rhodopsin knockout (*rho<sup>-/-</sup>*) mouse models (Humphries, Rancourt et al. 1997). Mice lacking both alleles for a functional *rds* gene have disrupted outer segments, which upon 2-3 months of age undergo further retinal degeneration leading to reactive gliosis. Similarly, disruption of the *rhodopsin* locus, leads to extensive retinopathy with signs of reactive gliosis. Cells derived from the CE of both the *rds* and the *rho<sup>-/-</sup>* mice were able to generate neurospheres, as found in wildtype animals. However, cells from the CNR of either genotype failed to produce neurospheres (Figure 4.3).

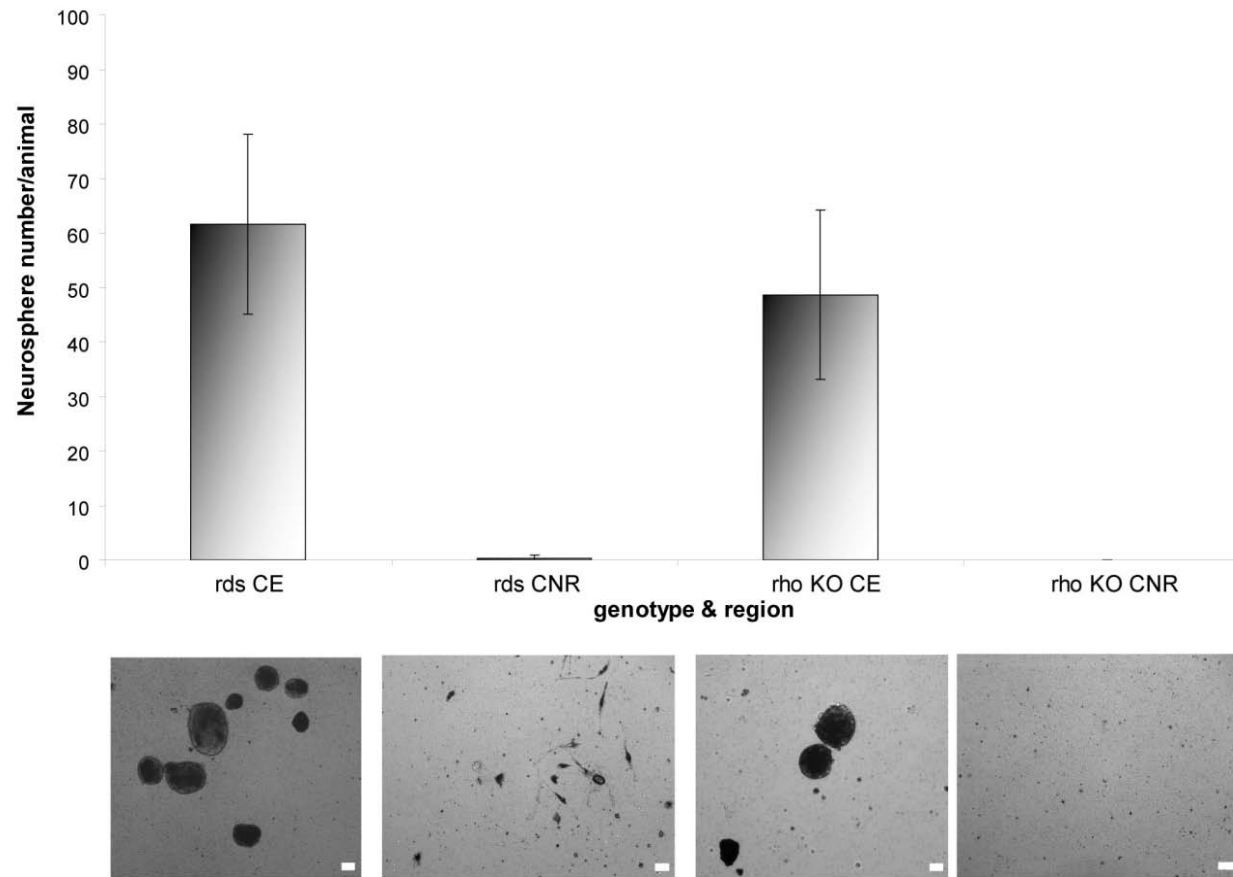
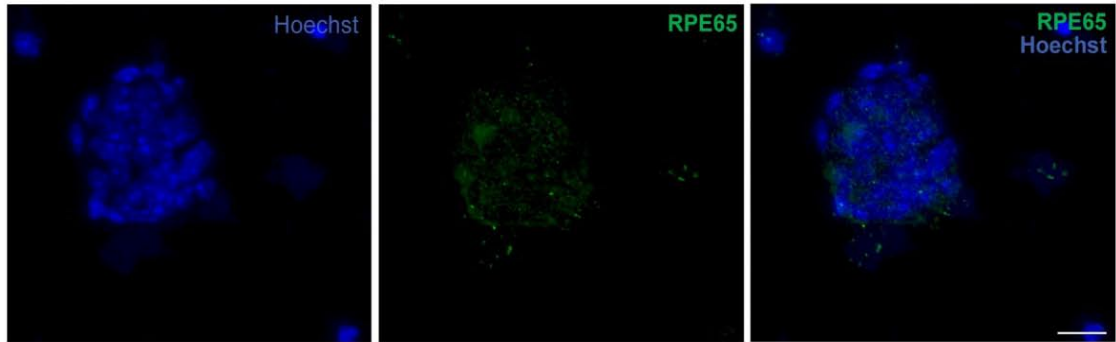


Figure 4.3 Neurosphere formation from rds and  $Rho^{-/-}$  CE and CNR. CE and CNR cells were isolated from rds<sup>-/-</sup> and Rho adult mice and cultured in vitro in the presence of NGM for 7 days. Histogram shows the number of neurospheres obtained per well per animal for each genotype and region. No neurospheres were observed in both CNR-isolated cell cultures of these retinal degeneration mouse models. N=4 independent experiments, n=4 eyes for each mouse model per experiment, error bars; SD, scale bars; 10  $\mu$ m.

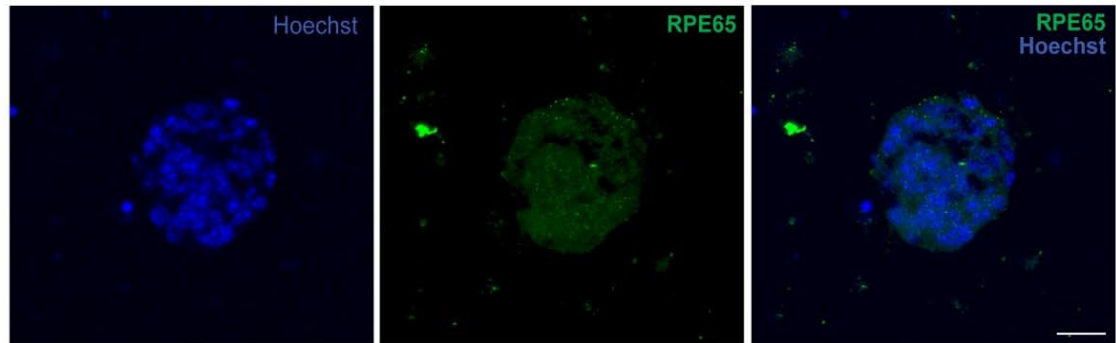
#### 4.2.2 Neurospheres from the adult Chx10<sup>orJ/orJ</sup> CNR rarely contain RPE cells

These findings suggest that the presence of neurosphere-forming cells in the CNR is caused by lack of Chx10 rather than an injury or degeneration response *per se*. therefore, based on the literature indicating trans-differentiation events occurring in the microphthalmic murine retina (Rowan S 2004), it was assessed if the de-pigmented neurospheres contain cells that have acquired an RPE-like cell fate. Seven day-old neurospheres deriving from the Chx10-deficient CE and CNR and wildtype CE were immunolabelled with RPE65, a marker expressed in RPE cells (Figure 4.4). Rarely, scattered RPE65<sup>pos</sup> cells were found in mutant CNR- but not in the CE- derived neurospheres (Figure 4.4b, c). Since no RPE65 expression was detected in the wildtype CE-derived neurospheres (Figure 4.4a), RPE cells are not strong candidates for giving rise to the mutant CNR neurospheres.

**a) Wildtype CE**



**b) *Chx10<sup>orJ/orJ</sup>* CE**



**c) *Chx10<sup>orJ/orJ</sup>* CNR**

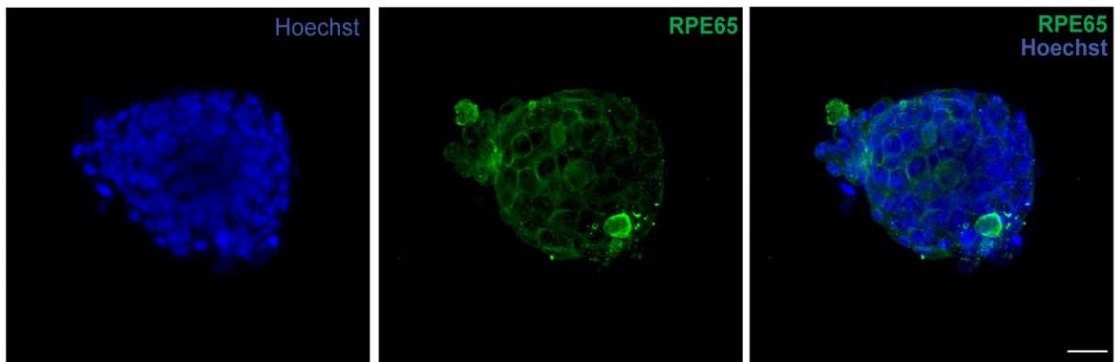


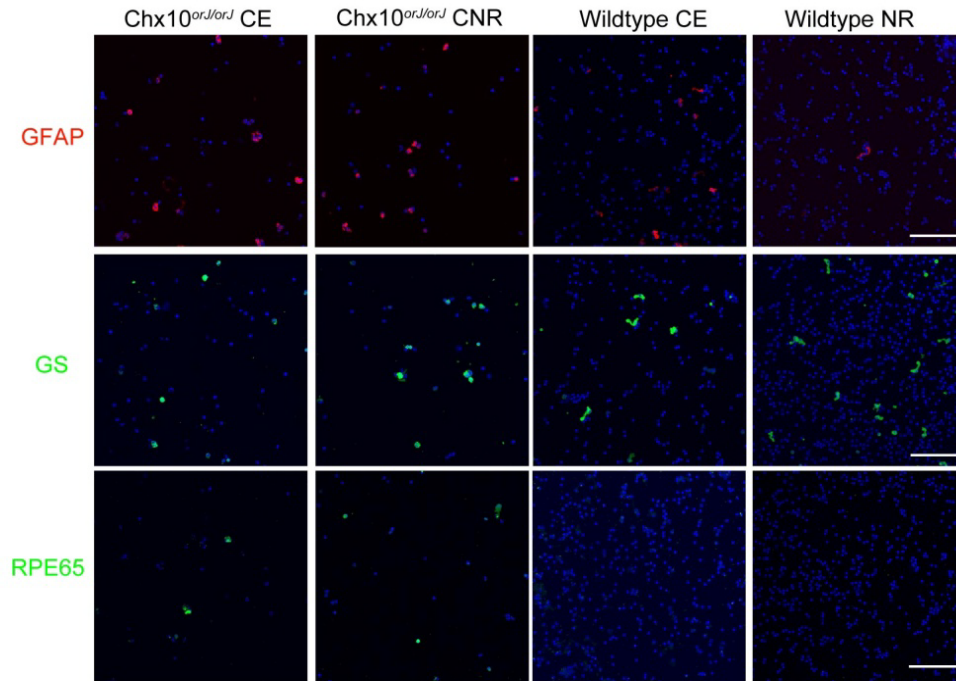
Figure 4.4 Adult neurospheres rarely express RPE markers. Adult wildtype (a), *Chx10<sup>orJ/orJ</sup>* CE (b) and wildtype and *Chx10<sup>orJ/orJ</sup>* CNR (c) cells were dissociated and allowed to form neurospheres for 7 days in NGM. Neurospheres were immunolabelled with  $\alpha$ -RPE65 (green) and counterstained with Hoechst dye.

N=2 independent neurosphere cultures for each genotype.

### 4.2.3 Adult $Chx10^{orJ/orJ}$ retina has an increased population of Müller/progenitor-like cells

In the light of the new results, it was essential to characterise the primary population that gave rise to neurospheres *in vitro*. Since mutant CNR cells that form neurospheres are de-pigmented, the presence of RPE65<sup>pos</sup> cells, even in very low numbers, primed me to investigate the proportion of GFAP, GS and RPE65 positive cell populations from wildtype CE and CNR as well as  $Chx10$ -deficient CE and CNR (Figure 4.5). The level of cells immune-reactive for RPE65 within the dissected CNR was  $7\pm 2\%$ , whereas RPE65 positive cells were not detected in the wild type CNR. Notably, mutant CE cells also expressed RPE65 ( $9\pm 2\%$ ), whereas the wildtype CE did not, suggesting that the expression of this marker does not tag cells with neurosphere forming potential. A markedly increased number of GFAP<sup>pos</sup> and GS<sup>pos</sup> cells from the acutely dissociated  $Chx10^{orJ/orJ}$  CNR compared to the wild type (GFAP:  $35\pm 6\%$  versus  $1\pm 1\%$ , respectively; glutamine synthetase:  $44\pm 7\%$  versus  $4\pm 1\%$ , respectively, Figure 4.5a, b) was observed. Increased numbers of glutamine synthetase- and GFAP-positive cells were also observed in the  $Chx10^{orJ/orJ}$  CE, compared with wildtype CE (GFAP:  $33\pm 4\%$  versus  $4\pm 1\%$ , respectively; glutamine synthetase:  $21\pm 2\%$  versus  $5\pm 1\%$ , respectively, Figure 4.5a, b).

**a**



**b**

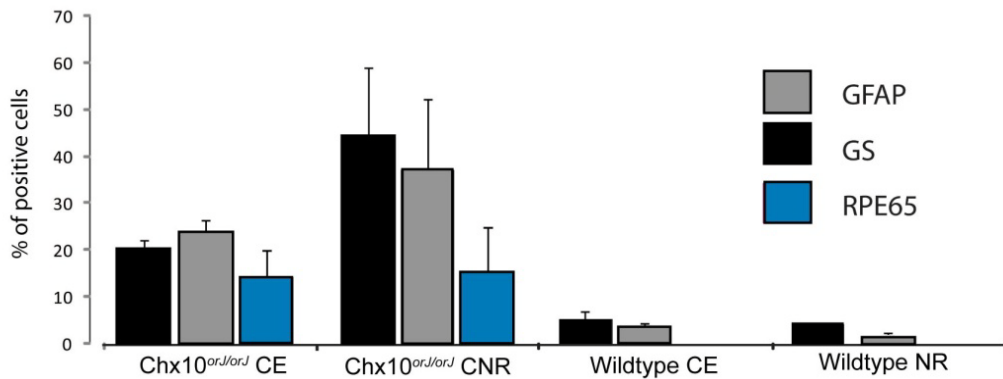


Figure 4.5 Neurosphere-forming cells in the CNR may be Müller glia.

(a) Adult wildtype, Chx10<sup>orJ/orJ</sup> CE and wildtype and Chx10<sup>orJ/orJ</sup> CNR cells were dissociated and fixed immediately on glass coverslips. Representative planes of view for cells immunolabelled for  $\alpha$ -GFAP (red, upper row),  $\alpha$ -GS (green, middle row) and  $\alpha$ -RPE65 (green, lower row). Scale bar: 10  $\mu$ m.

(b) Cell counts were performed and the percentage of cells was calculated from each region and genotype for cells scored positive for GFAP (grey bar), GS (black bar) and RPE65 (blue bar).

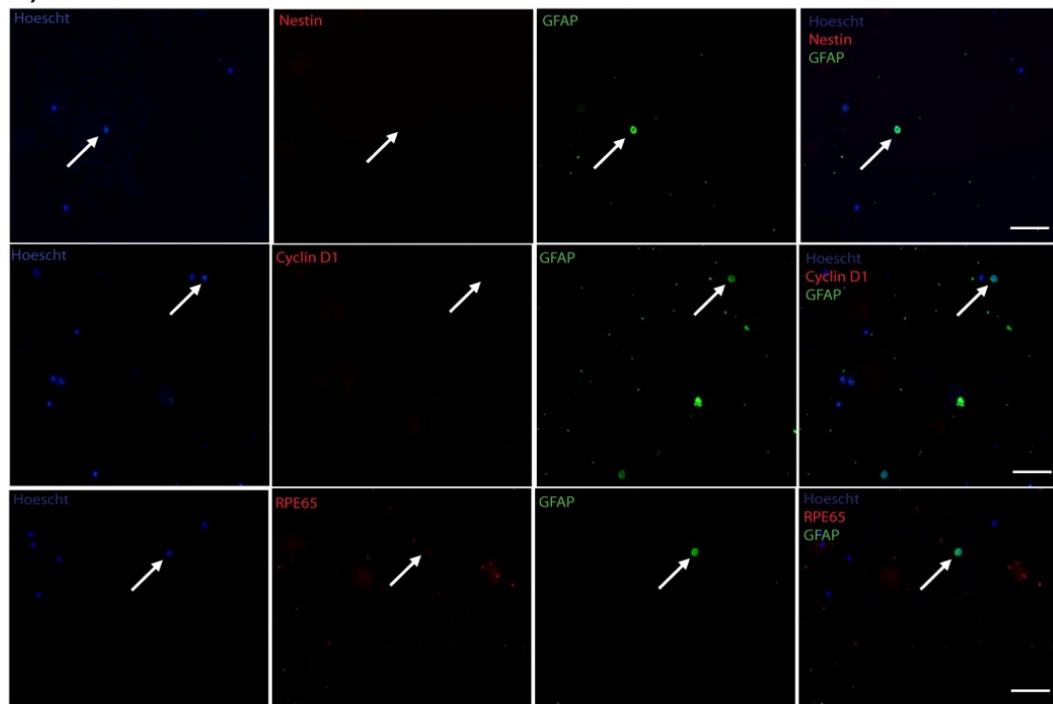
N=3, error bars; SEM.



Further characterising the neurosphere-forming cells, dissociated cells deriving from wildtype CE, Chx10<sup>orJ/orJ</sup> CE and Chx10<sup>orJ/orJ</sup> CNR were cultured in neurosphere-forming conditions for 24 and 48 hours, attempting to identify potential early neurosphere-forming progenitor cells *in vitro*. Wildtype CE cells, after being cultured in NGM for 24 hours, were co-immunolabelled with Nestin and GFAP, Cyclin D1 and GFAP and RPE65 and GFAP (Figure 4.6a). No double-labelled cells were detected under these conditions but I only observed GFAP<sup>pos</sup> cells. Similarly, cells that were in culture for 48 hours, showed clusters of GFAP<sup>pos</sup> only cells, but no RPE65<sup>pos</sup> or double-labelled cells were observed (Figure 4.6b). When Chx10<sup>orJ/orJ</sup> CE-derived cells were cultured for 24 hours under the same conditions, the same pattern of immune-reactivity was detected, with cells only being positive for GFAP with no evidence of co-localisation with RPE65, Cyclin D1 and/or Nestin (Figure 4.7a). After 48 hours in culture, cells also showed clusters of cells that were only GFAP<sup>pos</sup>, with no apparent RPE65 immuno-reactivity (Figure 4.7b). Strikingly, cells deriving from the Chx10<sup>orJ/orJ</sup> CNR, when cultured in neurosphere-forming conditions for 24 hours, were positive for GFAP and RPE65 but not for Cyclin D1 (Figure 4.8a). Interestingly, there were cells co-immunolabelled with Nestin and GFAP and also some clusters of cells showed RPE65<sup>pos</sup> cells adjacent to GFAP<sup>pos</sup> cells with no apparent co-localisation. Culturing Chx10<sup>orJ/orJ</sup> CNR cells for 48 hours conferred cell clusters positive for GFAP but not RPE65 and clusters of cells co-immunolabelled for Nestin and GFAP (Figure 4.8b). These data indicate the presence of neural progenitors with the central mutant retina of the adult mouse that also possesses Müller glia characteristics (see table 4.1 in discussion of this chapter).

## Wildtype CE

a)



b)

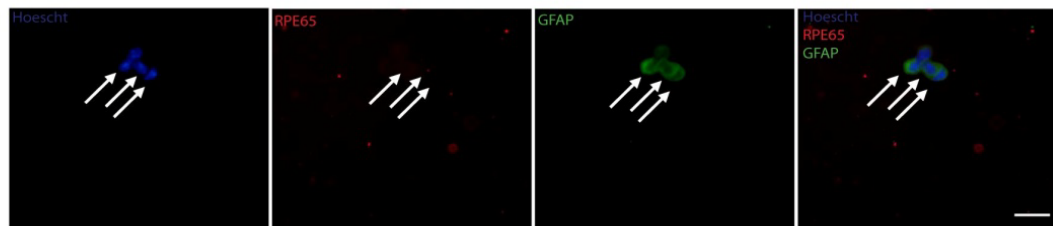


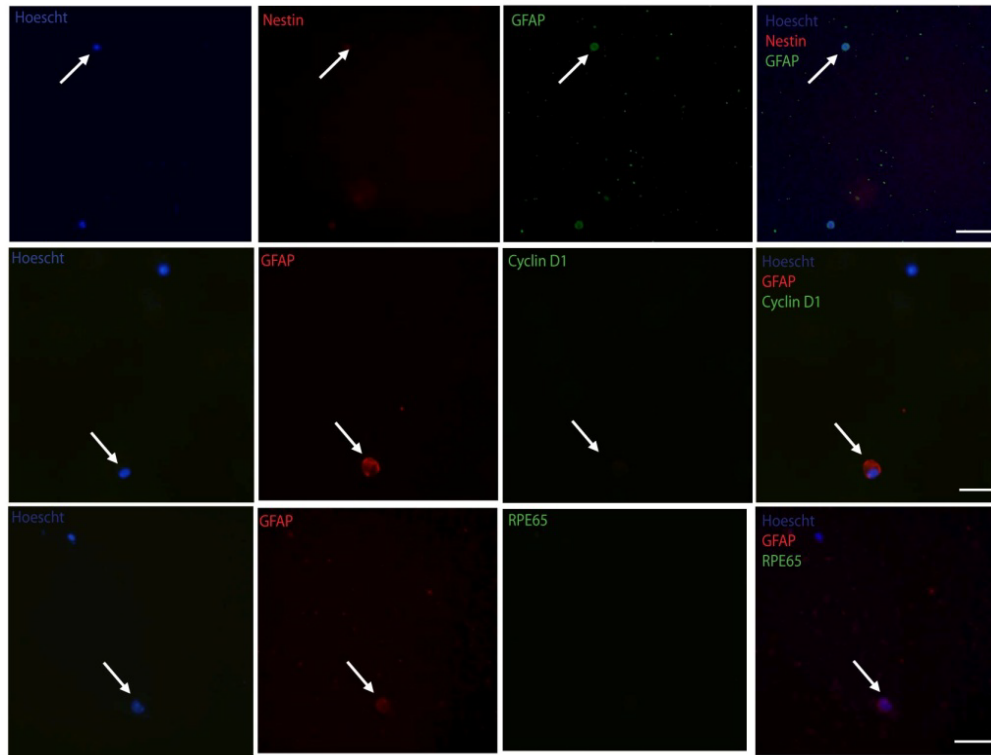
Figure 4.6 Wildtype CE-derived neurosphere-forming cells are potentially progenitors expressing Müller glia cells.

Wildtype adult CE neurosphere-forming cells were isolated from the CE were cultured for (a) 24 hours day and (b) 48 hours in vitro in the presence of NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for Müller glial/progenitor marker GFAP, but no co-localisation was observed with RPE65, CyclinD1 and Nestin.

Scale bar: 10  $\mu$ m

**Chx10<sup>orJ/orJ</sup> CE**

a)



b)

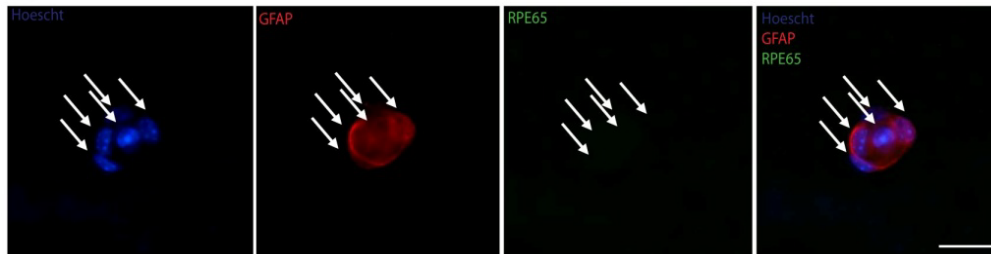


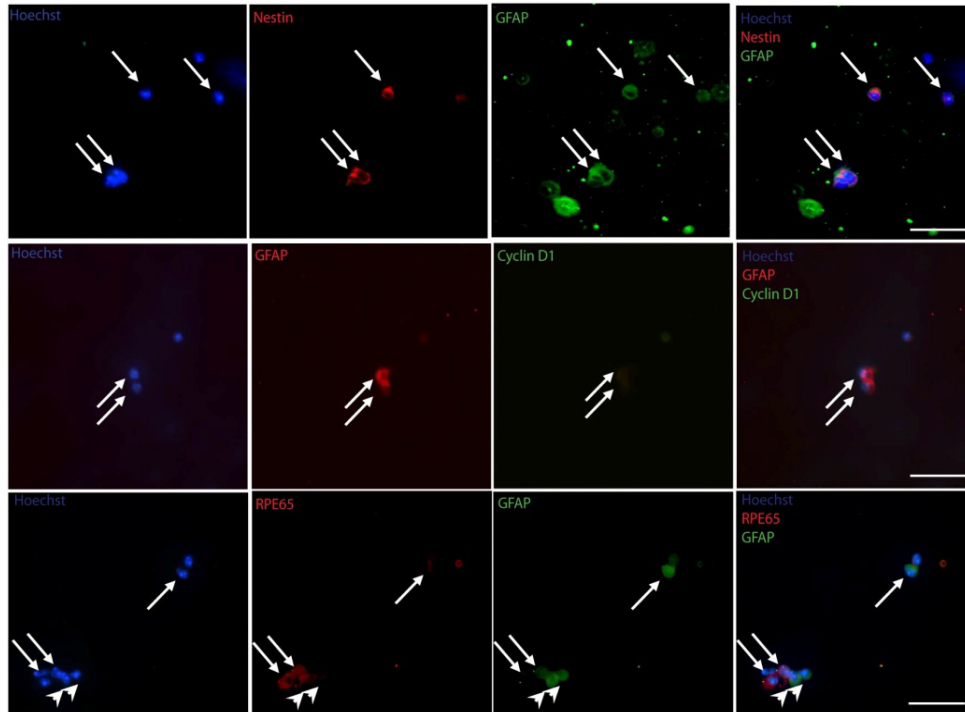
Figure 4.7 Chx10<sup>orJ/orJ</sup> CE-derived neurosphere-forming cells are potentially progenitors expressing Müller glia cells.

Chx10<sup>orJ/orJ</sup> adult CE neurosphere-forming cells were isolated from the CE and cultured for (a) 24 hours day and (b) 48 hours in vitro in the presence of NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed clusters of cells at 48 hours, positive for Müller glial/progenitor marker GFAP, but no co-localisation was observed with RPE65, CyclinD1 and Nestin.

Scale bar: 10  $\mu$ m

**Chx10<sup>orJ/orJ</sup> CNR**

a)



b)

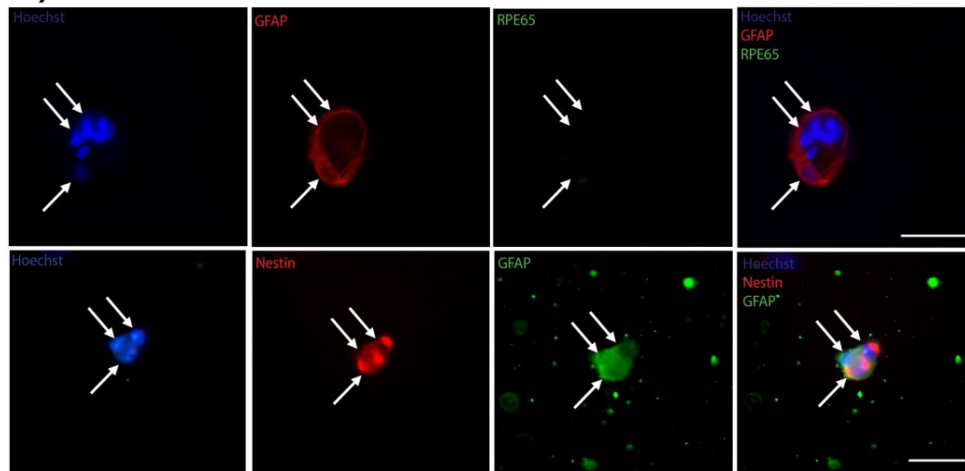


Figure 4.8 Chx10<sup>orJ/orJ</sup> CNR-derived neurosphere-forming cells are potentially progenitors expressing Müller glia cells.

Chx10<sup>orJ/orJ</sup> adult CNR neurosphere-forming cells were isolated from the CNR and cultured for (a) 24 hours day and (b) 48 hours in vitro in the presence of NGM. Indirect fluorescent-conjugated immunocytochemistry and confocal imaging showed cells positive for Müller glial/progenitor marker GFAP, and cells detected co-expressing GFAP and Nestin but not GFAP<sup>POS</sup> RPE65<sup>POS</sup>, or GFAP<sup>POS</sup> Cyclin D1<sup>POS</sup> cells. Scale bars: 10  $\mu$ m.

#### 4.2.4 Cell cycle regulators are altered in adult-derived Chx10<sup>orJ/orJ</sup> CNR neurospheres

So far, I have attempted to characterise the cells from mutant CE and CNR and to compare them with those from wildtype CE. In culture, cells from these regions proliferate extensively giving rise to neurospheres, but *in vivo*, these cells are apparently quiescent and halted in a cell cycle stage. Activation of the cell cycle might be due to intrinsic or extrinsic cues or a combination of both. Adult wildtype and Chx10-deficient CE as well as Chx10-deficient adult CNR cells could alter their cell cycle enhancer and inhibitor gene expression levels and protein levels upon culture in the presence of growth factors. To investigate this idea quantitative real-time PCR was performed for cell cycle inhibitors and enhancers that are known to be affected in Müller glia cell activation upon exposure to neurotoxin substances (Dyer MA 2000). These were p27<sup>Kip1</sup> and Cyclin D3, with the former being a cell cycle progression inhibitor while the latter an enhancer. In figures **4.9a** and **4.9b**, gene expression levels for the above-mentioned proteins were compared in RNA transcripts extracted from neurospheres formed from Chx10<sup>orJ/orJ</sup> CE and CNR as well as wildtype CE cells in the presence of NGM after 7 days in culture. Relative expression analysis showed a significant decrease for p27<sup>Kip1</sup> (relative expression for Chx10<sup>orJ/orJ</sup> CNR versus Chx10<sup>orJ/orJ</sup> CE and versus wildtype CE showed a 7-fold and 6-fold decrease, respectively) and Cyclin D3 (relative expression for Chx10<sup>orJ/orJ</sup> CNR versus Chx10<sup>orJ/orJ</sup> CE and versus wildtype CE showed a 1,025-fold and 193-fold decrease, respectively).

Also, it was hypothesised that since neurosphere formation can be considered as a mechanism of aberrant cell growth, there is potentially alteration in tumour suppressor genes expression. Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN) was considered an appropriate candidate since its absence cause brain neurospheres to have an increased proliferation rate linked

to larger neurospheres being formed *in vitro* (Li L 2002; Li L 2003; Stiles B 2004). In Figure 4.9c, I investigated whether *PTEN* was altered at the gene expression level. Messenger RNA transcripts were extracted from neurospheres formed from Chx10<sup>orJ/orJ</sup> CE and CNR as well as wildtype CE cells in the presence of NGM *in vitro*. Relative expression analysis showed an increase in *PTEN* transcripts between Chx10-deficient and wildtype (relative expression for Chx10<sup>orJ/orJ</sup> CNR and Chx10<sup>orJ/orJ</sup> CE versus wildtype CE showed a 1.9-fold and 2.8-fold increase, respectively).

Cyclin D1, another cell cycle enhancer protein showed a trend of upregulated gene expression but with no statistical significance (Figure 4.9d). In general, primary neurospheres cultured for 7 days in the presence of growth factors showed a decrease in gene expression levels of *Kip1* and *Cyclin D3* with an up-regulation of *PTEN* and a trend of *Cyclin D1* upregulation.

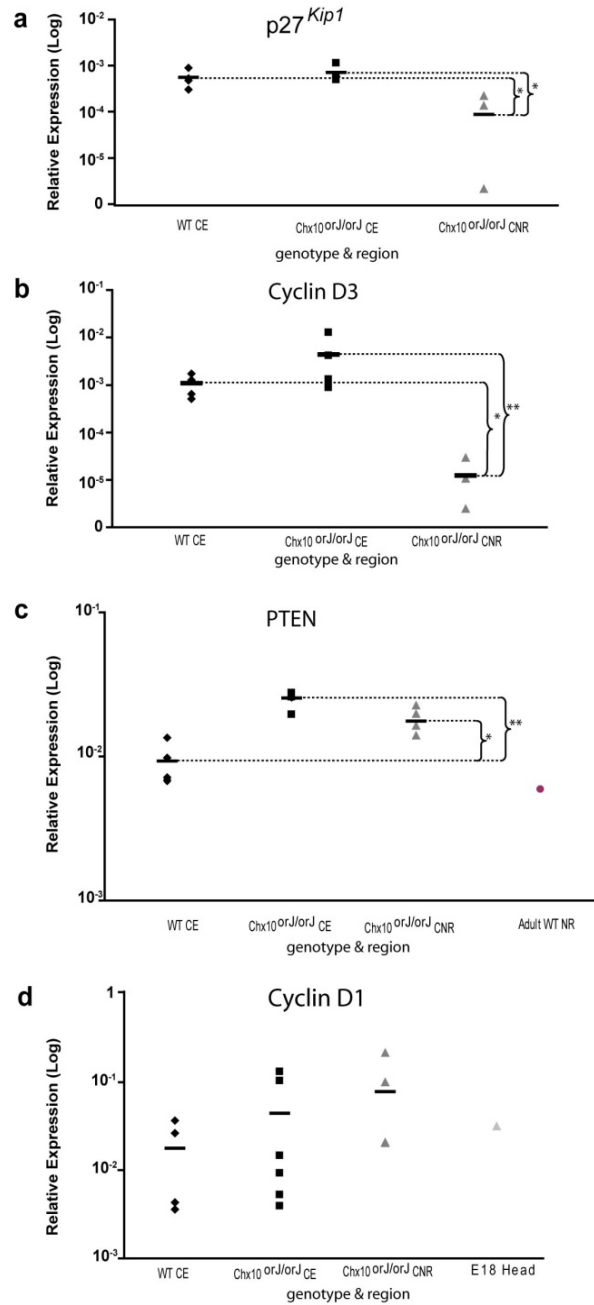


Figure 4.9 Neurospheres from Chx10-deficient adult CNR express gene expression levels similar to activated Müller glial/progenitor shown upon injury. Quantitative Real-Time PCR analysis for expression of *p27<sup>Kip1</sup>* (a), Cyclin D3 (b), PTEN (c) and Cyclin D1 (d) in neurospheres derived from Chx10<sup>orJ/orJ</sup> CE and CNR, and wildtype CE (cultured for 7 days in vitro as proliferating neurospheres in NGM), and adult neural retina or mRNA extracted from E18.5 head tissue for comparison. Graphs show relative expression levels from four independent experiments normalized to Gapdh (N=4 eyes from each genotype per experiment); Mann-Whitney and Independent Student's T-test \*\* P<0.05, \* P<0.01.

#### 4.2.5 Co-expression of CRALBP and p27<sup>Kip1</sup> in adult wildtype and Chx10<sup>orJ/orJ</sup> CNR

Downregulation of p27<sup>Kip1</sup> and Cyclin D3 were consistent with data from other researchers that identified a population of Müller glia to divide following retinal injury (Levine, Close et al. 2000). Since it was shown that Chx10<sup>orJ/orJ</sup> adult CNR progenitors are not part of a reactive gliosis effect I assessed if p27<sup>Kip1</sup> would be altered in the mutant retina both on the protein and gene expression levels as well as *Cyclin D3* gene expression levels (Dyer MA 2000). Figure 4.10a, shows p27<sup>Kip1</sup> protein expression profile in wildtype and Chx10<sup>orJ/orJ</sup> adult retinae, indicating its expression within the Müller glia CRALBP<sup>pos</sup> cells. Since protein levels for this protein appeared to be similar, both showing the same co-localisation of CRALBP and p27<sup>Kip1</sup>, quantitative analysis was performed by employing real-time PCR on *Cyclin D3* and p27<sup>Kip1</sup> gene expression levels on mRNA transcripts extracted from dissociated cells from adult wildtype and Chx10<sup>orJ/orJ</sup> CNR. Results indicated on Figure 4.10b, showed no apparent difference between p27<sup>Kip1</sup> gene expression levels between wildtype and Chx10<sup>orJ/orJ</sup>, a result resembling what it was indicated from protein immunoreactivity on Figure 4.10a.



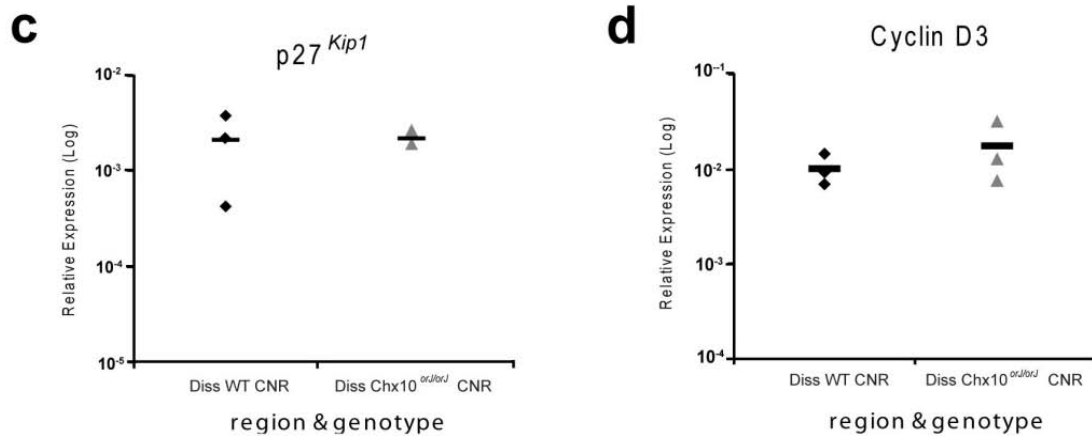
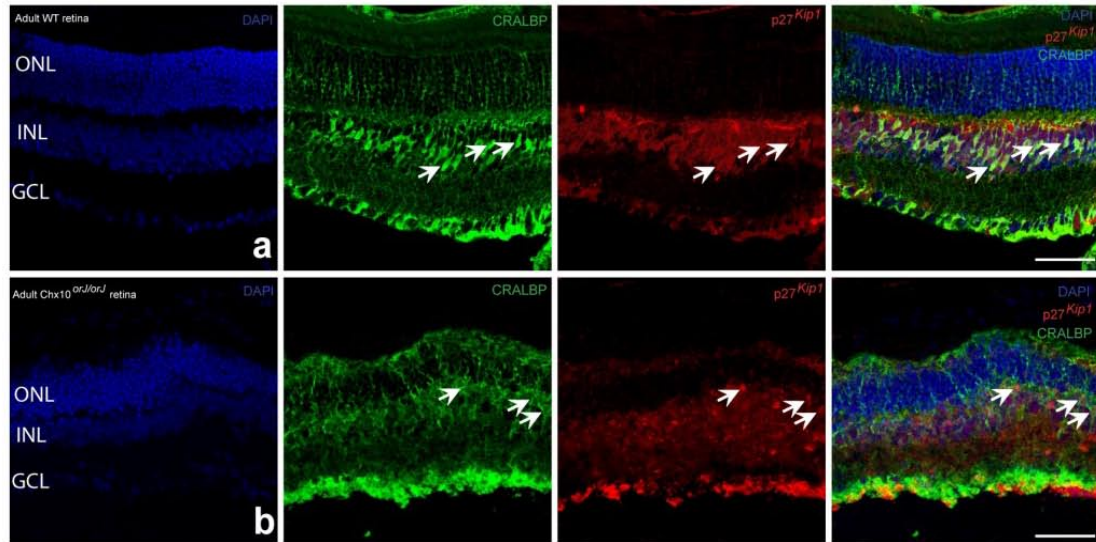


Figure 4.10 Protein and gene expression levels of p27<sup>kip1</sup> in the adult wildtype and Chx10-deficient adult retina. 12 μm saggital cryosections of adult wildtype (a) and Chx10-deficient (b) retina were co-immunolabelled with p27<sup>kip1</sup> (Cdkn1b) and CRALBP (Müller glia). Cells were counter-stained with DAPI. Gene expression analysis using quantitative Real-Time PCR on p27<sup>kip1</sup> (c), and Cyclin D3 (d) in dissociated wildtype and Chx10-deficient CNR. Scale bars = 10 μm. N=3-4 eyes from each genotype. Graphs show relative expression levels from four independent experiments normalized to Gapdh (N=4 eyes from each genotype per experiment).

Abbreviations: ONL=outer nuclear layer, INL=inner nuclear layer, GCL=ganglion cell layer.

#### **4.2.6 The adult Chx10<sup>orJ/orJ</sup> retina possesses a reactive Müller glia/progenitor cell population.**

Examination of the Müller glia cell population within the adult mutant and wildtype retina was performed through indirect immunolabelling of markers shown to preferentially be expressed in those cells. Figure 4.11 shows a montage of a whole adult mutant eye saggital cryo-section immunolabelled with GFAP. The latter is expressed at very low levels in the wildtype but lack of Chx10 cause an upregulation of this marker. Higher magnification photos show one of the parts of the retina where expression of GFAP is more intense. To investigate if these cells have the same characteristics as the ones obtained from acutely dissociated retina and the neurosphere-forming assay further co-immunolabelling experiments were performed on cells. Figure 4.12, shows representative comparative photos of saggital cryosections of adult wildtype and mutant CNR investigating the population of retinal cells immuno-reactive for Nestin (red) and CRALBP (green) [Figure 4.12a,b(i)] and Nestin (red) and GFAP (green) [Figure 4.12a,b(ii)] protein expression. Additionally, RPE65 and Vimentin expression was investigated in mutant retinae [Figures 4.12b (iii) & 4.12b (IV)].

Expression of GFAP and nestin within the adult wildtype CNR is very weak and often absent [Figure 4.12a (ii)]. Examination of adult saggital retinal section with nestin and CRALBP, as well as with nestin and GFAP, confirmed the low expression of nestin and GFAP and high expression of CRALBP in the CNR [Figure 4.12a (ii)]. Similarly, CRALBP was present within the inner nuclear layer [Figure 4.12b (i)]. In the central part of the mutant retina cells expressing both GFAP and RPE65 were observed [Figure 4.12b (iii)], while CRALBP<sup>pos</sup> cells were present, but no Vimentin<sup>pos</sup> cells were found [Figure 4.12b (IV)]. In general, in all sections examined cells expressed only Nestin or CRALBP or GFAP but never co-expressed these markers, according to the nuclear counterstaining.

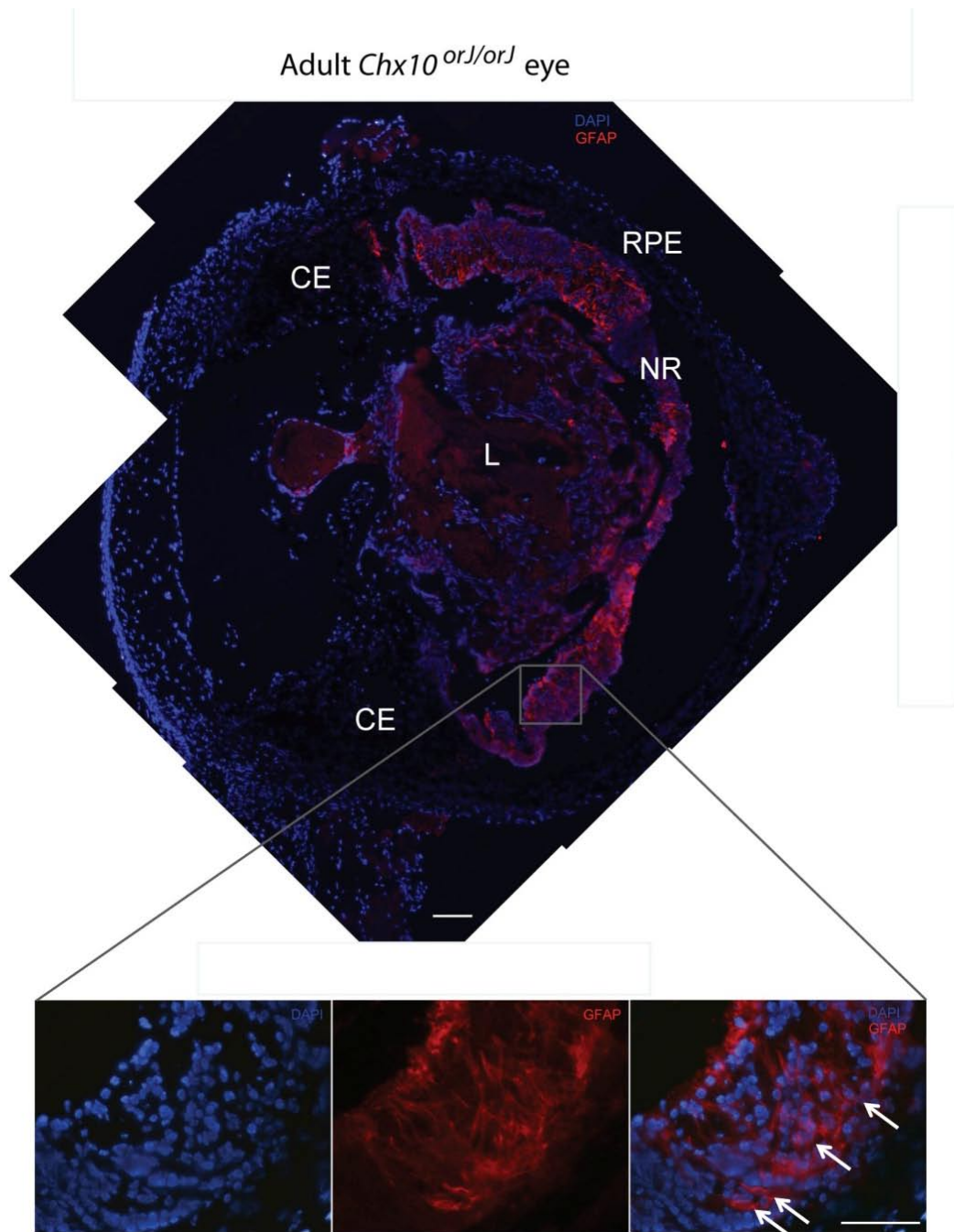
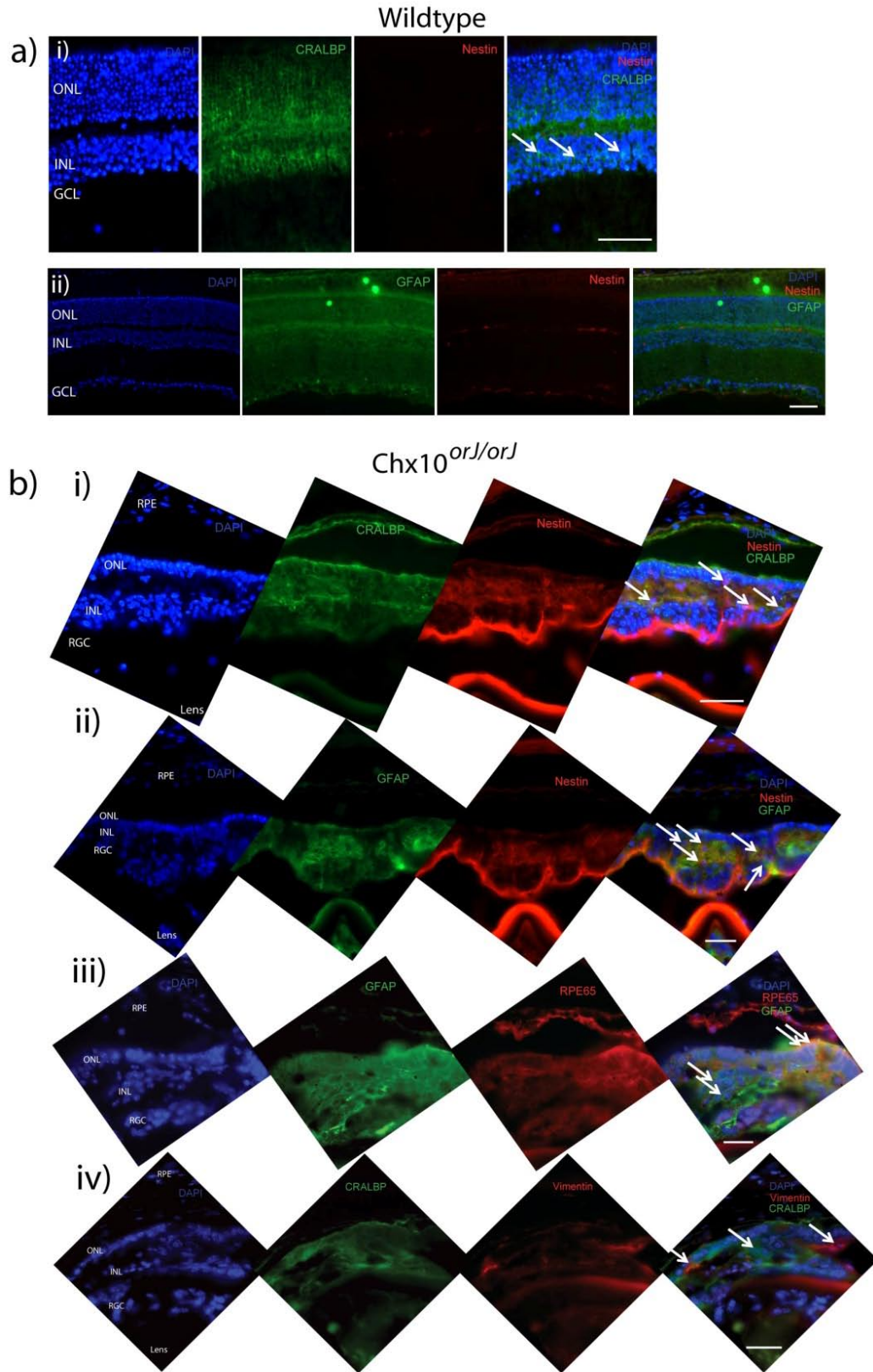


Figure 4.11 Expression of Müller glia markers in the adult *Chx10<sup>orJ/orJ</sup>* retina. A montage of an adult *Chx10<sup>orJ/orJ</sup>* retina immunolabelled for GFAP (red) protein expression. Higher magnification of a region of the central retina showing the high expression levels of GFAP. White arrows indicate cells positive for GFAP. Cells were counterstained with DAPI. Scale bars 10  $\mu$ m.

Abbreviations: CE=ciliary epithelium, RPE=retinal pigment epithelium, NR=neural retina, L=Lens





**Figure 4.12 Expression of Müller glia and progenitor markers in the adult wildtype and Chx10<sup>orJ/orJ</sup> CNR**

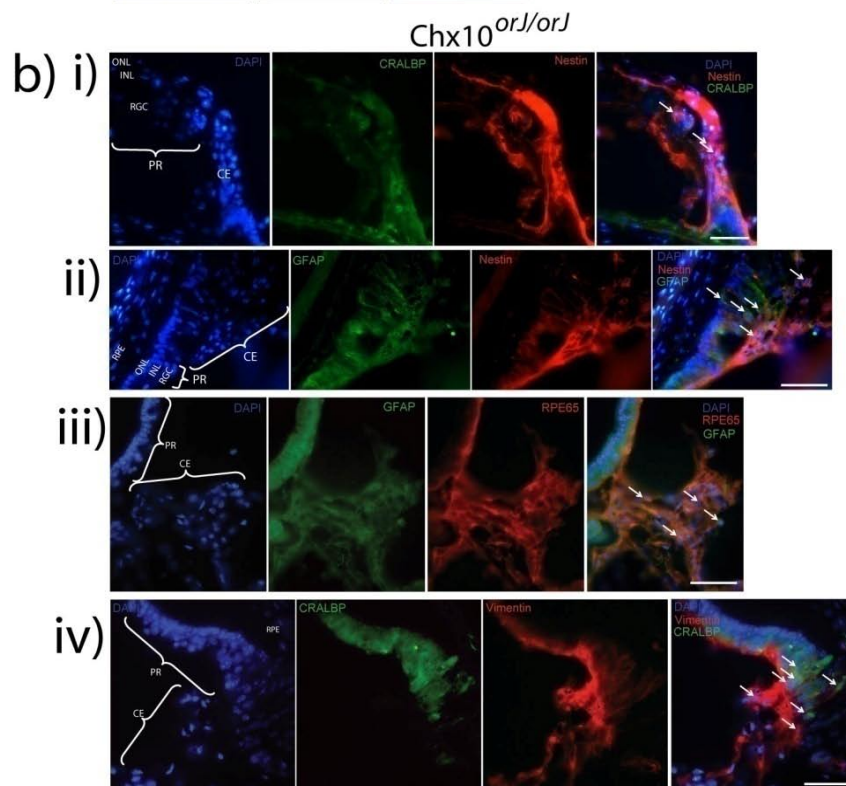
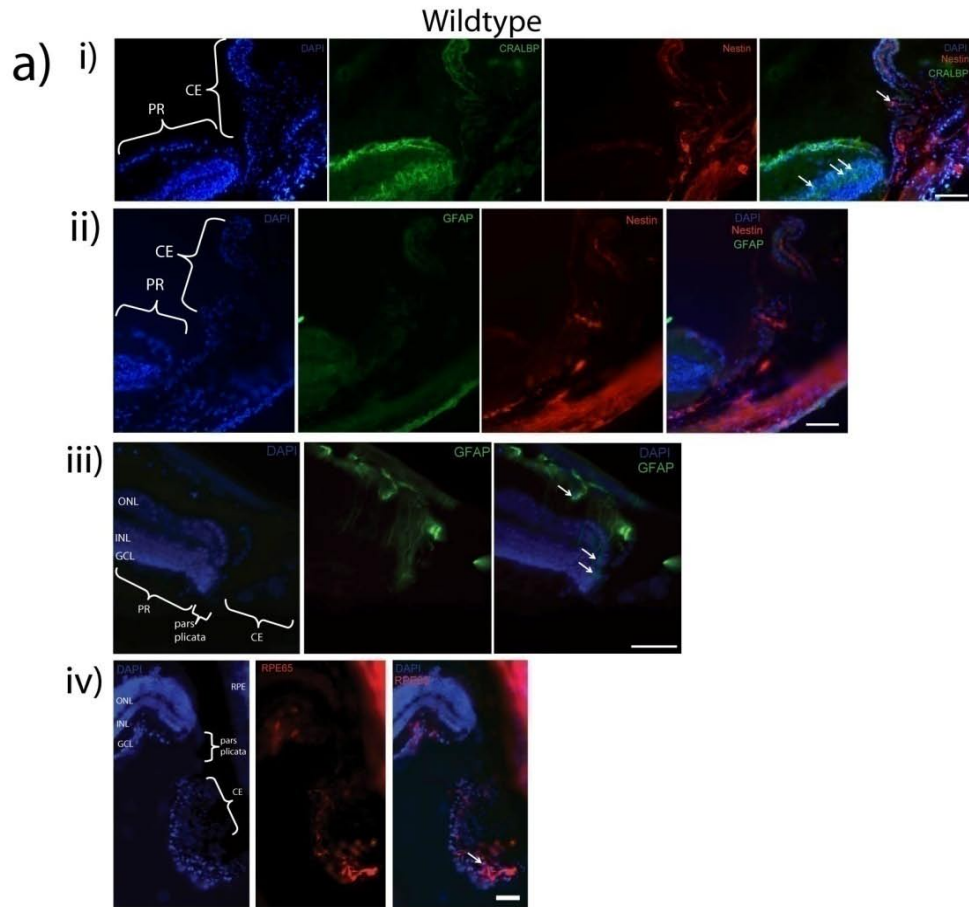
**(a)** Cryosections of adult wildtype central neural retina co-immunolabelled for Nestin (red) and CRALBP (green) **(i)** and Nestin (red) and GFAP (green) **(ii)**. No cells were observed expressing Nestin and GFAP, while CRALBP positive cells were located in the INL.

**(b)** Cryosections of adult Chx10<sup>orJ/orJ</sup> central neural retina co-immunolabelled for RPE65 (red) and CRALBP (green) **(i)**, Nestin (red) and GFAP (green) **(ii)**, RPE65 (red) and GFAP (green) **(iii)** and RPE65 (red) and CRALBP (green) **(iv)** protein expression. N=4 eyes from each genotype. Cells were counterstained with DAPI. Scale bars 10 µm. White arrows indicate cells positive for at least one of the two markers used per co-immunolabelling experiment. Cells readily expressed Nestin, GFAP and Vimentin, as well as CRALBP. RPE65 positive cells were rarely observed

Abbreviations: PR=peripheral retina, CE=ciliary epithelium, RPE=retinal pigment epithelium, ONL=outer nuclear layer, INL=inner nuclear layer, GCL=ganglion cell layer.

In a similar fashion, the margins of the adult wildtype and mutant retinae, as well as the CE were investigated for expression of retinal cells immuno-reactive for Nestin (red) and CRALBP (green) [Figure 4.13a,b(i)] and Nestin (red) and GFAP (green) [Figure 4.13a,b(ii), 4.13a(iii) for wildtype only] protein expression. Additionally, RPE65 and GFAP expression was investigated in mutant retinae [Figure 4.13b (iii) & 4.13a (iii) for wildtype] along with CRALBP (green) and Vimentin (red) [Figure 4.13b (IV)].

GFAP expression showed to be very weakly expressed within the wildtype retinal margins and ciliary processes [Figure 4.13a (i-iii)]. A number of coronal sections showed that GFAP is weakly but specifically expressed in cells at the extremes of the peripheral retina, the region resembling the CMZ in lower vertebrates. RPE65<sup>pos</sup> cells were found in the retinal pigment epithelium, as expected, while selective cells in the ciliary processes were positive for this marker along with possibly some astrocytes in the NFL [Figure 4.13a (IV)]. No cells were found positive for this marker in the peripheral retina. In contrast to the mutant central retina, the expanded mutant CE as well as the retinal margins showed an altered profile of protein expression (Figure 4.13b). RPE65 was expressed mainly in the CE and in the NFL, while GFAP was strongly expressed in the PR and weakly in the CE [Figure 4.13b (ii)]. Similarly, Vimentin was expressed more highly in the CE and astrocytic/nerve fibre layer of the retinal margin, with CRALBP staining a large number of cells within the peripheral retina [Figure 4.13b (IV)]. Interestingly, Nestin<sup>pos</sup> cells were found in the CE and NFL only, while CRALBP and GFAP immunoreactivity was again stronger in the retinal margins rather within the CE [Figure 4.13b (iii)].



**Figure 4.13 Expression of Müller glia and progenitor markers in the adult wildtype and  $Chx10^{orJ/orJ}$  peripheral retina and CE**

**(a)** Cryosections of adult wildtype peripheral neural retina and CE co-immunolabelled for Nestin (red) and CRALBP (green) **(i)** and Nestin (red) and GFAP (green) **(ii)** protein expression. Single immunolabelling with anti-GFAP in the peripheral margins of the retina **(iii)** and RPE65 (red) **(iv)**.

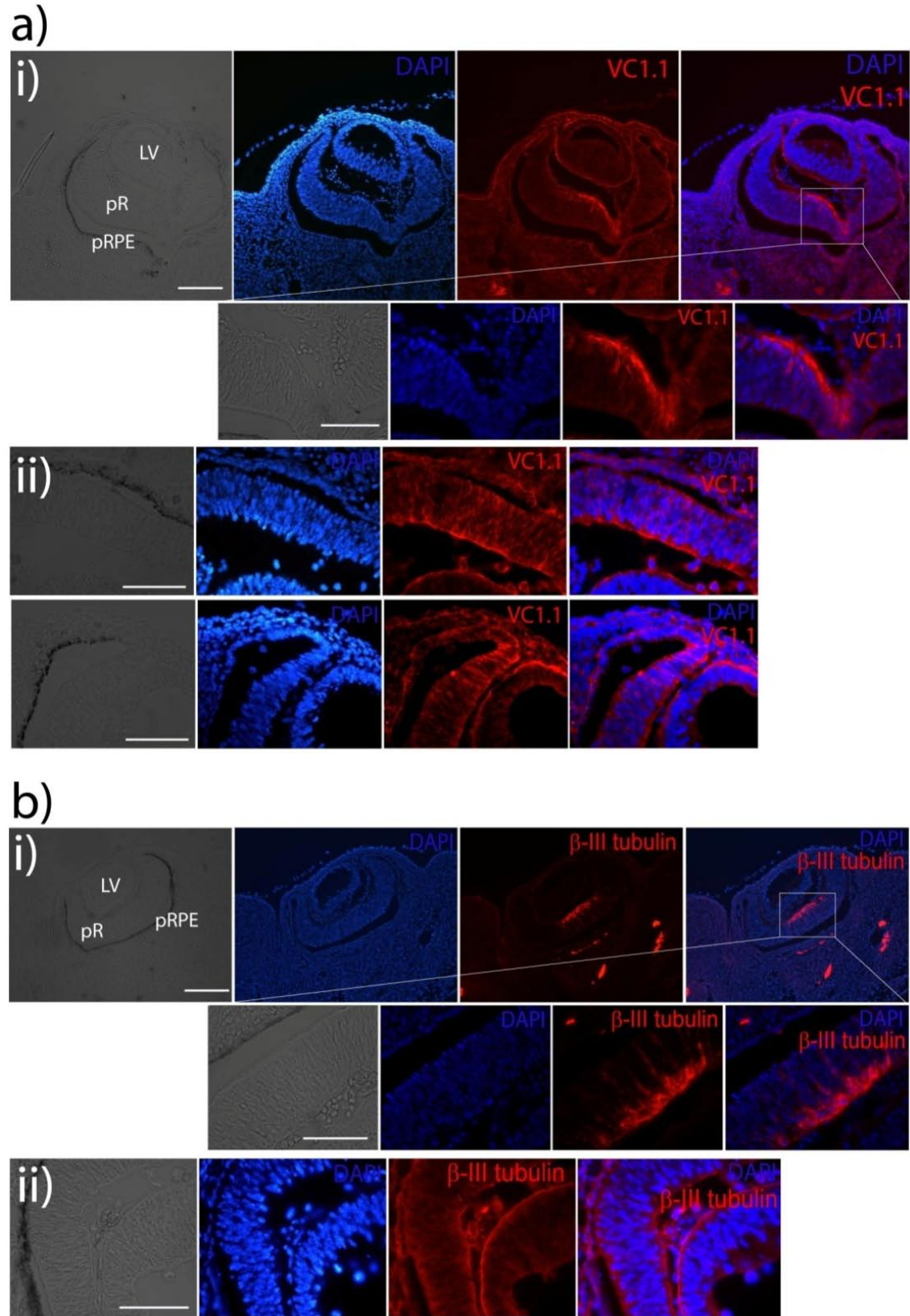
**(b)** Cryosections of adult  $Chx10^{orJ/orJ}$  peripheral neural retina and CE co-immunolabelled for Nestin (red) and CRALBP (green) **(i)**, Nestin (red) and GFAP (green) **(ii)**, RPE65 (red) and GFAP (green) **(iii)** and Vimentin (red) and CRALBP (green) **(iv)**. Cells were counterstained with DAPI. Scale bars 10  $\mu$ m. N=3 eyes from each genotype. White arrows indicate cells positive for at least one of the two markers used per co-immunolabelling experiment.

Abbreviations: PR=peripheral retina, CE=ciliary epithelium, RPE=retinal pigment epithelium, ONL=outer nuclear layer, INL=inner nuclear layer, GCL=ganglion cell layer.



#### **4.2.7 Abnormal presence of reactive Müller glia-like cells in the embryonic Chx10<sup>orJ/orJ</sup> retina.**

The aberrant GFAP expression in the adult mutant retina led me to hypothesise that since during retinal development, Müller glia appear postnatally, lack of Chx10 could involve the presence of this glial cell population to appear earlier than usual, possibly during embryonic retinogenesis; I chose an early embryonic E12.5 and a late E18.5 stage to compare the protein expression levels of Müller-expressing proteins to assess if lack of Chx10 causes an earlier-than-normal presence of these cells during retinal development. As Figure **4.14a(i)** indicates, upon immune-staining with VC1.1, a marker for amacrine and horizontal cells, there was expression of this marker on wildtype E12.5 in the centre and close to the optic nerve exit formation, while in the Chx10<sup>orJ/orJ</sup> retina, immuno-reactivity for this marker was weak Figure **4.14a(ii)**, and there was a consistent expression in the periphery. Similarly, immunolabelling of wildtype and Chx10<sup>orJ/orJ</sup> retinae with  $\beta$ -III tubulin, a retinal ganglion cell marker, was considerably weaker both in the central and peripheral mutant retina from the mutant while readily detected in the presumptive RGC layer of the wildtype [Figures **4.14b(i)** & **4.14b(ii)**].



**Figure 4.14 Expression of early retinal markers in the E12.5 embryonic wildtype and Chx10<sup>orJ/orJ</sup> retina**

**(a)** Coronal retinal cryosections of E12.5 wildtype **(i)** and Chx10<sup>orJ/orJ</sup> **(ii)** eyes immunolabelled for VC1.1 (red) protein expression. Inserts; higher magnification microphotographs of wildtype neuroblastic layer.

**(b)** Coronal retinal cryosections of E12.5 wildtype **(i)** and Chx10<sup>orJ/orJ</sup> **(ii)** eyes immunolabelled for  $\beta$ -III tubulin (red) protein expression. Inserts; higher magnification microphotographs of wildtype neuroblastic layer.

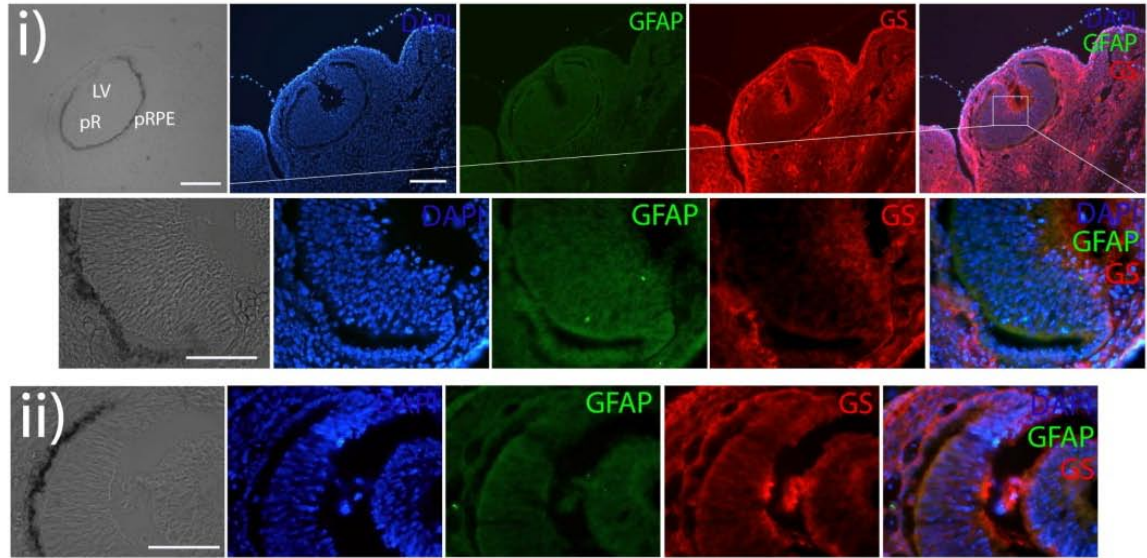
Cells were counterstained with DAPI. Scale bars 10  $\mu$ m. N=4 embryonic eyes from each genotype.

Abbreviations: LV= Lens vehicle, pR=presumptive retina, pRPE= presumptive retinal pigment epithelium.

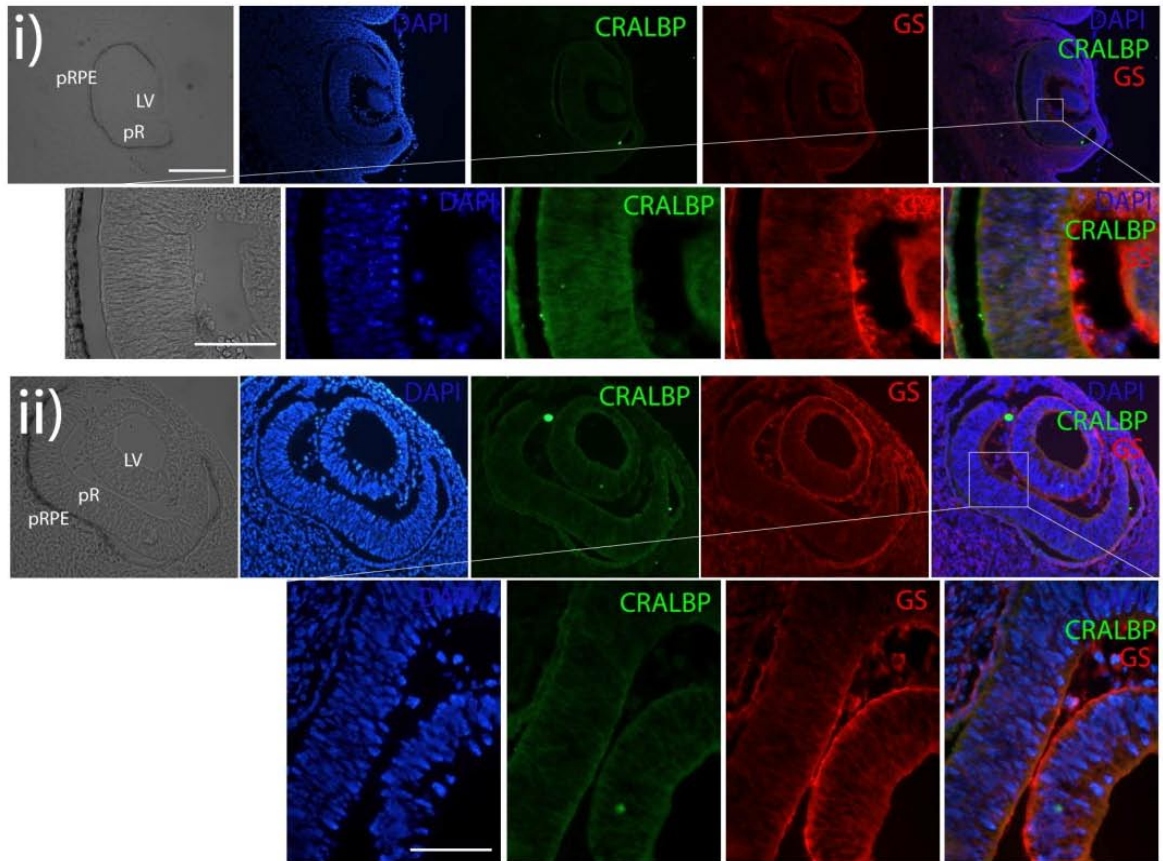
In addition, GFAP and GS expression was also absent from both wildtype and mutant retinae [Figures **4.15a (i)** & **4.15a (ii)**]. Expression of CRALBP was also examined in both wildtype and  $Chx10^{orJ/orJ}$  retinae during early embryonic development; representative photos from co-immunolabelling with GS and CRALBP ([Figures **4.15b (i)** & **4.15b (ii)**], showed no expression of either marker upon wildtype or  $Chx10$ -deficient early embryonic retinal development. As a conclusion, the above data show that absence of  $Chx10$  causes a delay in the expression of early-born retinal cells such as ganglion and amacrine cells.

The control used for E18.5 stage was VC1.1, showing again an abnormal expression pattern in the mutant in comparison to the adult retina (Figures **4.16a** & **b**). By examining the expression of Müller glia markers on the same embryonic stage as VC1.1, I found the presence of immunoreactivity against GFAP in the  $Chx10^{orJ/orJ}$  retina in comparison to its absence from the wildtype, but not for GS, while the wildtype showed no expression of either marker [Figure **4.17a (i)** & **4.17a (ii)**]. A closer examination showed the presence of GFAP<sup>pos</sup> cells in the retinal margins, with very weak or absent expression in the central retinal region. Expression of GS was limited only in the nerve fibre probably within developing astrocytes of the mutant and wildtype. Upon examination of CRALBP and GS immunoreactivity, at the same embryonic stage, no expression was detected in both wildtype and  $Chx10$ -deficient retinae, apart from in the NFL [Figure **4.17b (i)** & **4.17b (ii)**]. These results show that a GFAP-expressing population of cells is abnormally located in the retina of the  $Chx10^{orJ/orJ}$  late embryonic retina.

a)



b)



**Figure 4.15 Expression of Müller glia markers in the E12.5 embryonic wildtype and Chx10<sup>orJ/orJ</sup> retina**

**(a)** Coronal retinal cryosections of E12.5 wildtype **(i)** and Chx10<sup>orJ/orJ</sup> **(ii)** eyes co-immunolabelled for GS (red) and GFAP (green) protein expression. Inserts; higher magnification microphotographs of wildtype neuroblastic layer.

**(b)** Coronal retinal cryosections of E12.5 wildtype **(i)** and Chx10<sup>orJ/orJ</sup> **(ii)** eyes co-immunolabelled for GS (red) and CRALBP (green) protein expression.. Inserts; higher magnification microphotographs of wildtype neuroblastic layer.

Cells were counterstained with DAPI. Scale bars 10 µm. N=4 embryonic eyes from each genotype.

Abbreviations: LV= Lens vehicle, pR=presumptive retina, pRPE= presumptive retinal pigment epithelium.



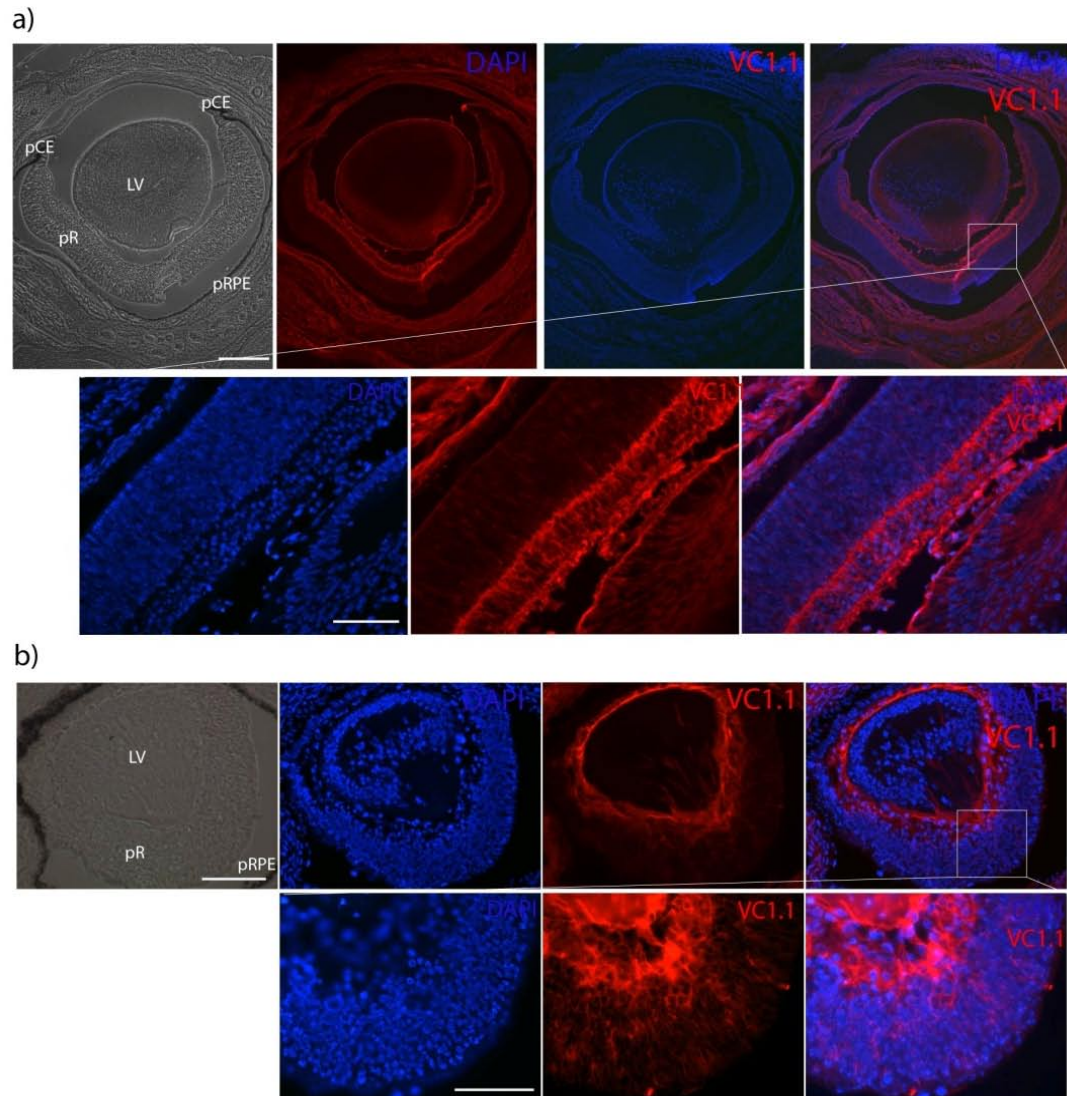


Figure 4.16 Expression of the retinal marker VC1.1 in the E18.5 embryonic wildtype and  $Chx10^{orJ/orJ}$  retina

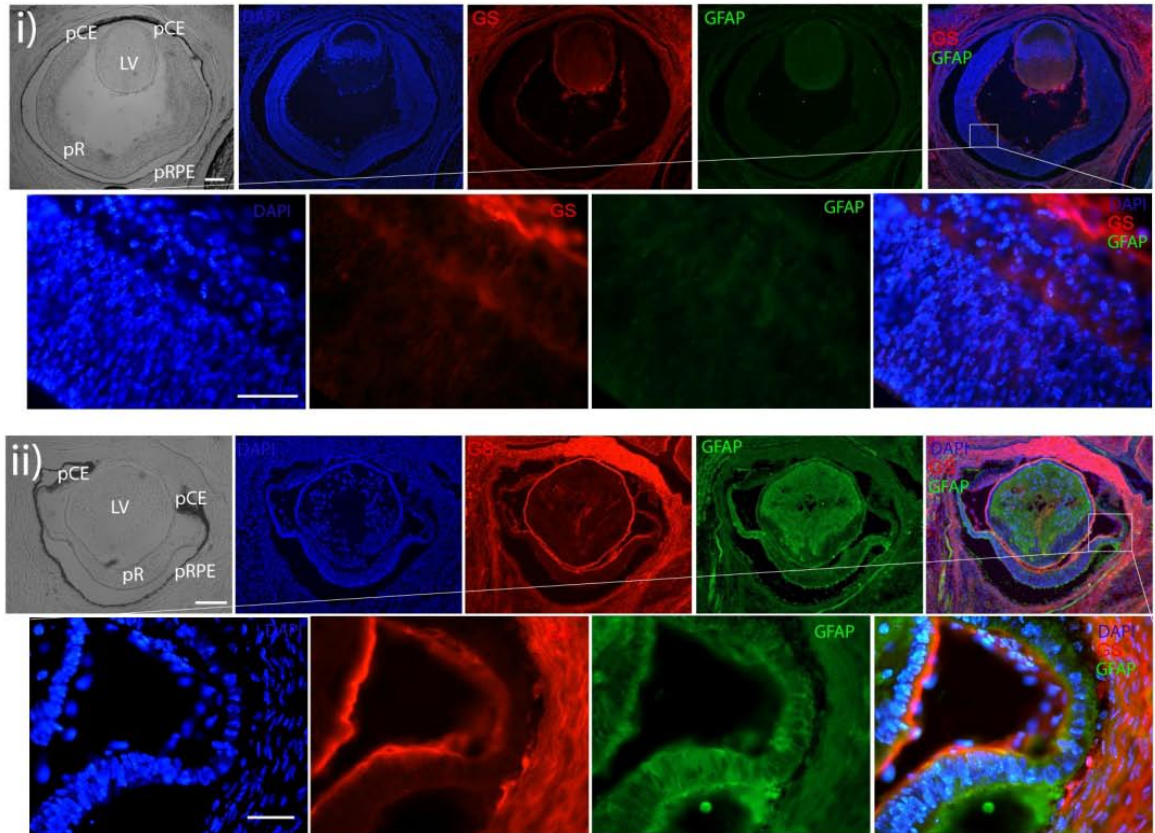
(a) Coronal retinal cryosections of E18.5 wildtype eye immunolabelled for VC1.1 (red) protein expression. Inserts; higher magnification microphotographs of wildtype neuroblastic layer.

(b) Coronal retinal cryosections of E18.5  $Chx10^{orJ/orJ}$  eye immunolabelled for VC1.1 (red) protein expression. Inserts; higher magnification microphotographs of mutant neuroblastic layer.

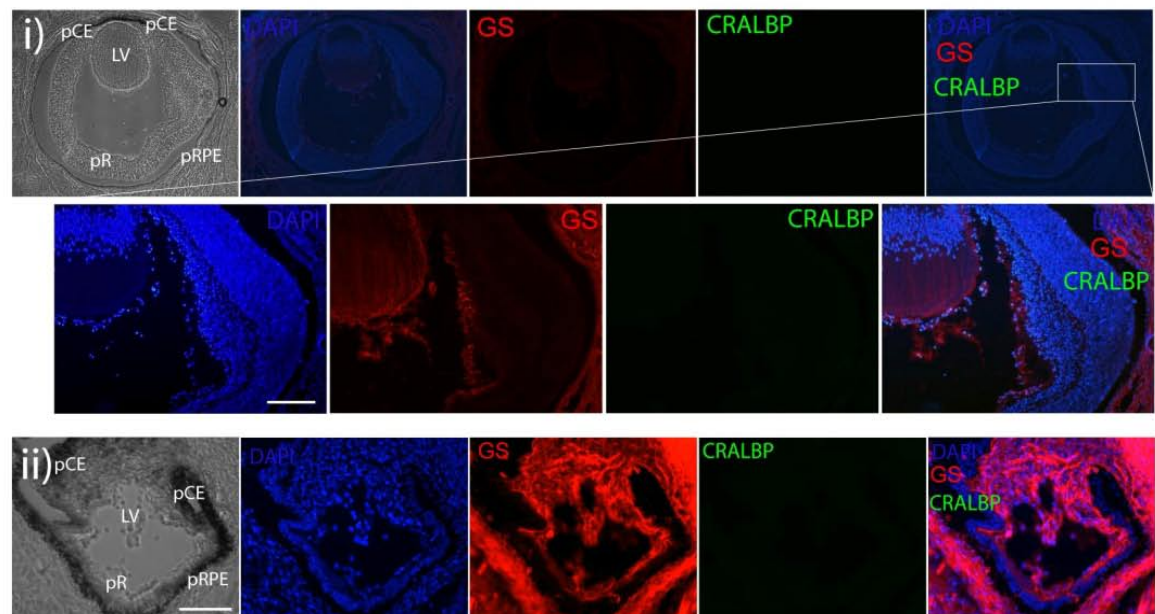
Cells were counterstained with DAPI. Scale bars 10  $\mu$ m. N=4 embryonic eyes from each genotype.

Abbreviations: LV= Lens vehicle, pR=presumptive retina, pRPE= presumptive retinal pigment epithelium, pCE= presumptive CE.

a)



b)





**Figure 4.17 Expression of Müller glia markers in the E18.5 embryonic wildtype and Chx10<sup>orJ/orJ</sup> retina**

**(a)** Coronal retinal cryosections of E18.5 wildtype **(i)** and Chx10<sup>orJ/orJ</sup> **(ii)** eyes co-immunolabelled for GS (red) and GFAP (green) protein expression. Inserts; higher magnification microphotographs of wildtype and mutant neuroblastic layers.

**(b)** Coronal retinal cryosections of E18.5 wildtype **(i)** and Chx10<sup>orJ/orJ</sup> **(ii)** eyes co-immunolabelled for GS (red) and CRALBP (green) protein expression. Inserts; higher magnification microphotographs of wildtype neuroblastic layer.

Cells were counterstained with DAPI. Scale bars 10 µm. N=4 embryonic eyes from each genotype.

Abbreviations: LV= Lens vehicle, pR=presumptive retina, pRPE= presumptive retinal pigment epithelium, pCE= presumptive CE.

## **4.3 Discussion**

### **4.3.1 Summary of findings**

In this chapter, I characterised the Chx10-deficient retinal CE and CNR progenitor cells with respect to their Müller glia-like profile, CE and CNR cells were examined after 1 and 2 days in neurosphere cultures in an attempt to characterise neurosphere-forming cells. Furthermore, adult, E12.5 and E18.5 wildtype and Chx10<sup>orJ/orJ</sup> retinal sections were examined in order to assess the altered nature of the mutant retinal progenitors.

From this study the following conclusions can be drawn;

- i. Neurospheres from the adult Chx10<sup>orJ/orJ</sup> CNR and CE express higher levels of Müller cell markers than wildtype CE.
- ii. The ability of the adult Chx10<sup>orJ/orJ</sup> CNR to give rise to neurospheres *in vitro* is not due to reactive gliosis.
- iii. Chx10<sup>orJ/orJ</sup> RPE cells do not form neurospheres
- iv. The adult Chx10<sup>orJ/orJ</sup> central neural retina possesses an abnormal Müller/progenitor population.
- v. The embryonic Chx10<sup>orJ/orJ</sup> neural retina shows a delayed production of early retinal populations but an abnormal presence of late-born Müller glia cells.

### **4.3.2 Increased number of Müller/progenitor cells within the neurospheres deriving from Chx10<sup>orJ/orJ</sup> CNR**

There is increasing evidence that glial cells in the brain and the Müller glia cells in the retina are progenitor-like (Doetsch et al. 1999; Dyer MA 2000; Fischer AJ

2001; Das, Mallya et al. 2006; Das AV 2006) and may give rise to neurons. Previously, our laboratory found that BrdU positive cells observed *in vivo* in the *Chx10<sup>orJ/orJ</sup>* CNR co-labelled with the Müller cell marker CRALBP (Dhomen NS 2006) and this prompted the examination of the expression of a number of Müller glial markers in the neurospheres. In this study, both CRALBP and GS were present in neurospheres arising from the CNR and CE of the *Chx10<sup>orJ/orJ</sup>* eye, consistent with their reported expression in Müller glia with progenitor-like properties in human foetal retina (Walcott JC 2003) while in the adult murine retina upon injury Müller glia re-enter the cell cycle and divide (Dyer MA 2000), an event occurring also in postnatal chicks (Fischer and Reh 2001; Fischer AJ 2003). Co-localisation with the neural progenitor markers was evident for these active Müller glia cells, like Pax6 with Nestin as well as with Chx10 and CASH-1, and CRALBP with Ki-67 (Walcott JC 2003; Rowan S 2004). In my system, CRALBP and GS were also expressed in wildtype CE-derived neurospheres, although staining was more limited. Neurospheres derived from all three regions also expressed the reactive glial marker, GFAP. Since labelling for GFAP and glutamine synthetase was consistently stronger in neurospheres derived from the *Chx10<sup>orJ/orJ</sup>* CNR, the differences in levels of Müller gene expression were examined by quantitative real time-PCR. Expression analysis confirmed the raised level of expression of two Müller glial markers, GFAP and ApoE (Shanmugaratnam et al. 1997), and a retinal stem cells marker, Sox2 (Ellis P 2004; Taranova OV 2006), in neurospheres derived from the *Chx10<sup>orJ/orJ</sup>* CNR, compared to those derived from either the mutant or the wildtype CE. These data indicate that while the majority of cells within neurospheres from each region expressed markers consistent with a retinal neural/progenitor identity, the CNR neurospheres were distinctive, in that a large number also displayed Müller glial cell characteristics.

### 4.3.3 Neurosphere-forming potential from the $Chx10^{orJ/orJ}$ CNR is not due to a reactive gliosis

The adult  $Chx10$ -deficient retina is characterised by poor lamination, abnormal or absent photoreceptor outer segments and optic nerve. It shows signs of progressive degeneration since with time it becomes more hypocellular, possibly due to atrophy (Burmeister M 1996). Previous work in our laboratory demonstrated the presence of  $BrdU^{pos}$  cells previously within the adult central neural retina (Dhomen NS 2006). Reactive gliosis is the result of many injuries of the central nervous system, mainly involving glial activation. In the mammalian retina, a strong characteristic of reactive gliosis upon degeneration or injury is the occurrence of a population of Müller glia expressing high levels of GFAP. Its main role has been shown to be protective for neighbouring retinal neurons but also acts as a promoter of extensive cell death and extensive degeneration (Bringmann et al. 2006). My data showed that neurosphere-forming cells deriving from the  $Chx10^{orJ/orJ}$  retina and cultured for 7 days in the presence of growth factors, showed an increased expression of GFAP and Apo E as well as GS, all expressed in reactive Müller glia (Amaratunga et al. 1996; Shanmugaratnam, Berg et al. 1997). Next, I examined whether the neurosphere-forming population residing in the mutant retina is simply a reactive glial population that gives rise to neurospheres. Central neural retinal and CE cultures were setup from two mouse models of retinal degeneration, the *rds* (retinal degeneration slow) mouse and the rhodopsin knockout (*rho*<sup>-/-</sup>) mouse. Both models undergo a progressive degeneration from around three weeks postnatally (Reuter and Sanyal 1984; Sanyal and Hawkins 1988; Humphries, Rancourt et al. 1997; Bone-Larson C 2000). Cells were dissociated and cultured for 7 days in NGM promoting neurosphere formation. Neurospheres were observed only in CE-cultures and not in the CNR cultures, excluding the possibility of activation of a resident murine Müller glia cell population upon degeneration giving neurospheres in culture.

#### 4.3.4 RPE cells are not the source of neurosphere-forming potential in the $Chx10^{orJ/orJ}$ CNR

The  $Chx10^{orJ/orJ}$  retina has been shown to abnormally express pigment genes, particularly in certain genetic backgrounds (Horsford DJ 2004; Rowan S 2004). This has been described as a trans-differentiation event, during which neural retinal cells are converted to pigmented cells. The genetic background used in my study did not show an overtly pigmented  $Chx10^{orJ/orJ}$  CNR. However, it is possible that trans-differentiating neural retinal cells are giving rise to neurospheres *in vitro*. Therefore, a marker known to be expressed by retinal pigment epithelium cells, RPE65, as well as several Müller glia markers such as GFAP, GS and CRALBP (the latter also expressed in RPE cells (Kennedy, Li et al. 2003), and a neural stem/progenitor marker, nestin were employed, in order to characterise the neurosphere-forming cells. In neurospheres formed from cells dissociated and cultured for 7 days *in vitro*, it was observed that a small proportion of cells within the neurospheres deriving from  $Chx10^{orJ/orJ}$  CNR were immune-positive for RPE65. In contrast, cells from the mutant or wildtype CE were rarely immuno-positive for RPE65. In all three neurosphere sources, cell immuno-positive for GFAP and GS were abundant and also there were cells co-expressing both markers.

Dissociated cells from wildtype CE and CNR as well as  $Chx10^{orJ/orJ}$  CE and CNR were also immuno-stained immediately after dissociation. Single immuno-staining for GFAP, GS and RPE65, indicated a considerable amount of mutant CNR cells expressing RPE65, whereas RPE65 positive cells was only detected in the  $Chx10^{orJ/orJ}$  CE but not in the wildtype CE and CNR. The absence of RPE65<sup>pos</sup> cells from the wildtype CE suggests that expression of this marker does not confer neurosphere forming potential. To confirm this I tested whether

dissociated Chx10<sup>orJ/orJ</sup> RPE cells, which are RPE65-positive, were able to generate neurospheres. No neurospheres were obtained from Chx10<sup>orJ/orJ</sup> RPE in contrast to the CNR and CE cultures that readily gave rise to neurospheres *in vitro*. Nonetheless, the percentage of GFAP- and GS- positive cells was higher within the Chx10<sup>orJ/orJ</sup> CE- and CNR- dissociated cells in comparison to wildtype CE and CNR. Intriguingly, the percentage of glutamine synthetase- and GFAP- positive cells in each tissue source correlated with its ability to generate neurospheres (Chx10<sup>orJ/orJ</sup> CNR > Chx10<sup>orJ/orJ</sup> CE > wildtype CE). From the above data, it was concluded that neurospheres derived from the Chx10<sup>orJ/orJ</sup> CNR do not arise from RPE cells, as these cells do not form neurospheres. However, RPE65<sup>pos</sup> CNR cells are a potential source of neurosphere-forming cell as they are only found in the mutant, and not the wildtype CNR.

To investigate which cells have the potential to give rise to neurospheres *in vitro*, I attempted to culture wildtype and Chx10<sup>orJ/orJ</sup> CE and Chx10<sup>orJ/orJ</sup> CNR cells for 24 and 48 hours in neurosphere-forming conditions before immunolabelling. These data showed that after 24 hours in culture, wildtype and Chx10<sup>orJ/orJ</sup> CE-derived cells did not express RPE65<sup>pos</sup>, Nestin<sup>pos</sup> or CyclinD1<sup>pos</sup> cells, while GFAP<sup>pos</sup> cells were frequently present. By contrast, Chx10<sup>orJ/orJ</sup> CNR-derived cell cultures showed cells co-immunolabelled with GFAP and Nestin but not Cyclin D1 as well as the presence of RPE65<sup>pos</sup> cells after 24 hours in culture. Interestingly, culturing cells for 48 hours in similar conditions showed that wildtype CE and Chx10<sup>orJ/orJ</sup> CE cells formed clusters, possibly early neurosphere structures, of GFAP<sup>pos</sup> cells with no apparent RPE65 protein expression. Strikingly, Chx10<sup>orJ/orJ</sup> CNR-derived cells showed cells co-immunolabelled for Nestin and GFAP but the RPE65 immuno-positive cells were no longer present. These data support the idea that in the CNR, the GFAP/Nestin double-positive cell gives rise to neurospheres rather than the RPE65<sup>pos</sup> cells as Table 4.1 indicates. It has been documented that the Chx10<sup>orJ/orJ</sup> retinal cells undergo transdifferentiation events (Rowan S 2004),

with retinal cells acquiring an RPE-like fate *in vitro*. Chx10<sup>orJ/orJ</sup> CNR-derived neurospheres show very low levels of pigmentation and only very rarely were RPE65<sup>pos</sup> cells observed in neurospheres, suggesting that the RPE fate is not preferentially adopted *in vitro*. Whether the cells observed by Cepko's research group to acquire RPE characteristics *in vitro* are the same population of cells that have been observed to adopt a glial/neural status *in vitro* is not entirely resolved (Rowan S 2004). It is possible that these are the same plastic cells and that different genetic backgrounds and administration of different growth factors *in vitro* lead to the different phenotypes.

		Dissociated retina	24-hour culture	<sup>†</sup> 48-hour culture	7-day culture
Chx10 <sup>orJ/orJ</sup> CNR	RPE65	+	+	-	-
Chx10 <sup>orJ/orJ</sup> CE	RPE65	+	-	-	-
Wildtype CE	RPE65	-	-	-	-
Chx10 <sup>orJ/orJ</sup> CNR	GFAP	+	+	+	+
Chx10 <sup>orJ/orJ</sup> CNR	GFAP	+	+	+	+
Wildtype CE	GFAP	+	+	+	+
Chx10 <sup>orJ/orJ</sup> CNR	GFAP/Nestin			+*	
Chx10 <sup>orJ/orJ</sup> CNR	GFAP/Nestin			-	
Wildtype CE	GFAP/Nestin			-	

Table 4.1 Expression of RPE65, GFAP and GFAP/Nestin in the neurosphere-forming assay. \*No cells co-expressed GFAP and RPE65. <sup>†</sup>clusters observed at that time point.

#### 4.3.5 Model of different origin of CE versus CNR neurospheres

Diagram 4.3.1 below represents schematically my findings of the behaviour of the newly-discovered progenitor population isolated from the Chx10<sup>orJ/orJ</sup> CNR



along with its characterisation from the neurosphere assay data. The proposed model represents the potential effect of Chx10 in cells isolated from the wildtype and Chx10<sup>orJ/orJ</sup> CE and Chx10<sup>orJ/orJ</sup> CNR. Cells isolated from the wildtype CE are able to form neurospheres *in vitro*. These cells are GFAP<sup>pos</sup>RPE65<sup>neg</sup>GS<sup>pos</sup> upon dissociation and after 7 days in culture have additionally acquired a phenotype of cells expressing neural, retinal and additional Müller glia markers. Interestingly, Chx10<sup>orJ/orJ</sup> CE- and CNR- derived cells had distinct characteristics upon culturing with growth factors. CNR cells were Nestin<sup>pos</sup> on all time-points investigated and also upon 7 days in culture showed a high reactive Müller like-phenotype with increased upregulation of GFAP and ApoE. In addition, the presence of Nestin<sup>pos</sup> cells within the mutant CNR coincides with the notion that Chx10 represses Nestin expression in early retinal progenitors (Rowan S 2005; Kohno et al. 2006) and perhaps is linked to the increased proliferation state reported in Chapter 3. Therefore, it is possible that the initial population residing in the mutant retina is an aberrant Müller/progenitor population. Data is consistent with the idea that the Chx10<sup>orJ/orJ</sup> CE could possess both CE stem as well as a Müller/progenitor populations.

In the absence of Chx10, as was shown in Chapter 3, cells deriving from the CE and CNR gave rise to larger and more neurospheres implying that Chx10 regulates cell division as suggested before in double knockout-mice for Chx10 and p27<sup>Kip1</sup> (Green ES 2003). The authors found a profound rescue in the microphthalmic phenotype and suggested that Chx10 regulates p27<sup>Kip1</sup> via a cyclin-D1-dependent mechanism. The presence of a starting population of aberrant arrested RPC combined with the additional absence of Chx10 in the neurosphere-forming assay might explain why mutant CNR-derived cells can give rise to even more and larger neurospheres *in vitro*.

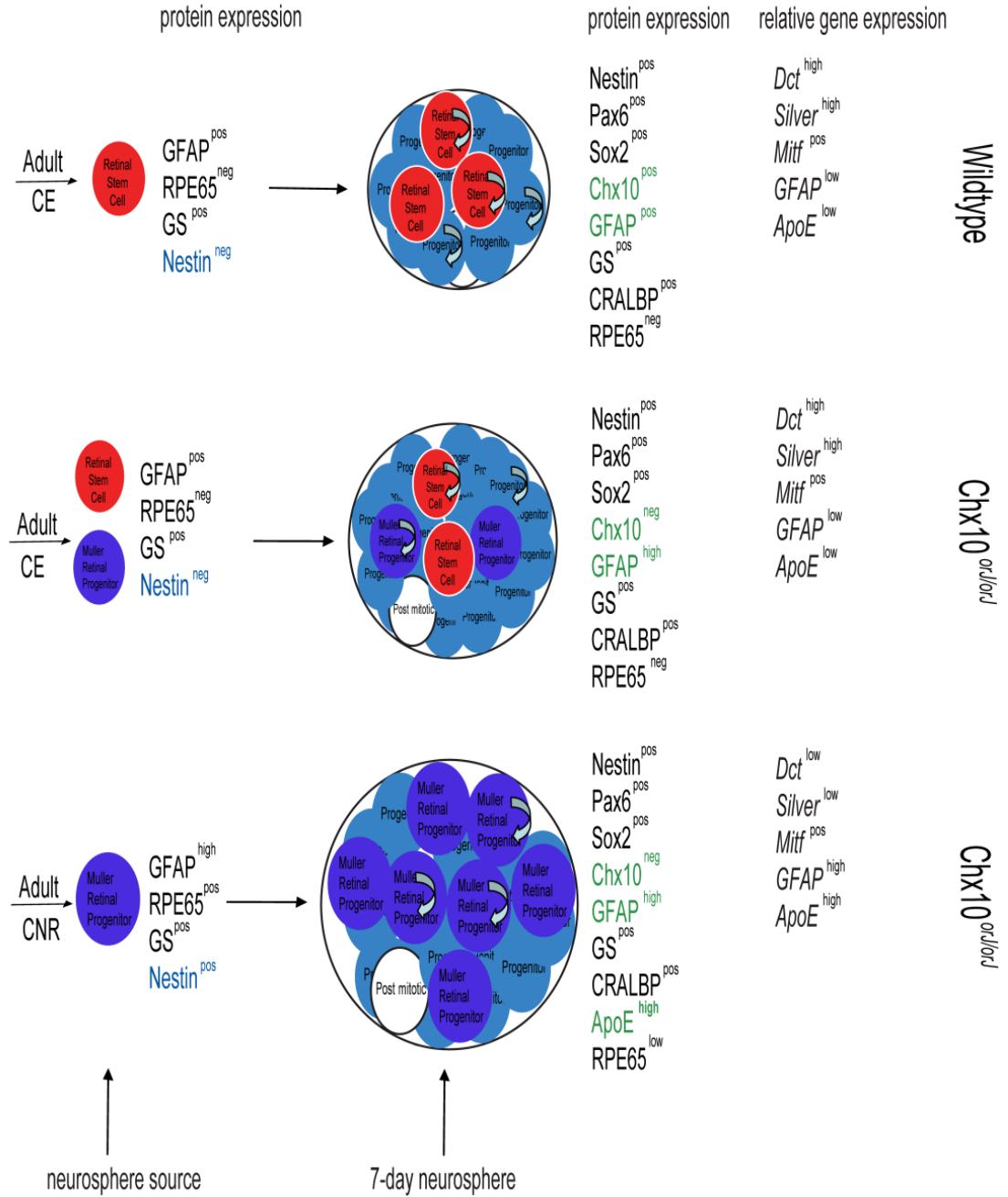


Diagram 4.3.1 Origin of the neurosphere-forming cell

#### 4.3.6 The adult Chx10<sup>orJ/orJ</sup> central neural retina possesses a population of proliferative Müller glia

Re-entry of Müller glial cells into the cell cycle and down-regulation of the tumour suppressor protein p27<sup>kip1</sup> and the cell cycle protein Cyclin D3 has previously been reported to occur after retinal injury (Dyer MA 2000). I investigated whether the increase in dividing Müller-like cells from the CNR correlated with a reduction in p27<sup>kip1</sup> and *Cyclin D3* expression. Quantitative real time-PCR analysis of neurospheres demonstrated that the Chx10<sup>orJ/orJ</sup> CNR cells expressed significantly reduced levels of these cell cycle regulators compared with the mutant or wildtype CE neurospheres. These data show that Müller/progenitor cells within neurospheres, cultured for 7 days *in vitro*, have acquired a cell cycle profile like that of the previously reported glial population found within the adult mammalian retina after injury (Dyer MA 2000). In addition, by examining *PTEN*, a tumour suppressor gene, and *Cyclin D1*, another cell cycle enhancer, a significant upregulation of *PTEN* was observed, especially in Chx10<sup>orJ/orJ</sup> CE-derived neurospheres, and a trend for increasing relative gene expression of *Cyclin D1* especially on Chx10<sup>orJ/orJ</sup> CNR-derived neurospheres, in comparison to wildtype CE-derived neurospheres. It has been suggested that the effect of p27<sup>kip1</sup> on the cell cycle is regulated by Chx10 via Cyclin D1 (Green ES 2003). In the *in vitro* culture system, gene expression assays showed that absence of Chx10 led to a marginal upregulation of Cyclin D1, while p27<sup>kip1</sup> is down-regulated the Chx10<sup>orJ/orJ</sup> CNR derived neurospheres proliferate. This is consistent with the increased proliferation observed in mice lacking both Chx10 and p27<sup>kip1</sup> proteins leading in a partial rescue of the severe hypoplastic retina by P19 (Green ES 2003). Furthermore, it suggests that the *in vitro* culture system releases the *in vivo* cell cycle block, possibly via growth factor stimulation since it has been reported that RPC which exit the cell cycle up-

regulate Kip1, while the opposite effect was observed *in vitro* (Levine, Close et al. 2000).

It has been shown that mice lacking p27<sup>Kip1</sup> show a constitutive form of reactive gliosis, which leads to retinal dysplasia (Dyer MA 2000). In addition, Green *et al.* found that at P0, p27<sup>Kip1</sup> was expressed by more progenitors in the Chx10<sup>orJ/orJ</sup> retinas compared to wild type retina (75% versus 50%) and concluded that its ectopic localization is responsible for the proliferation defect in the microphthalmic retina (Green ES 2003). Our examination of the microphthalmic adult retina both on the protein and gene expression levels, showed no apparent difference in p27<sup>Kip1</sup>. It was not possible to detect a difference of p27<sup>Kip1</sup> protein in CRALBP<sup>POS</sup> cells in the INL between the adult wildtype and microphthalmic retina. Interestingly, although p27<sup>Kip1</sup> gene expression levels were similar between wildtype CE, mutant CE and mutant CNR, there was a significant increase in Cyclin D3 expression in the mutant CE in comparison to its wildtype counterpart, which might partially explain why in the microphthalmic retina this region is expanded (Tropepe V 2000).

These results support the existence of a dormant Müller-like progenitor cell population within the central neural retina of adult mice lacking functional Chx10 TF. While most of the accumulated data presented here and elsewhere (Dhomen NS 2006) supports the idea that this population is unique to the microphthalmic retina, there is some indication that this cell population may be close to a Müller-like progenitor cell resident in the normal retina. Importantly, a recent study showed that when enriched from the normal rat retina, Müller cells, like their radial glial counterparts in the central nervous system, display features of neural stem cells, and are able to form neurospheres and generate new retinal neurons (Das AV 2006). Das *et al.*, propose that these Müller-like cells are latent neural stem cells in the mammalian retina and may serve as potential targets for manipulation for the treatment of retinal degeneration.

Significantly, by using a *Chx10.Gfp* BAC transgenic reporter line to reveal *Chx10* expression at high resolution, its expression has been identified in a subset of Müller cells (Rowan S 2004). In the *Chx10<sup>orJ/orJ</sup>* retina at postnatal day 14, increased levels of *Chx10.Gfp* reporter cells, co-expressing glutamine synthetase, CRALBP and *Pax6* were observed, all markers that are considered hallmarks of progenitors and/or Müller glial cells (Rowan S 2004). Our lab also previously reported CRALBP and nestin labelling of the dividing BrdU positive cells observed in the *Chx10<sup>orJ/orJ</sup>* CNR *in vivo* (Dhomen NS 2006). Here, I show that this glial population express high levels of the marker GFAP which has recently been shown to be expressed in CE stem cells (Das, Mallya et al. 2006; Xu et al. 2007), Therefore, it can be concluded that a proliferative Müller/progenitor cell exists in the adult mutant CNR.

#### **4.3.7 Production of Müller-like cells in the embryonic *Chx10<sup>orJ/orJ</sup>* retina**

Our *in vitro* culture system has revealed that in the *Chx10<sup>orJ/orJ</sup>* adult retina, a progenitor population resides with Müller glia characteristics. During mammalian retinogenesis, retinal progenitors will undergo a finite number of cell divisions and change their competence during development. During prenatal histogenesis, the production of early-born neurons like retinal ganglion cells, cone photoreceptors, amacrine and horizontal cells are formed, while rod photoreceptors along with bipolar cells begin to appear after E18.5 and closer to birth-time. Müller glia cells are the last cell type being born in the murine retina. Lack of *Chx10* shows a phenotypic difference early in retinal development from E13.5 onwards with a reduced RPC proliferation index (Dhomen NS 2006) and a delay in the production of rod photoreceptors (Rutherford AD 2004). I investigated the possibility of the adult mutant retina to show an increased expression of Müller glia cells and indeed when adult retinal sections were

immunolabelled with GFAP, there were clusters of GFAP positive regions within the central retina, while in the wildtype only rarely cells immuno-reactive for this marker were found and only at the retinal margins. By co-immunolabelling with additional glia markers, I found that there were also cells positive for vimentin in the mutant central retina, while CRALBP protein expression was similar to wildtype central retina. Of interest, nestin expressing cells were found in the mutant central retina but never in the wildtype, suggesting the close link between Chx10, nestin and possibly GFAP as previously reported (Rowan S 2005). Some cells were also co-immunolabelled for GFAP and nestin and CRALBP and nestin, while REP65<sup>pos</sup> cells were present but not co-localising with GFAP<sup>pos</sup> cells, similar to what was observed in our dissociated culture. A closer viewing of the peripheral retina and ciliary epithelium showed a distinct expression pattern to the central retinal region; expression of GFAP and CRALBP were strong in the periphery and CE, but nestin, Vimentin and RPE65 were not expressed in either the mutant or wildtype peripheral retina but only in the mutant CE and more strongly and less organised than in the wildtype CE. The question arising from these data is where do these cells derive from and is their progenitor/Müller profile acquired during the proper stage of glial development in the retina or earlier? To answer this question I investigated the possibility of an abnormal early production of Müller glia in the Chx10-deficient retina. At E12.5 a number of RPC have differentiated to retinal ganglion cells and subtypes of interneurons such as amacrine and horizontal cell populations. I used two antibodies against  $\beta$ -III tubulin and HPC-1 (VC1.1) to identify those populations in coronal cryosections of Chx10<sup>orJ/orJ</sup> and wildtype cryosections co-immunolabelled with CRALBP and GFAP, respectively. It was found that in early embryonic retinogenesis, there was a delay of RGC and interneuron production due to lack of Chx10 but no apparent presence of neither GFAP-, CRALBP- nor GS- positive cells. Interestingly, it was observed a shift for VC1.1 immuno-reactivity from the central neuroblastic layer to the periphery in the mutant developing eye. Therefore, RPC at early retinal histogenesis do not show a shift

to a Müller-like phenotype, although there is a delay in production of INL- and RGL- destined cells.

Previous studies have shown lack of Chx10 seems to affect late-born rather than early-born retinal cell types (Rutherford AD 2004) and so I assessed if lack of Chx10 directly affects the production of RPC. Upon co-immunolabelling of GFAP, GS and CRALBP in E18.5 retinal section of Chx10<sup>orJ/orJ</sup> and wildtype whole eyes, there is a strong expression of GFAP but not GS or CRALBP in the mutant retina, while the wildtype, as expected, did not show any expression of GFAP, GS or CRALBP. By examining closely, there was a stronger expression of GFAP, especially in the peripheral developing retina, while in the central mutant retina there were cells expressing GFAP faintly. Note that GS is expressed in developing astrocytes within the nerve fibre in the developing RGC. The glial intermediate filament protein is found primarily in astrocytes both in the embryonic and postnatal tissues and is one of the major components of the glial intermediate filaments (Steinert et al. 1985). It is not normally expressed in the embryonic or postnatal mammalian retina unless there is traumatic injury or neuronal degeneration (Bjorklund et al. 1985; Sarthy et al. 1991). Its presence in the microphthalmic eye, both during late embryonic development and in the adult indicates a degenerative retina (Sarthy and Egal 1995). My data showed that absence of Chx10 leading to microphthalmia does not follow the same pattern of Müller glia activation as reactive gliosis occurring in injured and degenerated retinae, since cells cultured from the adult central orJ retina behave as retinal progenitors. The presence of GFAP is due possibly to a different effect; rather, as shown recently, GFAP<sup>POS</sup> Müller glia do not re-enter in the cell cycle (Fischer AJ 2003). Therefore, these cells will not proliferate *in vivo* but once put in culture, under certain conditions, will probably initiate a mitotic program and divide, forming neurosphere-like structures.

Two assumptions can be drawn by these observations; Chx10 is expressed in Müller glia (Rowan S 2004) and is likely to be involved with at least one subpopulation of this retinal cell type. The presence of GFAP<sup>pos</sup> cells in the adult mutant retina show that probably the BrdU<sup>pos</sup> cells found in the CNR are the ones that express GFAP and preferably are the ones that give rise to neurospheres *in vitro*. With that in mind, it is noticeable to GFAP<sup>pos</sup> cells found in the late but not in early embryonic retinal histogenesis is indicative of an RPC cell population which due to lack of a functional Chx10 TF has converted to a reactive Müller glia cell-like phenotype with possible progenitor characteristics. By linking this to the reduced mitotic index in the periphery commencing from E13.5 and onwards (Dhomen NS 2006) and the fact that Müller glia in the vertebrate retina can become activated and undergo a limited number of cell divisions *in vivo* (Fischer and Reh 2001; Fischer AJ 2003; Ooto S 2004), it is possible that RPC that lack Chx10 will acquire a less primitive progenitor stage, that of a Müller-like cell earlier in retinogenesis, which exposed to inhibitory extrinsic and additional unknown intrinsic cues, will not proliferate properly. This shift to a more late-progenitor cell type seems to be dictated possibly by the proliferation state of the cell affected. In the adult nestin expression was shown to be stronger in the central retina, while in the periphery was absent.

Another possibility is based on two recent publications indicating that inhibiting Müller glia cell division in adult zebrafish blocks photoreceptor regeneration (Thummel et al. 2008) and Müller glia-secreting factors increased BrdU<sup>pos</sup> cells extracted from E14 and E18 retinæ by 2-fold (Yao et al. 2006). In addition, the authors observed that the presence of more Müller glia induced the formation of more ganglion cells by 2.5-fold and almost 2-fold in E14 and E18 retinal cultures, respectively. Therefore, we can assume that in the mutant E18 embryonic retina, lack of Chx10 induces expression or upregulation of GFAP implying the formation of a reactive Müller/progenitor population with an increased proliferation potential which is suppressed by the mammalian retinal



microenvironment. This aberrant progenitor population might be an attempt of the hypocellular retina to induce a higher proliferation environment compensating for the low mitotic index observed in the earlier embryonic retinal histogenesis.

**Chapter 5 – Retinal Stem Cells as a Source of Rod  
Photoreceptor Precursors**

## **5.1. Introduction**

Transplantation techniques using stem cells for the treatment of inherited degenerations and dystrophies of the retina are currently under experimentation, but are still far from a clinical treatment. Retinal progenitors can be identified and manipulated to give rise to cell population(s) of interest, but only in small numbers. (Ahmad I 2000; Belliveau MJ 2000; Yang P 2002; Akagi T 2003; Engelhardt M 2004; Klassen HJ 2004; Qiu et al. 2004; Das AV 2005; Gamm et al. 2005; Qiu, Seiler et al. 2005; Klassen et al. 2007). The overall goal is to isolate a genuine progenitor/stem cell and using *in vitro* specified conditions to drive its differentiation to a favourable state such that when transplanted to the patient, it will integrate and correct any abnormalities in the retina. The immune system that has become a burden for transplantation scientists in other non-immune privileged sites, may play a more minor role in retinal transplantation, but this is still controversial (Klassen HJ 2004). Specifically, two studies showed that adult brain-isolated NPC could develop into photoreceptors after transplantation into the sub-retinal space of retinal dystrophic mice. These cells expressed photoreceptor markers such as recoverin and/or rhodopsin (Pressmar S 2001). Nevertheless, cells did not acquire photoreceptor-like morphology and were not able to integrate in the ONL. Recently, it was shown that the adult murine retina is permissive to post-mitotic precursors but not fully differentiated photoreceptor cells, enabling researchers to focus on how to efficiently obtain rod photoreceptor precursors (MacLaren, Pearson et al. 2006). In addition, human RSC, when xeno-transplanted to SCID mouse and embryonic chick eyes were able to survive, integrate and differentiate into the neural retina, and express photoreceptor markers (Coles BL 2004). Over the last 25 years, cell transplantation has been pursued for the restoration of neural function in degenerative brain diseases and currently CNS stem cells are considered the most promising candidates. Some studies have argued that the

lack of neuronal regeneration that exists in mammals is not due to the absence of an appropriate cellular machinery, but rather to inhibitory processes that have been added over the course of evolution (Coles BL 2004). Nevertheless, the demonstration by MacLaren *et al.* that transplanted photoreceptor precursor cells are able to differentiate into functional photoreceptors that make functional connections in the adult retina indicates that the mature retina is permissive to the generation and integration of new photoreceptors.

Extraction of retinal progenitors from the CE involves micro-dissection procedures and yields only low cell numbers. For this reason an important aim is to identify conditions for expanding the retinal progenitor pool prior to differentiation and transplantation. This chapter presents an investigation of culture conditions designed in order to:

- i) Expand the retinal progenitor cell number
- ii) Prompt retinal neurosphere-forming cells from wildtype and  $Chx10^{orJ/orJ}$  CE and the newly discovered  $Chx10^{orJ/orJ}$  CNR progenitors to differentiate into retinal neurons and glia

## **5.2. Results**

### **5.2.1 Differentiation potential of progenitor cells from the adult Chx10<sup>orJ/orJ</sup> central neural retina**

One of the cardinal features of stem cells is their multipotentiality. Retinal stem/progenitors from the developing retina have shown, under certain conditions, the ability to differentiate giving rise to cells that express markers of all 6 major retinal cell types and Müller glia (Young 1985; Young 1985). My differentiation conditions, initially, included a one-step differentiation assay in high serum on PLL/Laminin-coated glass coverslips, after a 7-day neurosphere formation step in the presence of growth factors (Figure **5.1a**). I sought to determine whether the CNR neurosphere cells were able to differentiate, and whether the absence of Chx10 protein affected the differentiation potential of CNR and CE derived cells. As figure **5.1b** illustrates, during the one step differentiation assay, neurospheres from the Chx10-deficient CNR flatten down losing their typical spherical shape and cells migrate away from the neurosphere site. This observation was similar to reported results (Tropepe V 2000).

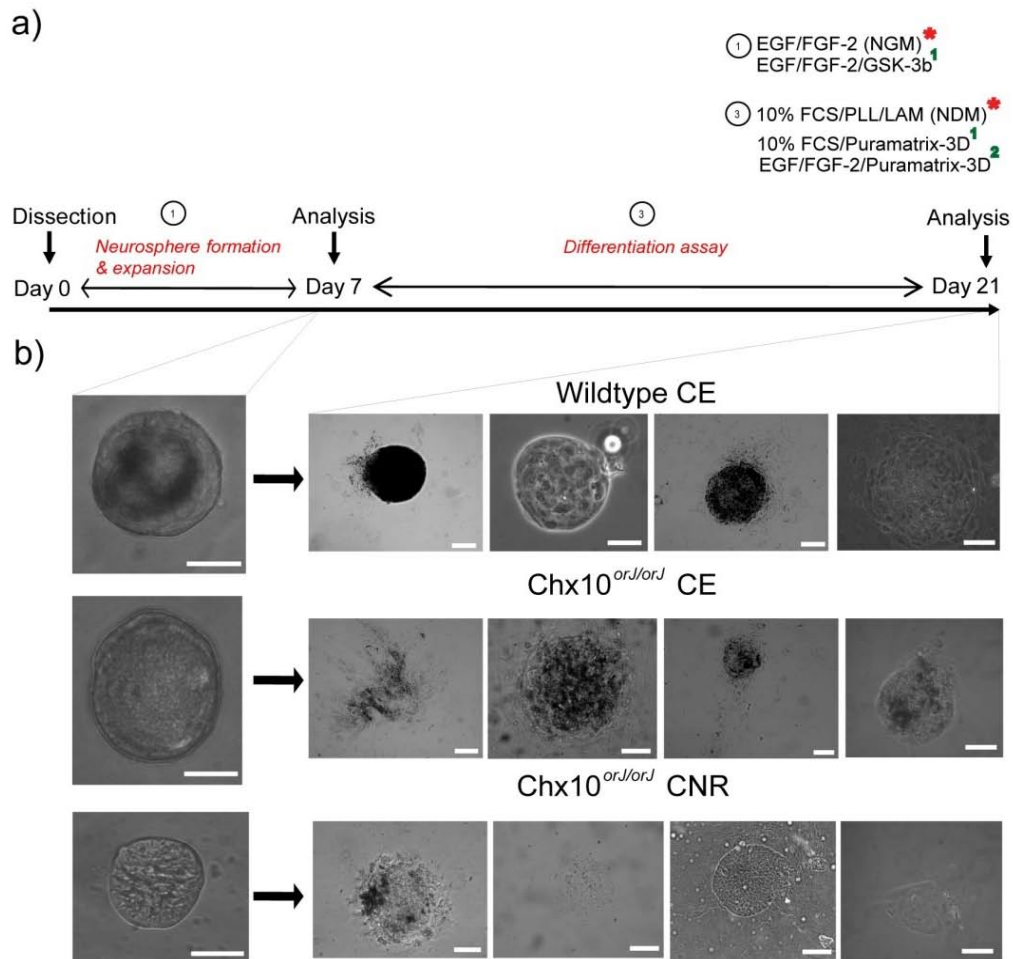
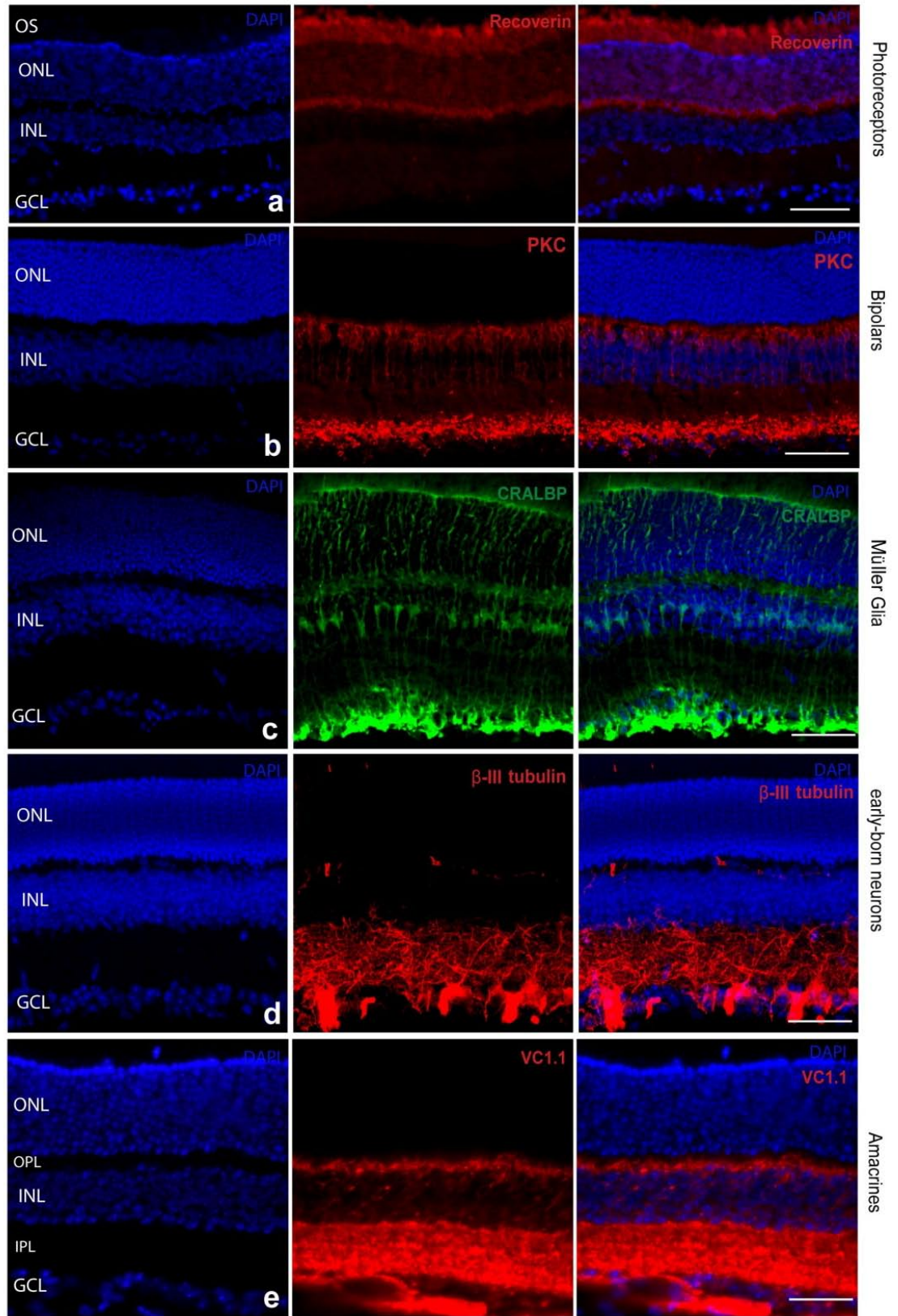


Figure 5.1 Direct differentiation of adult-derived wildtype and Chx10<sup>orJ/orJ</sup> retinal neurospheres

(a) Timeline of conditions used in culture for obtaining and differentiating wildtype and Chx10-deficient neurospheres. Eyes were dissected on day 0 and after dissociation cells were exposed in NGM (\*standard protocol) or NGM with GSK-3b [25 μM, (<sup>1</sup>altered protocol)] for 7 days, for neurosphere formation and potential expansion. Neurospheres were then exposed to 10% FCS in a substrate of PLL and Laminin [\*standard protocol, designated neurosphere differentiating medium, (NDM)] or 10% FCS with Puramatrix-3D (<sup>1</sup>altered protocol), or NGM with Puramatrix 3D (<sup>2</sup>altered protocol), for 14 days to induce differentiation. Analysis points were before exposing neurospheres to high serum-containing medium and at the end of the differentiation assay.

(b) Representative images of neurospheres in an undifferentiated state on day 7 in vitro in the presence of NGM. Representative images of a selection of neurospheres that have undergone differentiation after 14 days in vitro in the presence of 10% FCS PLL/Lam (NDM), showing heterogeneity. Neurospheres lose their typical spherical shape and usually adhere on the bottom of the culture plate with cells migrating away from the neurosphere site. Scale bars; 10 mm.

When primary antibodies against Recoverin (photoreceptors), PKC (bipolar cells), CRALBP (Müller glia),  $\beta$ -III tubulin (early-born neurons/retinal ganglion cells) and VC1.1 (amacrines/hizontals) were used on adult wildtype murine retinal sections, the secondary fluorescence-conjugated antibodies used detected the expected populations of retinal and glial neurons (Figure **5.2A**). It was essential to assess the specificity of the antibodies used in this study, so I used an osteoblast cell-line, MC3T3, which I used primary antibodies against  $\beta$ -III tubulin (early-born neurons), VC1.1 and Syntaxin (amacrines/hizontals), Recoverin (photoreceptors), PKC (bipolars) and Nestin (neural progenitors) using similar secondary fluorescence-conjugated antibodies as in retinal cultures (Figure **5.2Ba**). Control immunoglobulins (IgG1/IgG2a and IgM) were used for immunolabelling proliferative wildtype CE-derived neurospheres and differentiated wildtype CE-neurosphere-derived cells for assessing the background produced by the secondary fluorescence-conjugated antibodies used (Figure **5.2Bb**). From these two control experiments, no unspecific signal was detected either for our primary or secondary antibodies used, concluding that our findings from indirect fluorescence immunolabelling show a real expression of the protein markers investigated. Control experiments were also routinely conducted alongside experimental samples.



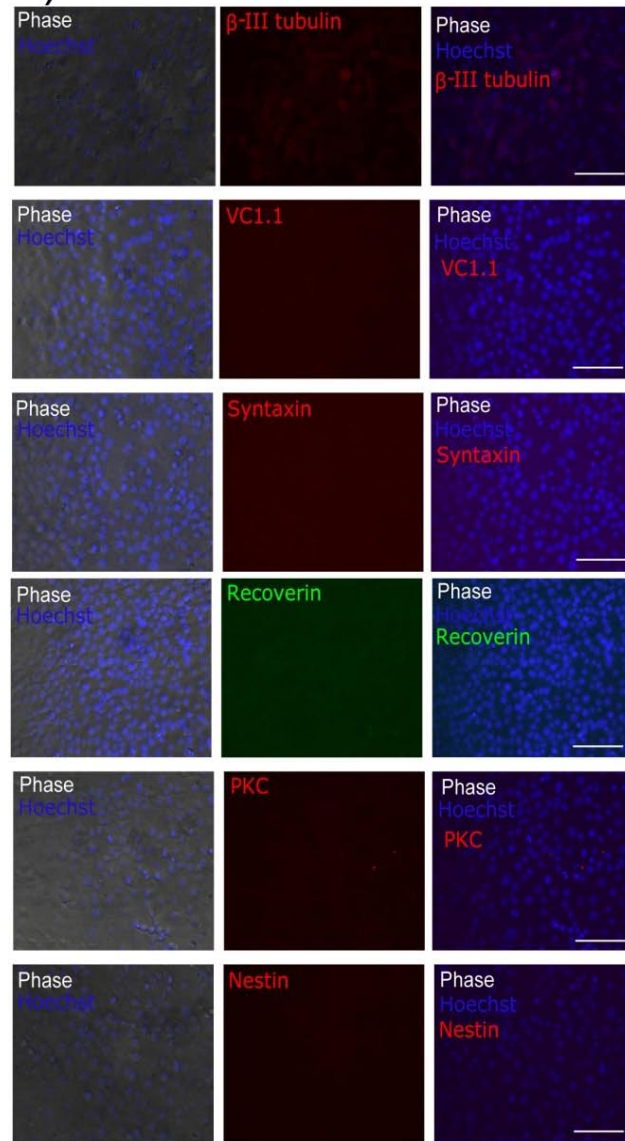


**Figure 5.2A Validation of indirect immunolabelling**

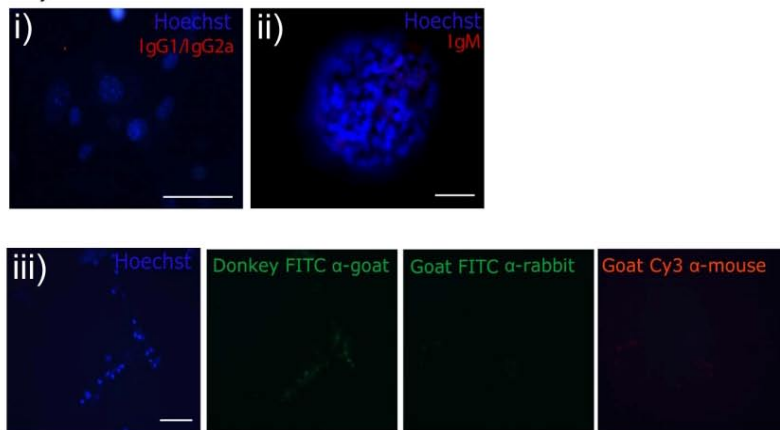
The specificity of the primary and secondary antibodies used for assessing the differentiation potential of the neurosphere-derived cells was tested in adult retinal cryosections. Cryosections of adult retina were immunolabelled for recoverin (photoreceptors) **(a)**, PKC (bipolar cells) **(b)**, CRALBP (Müller glia) **(c)**,  $\beta$ -III tubulin (inner retinal neurons) **(d)** and VC1.1 (amacrine cells) **(e)**. Cells were counter-stained with DAPI. Scale bars = 10  $\mu$ m.

Abbreviations: OS=outer segment ONL=outer nuclear layer, OPL=outer plexiform layer, INL=inner nuclear layer, IPL= inner plexiform layer, GCL=ganglion cell layer.

a)



b)



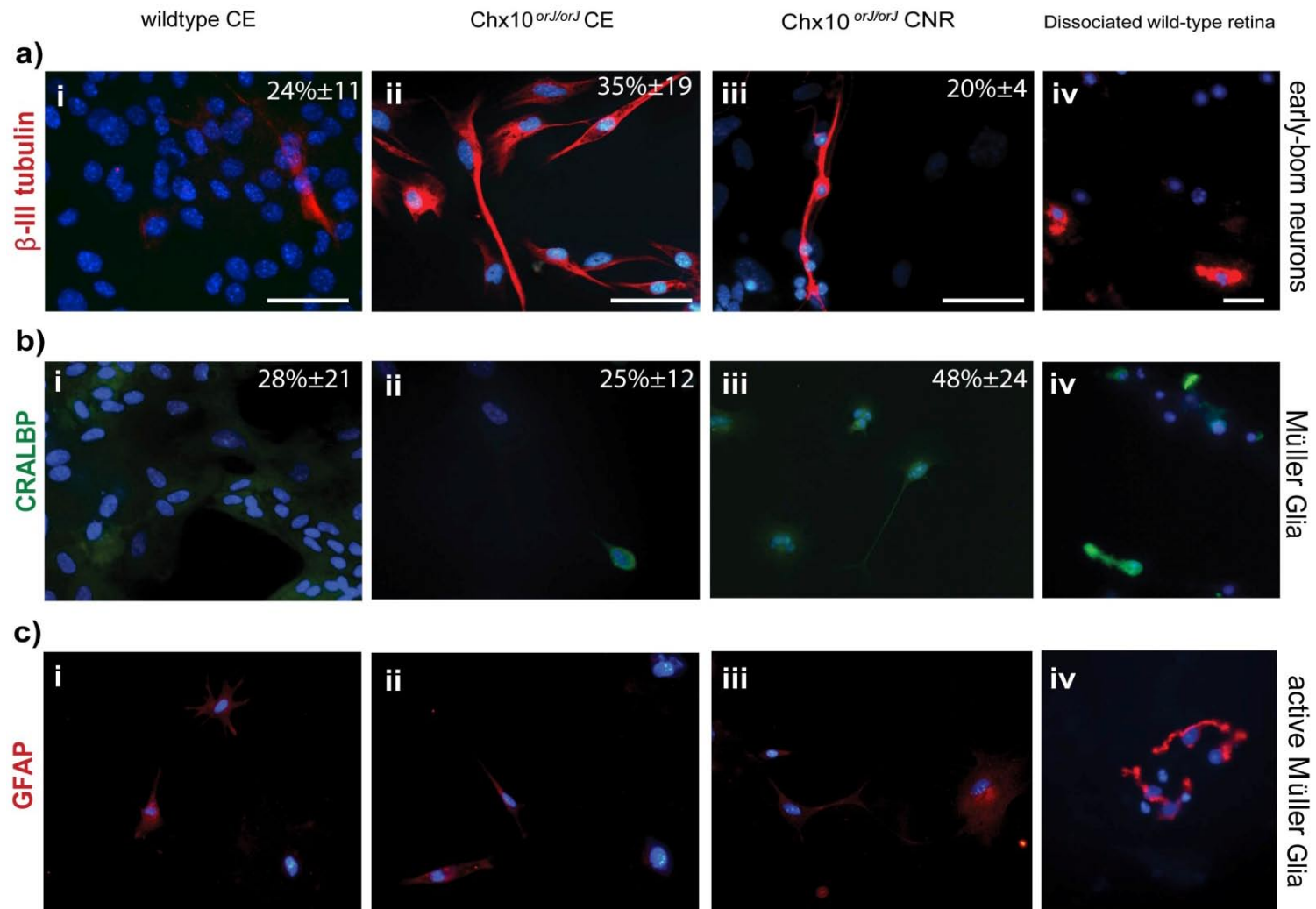
**Figure 5.2B Validation of indirect immunolabelling**

**(a)** The specificity of primary antibodies used for identifying proteins expressed in retinal neurons was analysed by indirect immunolabelling an osteoblast cell line MC3T3 known not to express any neuronal markers.

**(b)** The specificity of the secondary fluorescence-conjugated antibodies used in our assays was tested by immunolabelling in differentiated cells **(i)** and undifferentiated neurospheres **(ii)** by incubation and visualisation of secondary-conjugated IgG1/IgG2a and IgM antibodies. Also, differentiated retinal cells were immunolabelled with Donkey FITC anti-goat, Goat FITC anti-rabbit and goat Cy3 anti-mouse without primary antibody.

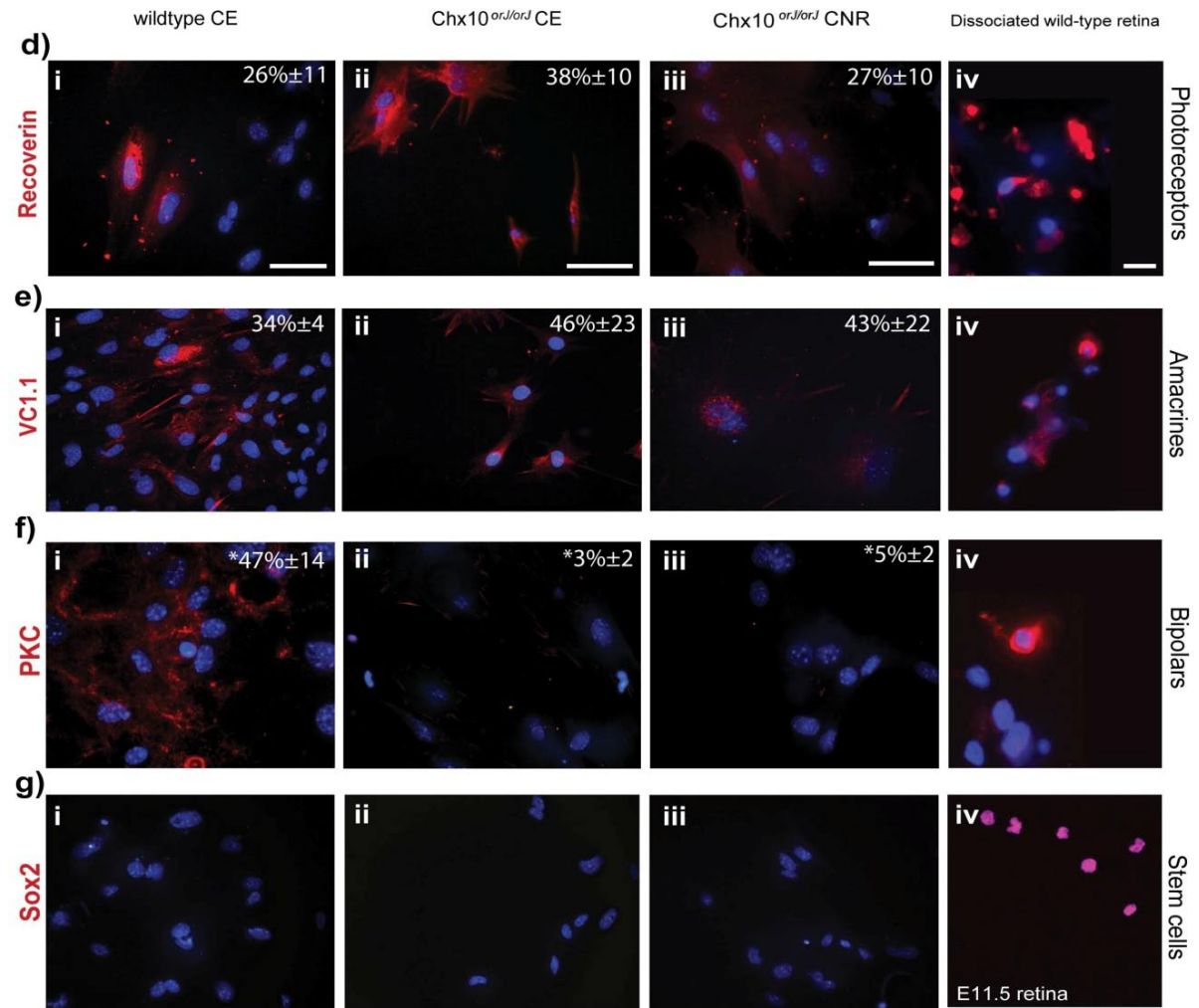
When placed on an adhesive substrate (PLL/ Laminin) in differentiation-promoting conditions, cells from both wildtype and  $Chx10^{orJ/orJ}$  CE, and  $Chx10^{orJ/orJ}$  CNR neurospheres migrated away from the neurosphere and differentiated, expressing both neuronal and glial markers, characteristic of retinal cell types (Figs. **5.3A** & **5.3B**). Wildtype CE neurospheres gave rise to cells that were immuno-positive for markers of early born neurons/retinal ganglion cells ( $\beta$ -III-tubulin; **a(i)**), Müller glia (CRALBP; **b(i)**), reactive Müller glia (GFAP; **c(i)**), photoreceptors (recoverin; **d(i)**), amacrine cells (VC1.1; **e(i)**) and bipolar cells (Protein Kinase C; **f(i)**).

Neurospheres from both the  $Chx10^{orJ/orJ}$  CE and CNR gave rise to cells that were immuno-positive for markers of early born neurons/retinal ganglion cells [**a(ii,iii)**], Müller glia [**b(ii,iii)**], and reactive Müller glia [**c(ii,iii)**]. Photoreceptors [**d(ii,iii)**] and amacrine cells [**e(ii,iii)**], but not bipolar cells [**f(ii,iii)**], were positive while all cells were negative for the neural progenitor marker Sox2 [**g(ii,iii)**]. Positive staining was confirmed by staining dissociated wildtype neural retina [**g(i-iii)**] and on E11.5 neural retina [**(g)iv**]. When cells positive for each protein marker were counted no significance was found between genotypes and regions. Notably, more Recoverin<sup>pos</sup> cells were found from  $Chx10^{orJ/orJ}$  CE-derived neurospheres in comparison to wildtype CE and  $Chx10^{orJ/orJ}$  CNR, but due to an increased standard deviation, no significance was found. The absence of cells expressing bipolar markers amongst the progeny of  $Chx10^{orJ/orJ}$  CE and CNR neurospheres *in vitro* corresponds to the absence of mature bipolar neurons *in vivo* in the  $Chx10^{orJ/orJ}$  retina (Burmeister M 1996).



**Figure 5.3A Differentiation potential of adult-derived wildtype and  $Chx10^{orJ/orJ}$  retinal neurospheres**

Adult-derived wildtype CE and  $Chx10^{orJ/orJ}$  CE and CNR cells were induced to form neurospheres for 7 days in the presence of NGM and then exposed to high-serum medium in the absence of growth factors in PLL and Laminin-coated glass coverslips for 14 days (NDM). Differentiated cells migrating out of the neurosphere site were then immunolabelled for  $\beta$ -III tubulin (neuronal marker/early-born retinal neurons) **(a)**, CRALBP (Müller glia) **(b)** and GFAP (reactive Müller glia) **(c)**. Positive controls were provided by staining dissociated adult wildtype neural retina [**a(iv)**, **b(iv)**, **c(iv)** and **d(iv)**]. Cells were counter-stained with Hoechst 33342. Images are representative of four independent experiments. Scale bars = 10  $\mu$ m.

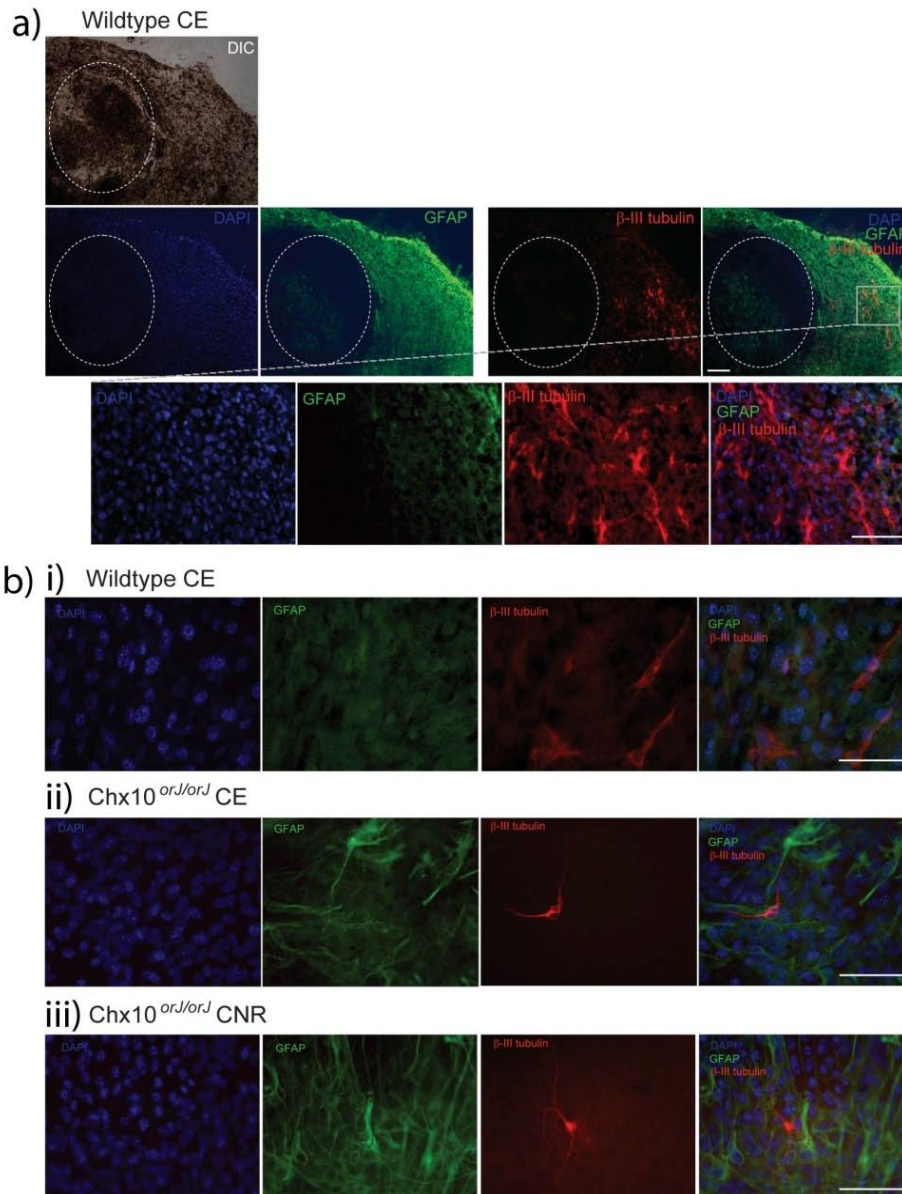


**Figure 5.3B Differentiation potential of adult-derived wildtype and Chx10<sup>orJ/orJ</sup> retinal neurospheres**

Adult-derived wildtype CE and Chx10<sup>orJ/orJ</sup> CE and CNR cells were induced to form neurospheres for 7 days in the presence of NGM and then exposed to high-serum medium in the absence of growth factors in PLL and Laminin-coated glass coverslips for 14 days (NDM). Differentiated cells migrating out of the neurosphere site were then immunolabelled for recoverin (photoreceptors) (**d**), VC1.1 (amacrine cells) (**e**) and PKC (**f**). Note that PKC is not expressed in Chx10-deficient cells since there is an absence of bipolar cells in these mice. The neural stem cell marker Sox2 was absent from all differentiated cultures (**g**). Positive controls were provided by staining dissociated adult wildtype [**d(iv)**, **e(iv)**, **f(iv)**] and E11.5 neural retina [**g(iv)**]. Cells were counter-stained with Hoechst 33342. Images are representative of four independent experiments. Scale bars = 10  $\mu$ m.



As the immunocytochemical data in chapter 3 indicated that neurospheres contain NPC, I tested whether in differentiating conditions cells expressed both neuronal and glial markers. When neurospheres are placed in a serum-free medium onto an adhesive substrate, cells begin to differentiate and migrate away from the original neurosphere adherent point (Figure **5.4A**). After 14 days *in vitro*, cells were co-immunolabelled with antibodies against  $\beta$ -III tubulin (neuronal marker) and GFAP (reactive Müller glia/astrocytes) [Figures **5.4A(a, b)**]. No cells were found positive for both markers on wildtype and mutant CE as well as mutant CNR cultures, indicating that cells with a dual glial/neuronal phenotype were not present. A graph was plotted indicating the percentage of differentiated cells found positive for neuronal or Müller glial protein markers (Figure **5.4B**). As illustrated, the same amount of  $\beta$ -III tubulin was found for wildtype and mutant CE as well as mutant CNR-derived cells, while there was a statistically significant increase in the amount of GFAP<sup>pos</sup> cells with the highest being in the mutant CNR, followed by the mutant CE and lastly, wildtype CE-derived cells. The multipotentiality of neurosphere-derived cells might also derive from the possibility that a cell cultured *in vitro* shows a potentially dual phenotype which might express both neuronal and glial proteins.



**Figure 5.4A** Neuronal versus glial differentiation potential of adult-derived wildtype and *Chx10<sup>orJ/orJ</sup>* retinal neurospheres  
 Adult-derived wildtype CE and *Chx10<sup>orJ/orJ</sup>* CE and CNR cells were induced to form neurospheres for 7 days in NGM and then exposed to NDM in PLL and Laminin-coated glass coverslips for 14 days. Differentiated cells tend to migrate away from the neurosphere site (a) migrating out of the neurosphere site were then co-immunolabelled for  $\beta$ -III tubulin (neuronal marker/ early-born retinal neurons) and GFAP (reactive Müller glia). Inserts showing cells migrated away from the initial neurosphere site (fragmented-circle). (b) Representative photomicrographs of wildtype CE (i) and *Chx10<sup>orJ/orJ</sup>* CE (ii) and CNR (iii) differentiated cells that were co-immunolabelled for  $\beta$ -III tubulin (red) and GFAP (green). Cells were counter-stained with DAPI. Images are representative of three independent experiments.

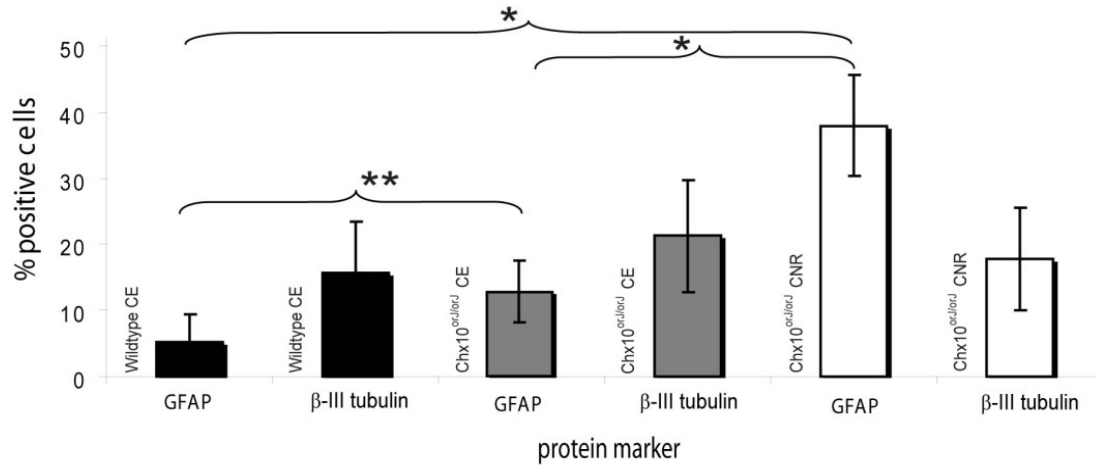


Figure 5.4B Graphical representation of neuronal versus glial differentiation potential of adult-derived wildtype and Chx10<sup>orJ/orJ</sup> retinal neurospheres  
 Bar chart representing the percentage of cells positive for the neuronal marker β-III tubulin and the reactive Müller glia marker GFAP, in differentiated cultures deriving from wildtype CE (black bars), Chx10<sup>orJ/orJ</sup> CE (grey bars) and Chx10<sup>orJ/orJ</sup> CNR (white bars). N=3 independent experiments. Scale bars = 10 μm. Error bars; SD. ANOVA with Dunnett's T3 post-hoc. \*P<0.01, \*\*P<0.05.

Quantitative Re-Ti PCR was also used to characterise the gene expression of retinal markers in undifferentiated and differentiated cultures (Figure 5.5). Transcripts for *β-III-tubulin* (Figure 5.5a) were detected in undifferentiated as well as differentiated cultures of cells deriving from both wildtype and *Chx10<sup>orJ/orJ</sup>* CE as well as *Chx10<sup>orJ/orJ</sup>* CNR neurospheres. No significant difference in mRNA transcripts was observed between undifferentiated and differentiated states, as well as between genotypes and retinal regions. In addition, the expression of another retinal ganglion cell marker, *Gap43* (Ivanov et al. 2006) was investigated and found to also be present in both undifferentiated and undifferentiated cultures of cells from both wildtype and *Chx10<sup>orJ/orJ</sup>* CE as well as *Chx10<sup>orJ/orJ</sup>* CNR neurospheres with no significant alteration between differentiated states, as well as between genotypes and retinal regions (Figure 5.5b). Finally *Sox2* mRNA transcript levels were investigated in both undifferentiated and undifferentiated cultures of cells from wildtype and *Chx10<sup>orJ/orJ</sup>* CE as well as *Chx10<sup>orJ/orJ</sup>* CNR neurospheres (Figure 5.5c). Interestingly, even if this TF has a dual developmental expression pattern by being expressed in both retinal stem/progenitor as well as in amacrine and horizontal cell populations, there was a non-statistically significant decrease of expression in my differentiated CE-derived cultures, while mRNA transcript levels between cells derived from undifferentiated and differentiated *Chx10<sup>orJ/orJ</sup>* CNR neurospheres remained constant.

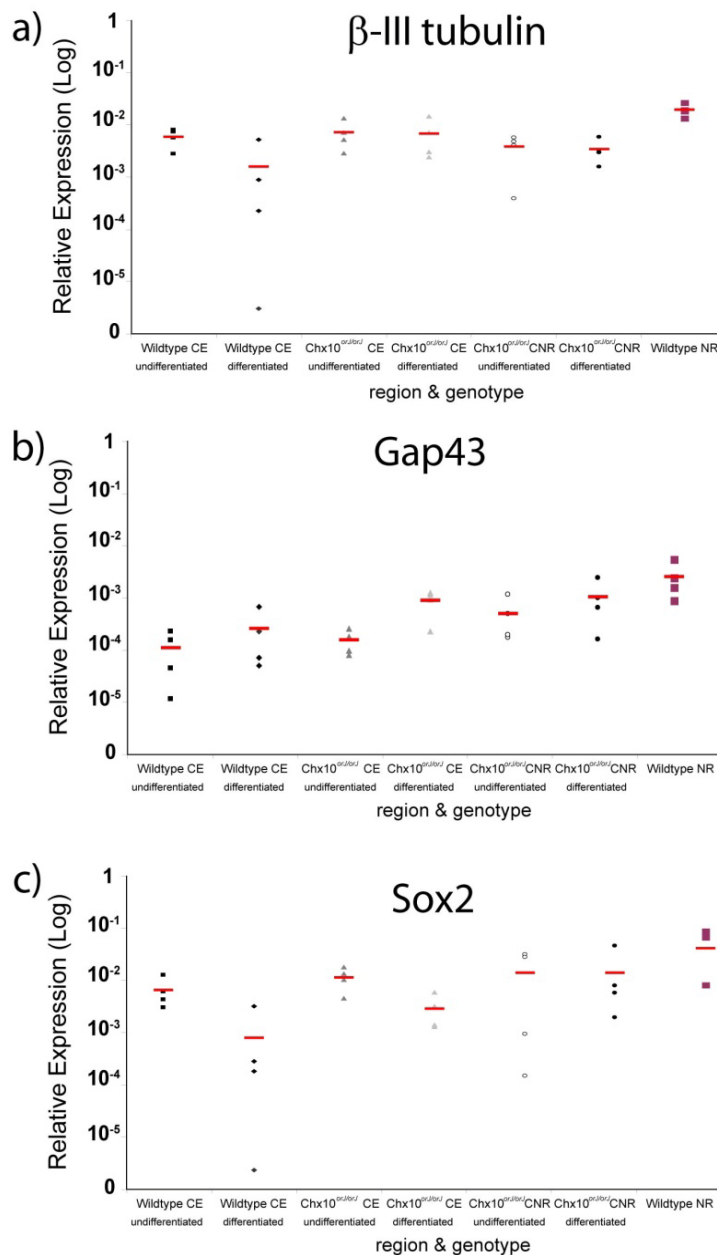


Figure 5.5 Quantitative Real-Time PCR analysis of retinal gene expression in undifferentiated and differentiated neurosphere-derived cells. Expression of  $\beta$ -III tubulin (a), Gap43 (b), and Sox2 (c) in undifferentiated (cultured for 7 days in NGM) and differentiated cells (cultured for 14 days in vitro in NDM) derived from Chx10<sup>orj/orj</sup> CE and CNR, and wildtype CE neurospheres, and adult neural retina for comparison. Graph shows relative expression levels from four independent experiments normalized to Gapdh (N=4 eyes from each genotype per experiment).

Where no values are presented on graph, means that Gapdh transcripts were detected normally, while the gene of interest was not observed.

Since photoreceptor protein expression was observed in the mutant in similar levels to the wildtype, I investigated the mRNA levels of three photoreceptor genes. *Crx* shows a restricted expression in developing and mature photoreceptors (Furukawa et al. 1997) (Figure 5.6a). A significant increase (more than 25 fold; \*P<0.05) in mRNA levels in *Crx* was detected in *Chx10<sup>orJ/orJ</sup>* CE-derived cells compared to wildtype CE and *Chx10<sup>orJ/orJ</sup>* CNR cells indicating a potential difference in the differentiation bias of adult-derived progenitor cells lacking *Chx10*. The *Chx10<sup>orJ/orJ</sup>* CNR derived cells also showed a trend of increased expression of these photoreceptor genes in comparison to wildtype CE but with no statistical significance. *Recoverin* mRNA levels also showed a statistically significant increase in *Chx10<sup>orJ/orJ</sup>* CE derived cells compared to wildtype CE and *Chx10<sup>orJ/orJ</sup>* CNR cells (more than 68 fold in comparison to wildtype CE; \*P<0.05, Figure 5.6b). Differentiated cells deriving from the *Chx10<sup>orJ/orJ</sup>* CNR neurospheres showed no alteration in *Recoverin* mRNA transcript levels although a trend of increase could be noted in comparison to wildtype CE. Interestingly, in both *Crx* and *Recoverin* gene expression assays, only the *Chx10<sup>orJ/orJ</sup>* CE showed an alteration between undifferentiated and differentiated cell cultures. Finally, to assess the expression of cone photoreceptor genes in our differentiated cultures, I investigated the mRNA transcripts of *Arrestin 3*, which is specific for this cell type (Craft et al. 1994) in both undifferentiated and differentiated wildtype and mutant CE as well as mutant CNR (Figure 5.6c). I could not identify any transcripts in my analysis for this gene except in undifferentiated wildtype CE and mutant CNR, possibly indicating very low or absence of gene expression for cone photoreceptors.

Taken together these data suggest that with the exception of the expression of bipolar cell markers, the progenitor cells from the adult *Chx10<sup>orJ/orJ</sup>* CNR show similar differentiation properties to the wildtype CE derived cells, but with an increase in photoreceptor gene expression.

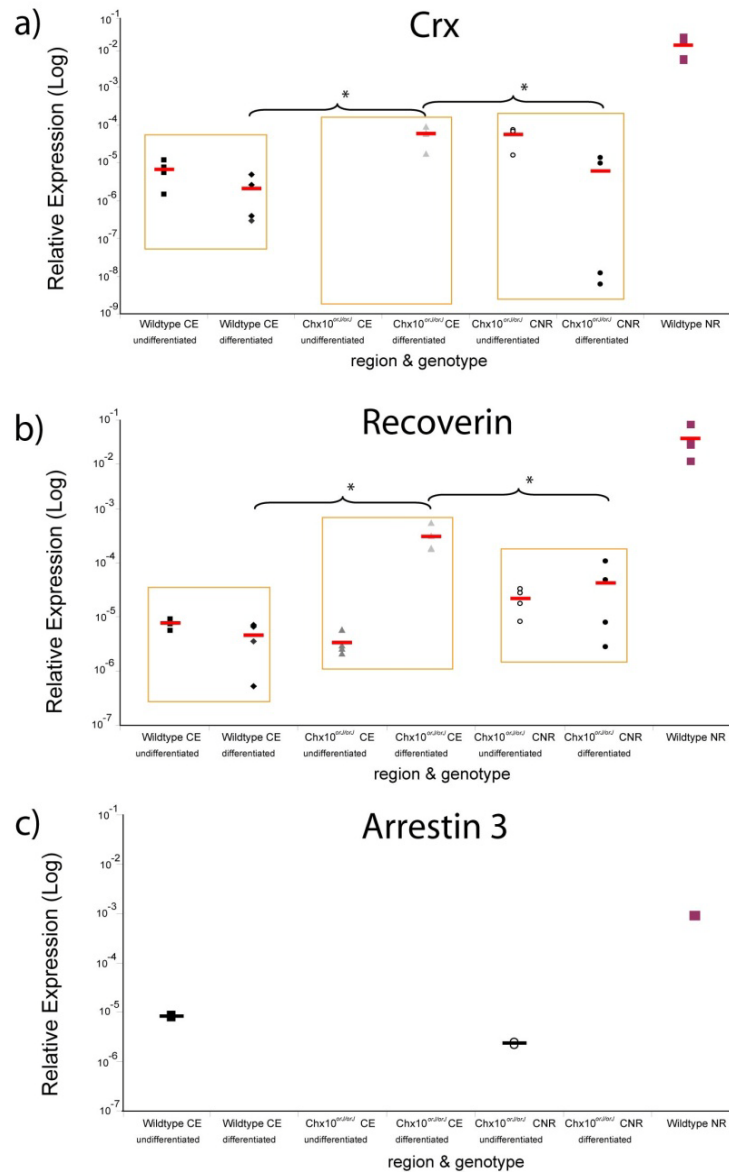


Figure 5.6 Quantitative Real-Time PCR analysis of rod and cone photoreceptor gene expression in undifferentiated and differentiated neurosphere-derived cells

Expression of Crx (a), Recoverin (b), and Arrestin 3 (c) in undifferentiated (cultured for 7 days in NGM) and differentiated cells (cultured for 14 days in vitro in differentiation conditions) derived from Chx10<sup>orj/orj</sup> CE and CNR, and wildtype CE neurospheres and adult neural retina for comparison. Graphs show relative expression levels from four independent experiments normalized to Gapdh (N=4 eyes from each genotype per experiment); Orange boxes indicate the pairs of differentiated and undifferentiated cells deriving from the same region and genotype. Independent samples T-test \* P<0.05.

Where no values are presented on graph, means that Gapdh transcripts were detected normally, while the gene of interest was not observed.



### 5.2.2 Analysis of the differentiation status of wildtype and Chx10-deficient neurospheres

The differentiation assay for neurosphere-derived cells relies on the conditions used providing the necessary signals which will allow progenitors to exit the cell cycle and become post-mitotic. I investigated if neurosphere-derived cells after treatment for 14 days *in vitro* with 10% FCS in an adhesive substrate of PLL and Laminin still undergo mitosis (Neurosphere differentiation medium, NDM) (Figure 5.7). Phospho-Histone 3 (PH3) was used, that labels cells in anaphase, from wildtype and mutant CE as well as mutant CNR (Figure 5.7a). As illustrated, withdrawal of growth factors and supplementing with high amount of serum in undifferentiated cultures for 14 days does not confer complete differentiation of neurosphere-derived cells from all regions and genotypes. Approximately, 2% of wildtype and mutant CE cells were undergoing mitosis in the differentiated culture conditions while the mutant CNR showed a higher proportion of cells undergoing mitosis (4-fold difference, ~8%). This experiment shows that withdrawal of growth factors and the presence of high amounts of FCS does not lead to complete differentiation.

The presence of p27<sup>kip1</sup>, a cyclin-dependent kinase inhibitor, has been reported to affect proliferating retinoblastoma cells and controls the decision of multipotent CNS progenitors to withdraw from the cell cycle (Levine, Close et al. 2000; Kase et al. 2006). A smaller number of cells was found positive for Kip1 in the mutant CNR in comparison to both wildtype and mutant CE (2-fold difference, respectively). Interestingly, the proportion of cells positive for both Kip1 and PH3 was the same between wildtype CE and mutant CNR, while mutant CE showed a 5-fold decrease in comparison to wildtype CE and mutant CNR (Figure 5.7a,b). Apart from the presence of Kip1, its distribution within the cell is important for correct cell cycle exit. Kip1 is nuclear when in G0 and early G1 phases and appears in the cytoplasm at the G1/S transition stage (Connor et



al. 2003). Therefore a downregulation or absence of Kip1 cannot be indicative of an abnormal cell proliferation since increased Kip1 degradation will cause cells not to enter the M-phase of the cell cycle (Kase, Yoshida et al. 2006).

a)

Wildtype CE		
	cells positive/total cells	% of total cells
H3	9/450	2.0
	3/213	1.4
Kip1	24/250	9.6
	21/196	10.7
colocalised	15/363	4.1
	12/342	3.5

Chx10 <sup>orJ/orJ</sup> CE		
	cells positive/total cells	% of total cells
H3	9/384	2.3
	10/336	3.0
Kip1	20/250	8.0
	21/285	7.4
colocalised	2/345	0.6
	2/234	0.9

Chx10 <sup>orJ/orJ</sup> CNR		
	cells positive/total cells	% of total cells
H3	21/260	8.1
	26/300	8.7
Kip1	10/231	4.3
	12/301	4.0
colocalised	9/266	3.4
	9/287	3.1

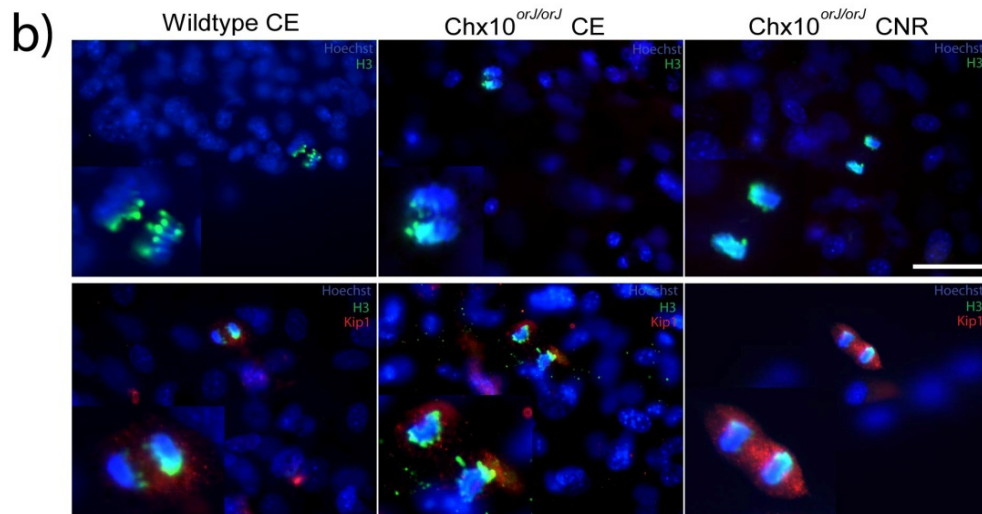


Figure 5.7 Mitotic index of differentiated cells from wildtype and Chx10<sup>orJ/orJ</sup> retinal neurospheres  
 Differentiated cells were immuno-stained for Phosphohistone-3 (H3, green) and p27<sup>kip1</sup> (Kip1, red) for assessing their mitotic activity.  
 (a) A table showing the number of cells per total cell counted and the percentage of cells found positive for H3 and Kip1 as well as cells co-immunolabelled for the above markers. (b) Representative photomicrographs of cells showing the positive immunolabelling and co-immunolabelling for H3 and H3/Kip1, respectively in differentiated cells. Inserts show higher magnification of mitotic cells during anaphase. Cells were counter-stained with Hoechst 33342. Scale bars= 5 mm. N=2 independent experiments.

### 5.2.3 Expansion of neurosphere-forming cells from wildtype and Chx10-deficient neurospheres

The Wnt signalling pathway is important for neural stem/progenitor cell proliferation. Specifically, Wnt3a ligand has been shown to increase neurosphere size and number from noggin-primed ESC (Davidson et al. 2007). GSK-3 $\beta$  inhibitor was added to the neurospheres at Day 0 of culture to test if this treatment, which activates the endogenous Wnt3a signalling pathway, will give rise to more and larger neurospheres in my culture system (Figure **5.8A** and **appendix - movies 5.8**). A concentration-dependent assay was performed for this inhibitor and its effect on neurosphere number from wildtype CE cells was plotted (Figure **5.8a**). From the graph, it was shown that there was not an overall alteration in the presence of 0 to 25  $\mu$ M of the inhibitor added.

The effect of the maximum amount of this inhibitor (25  $\mu$ M) in wildtype CE neurosphere cultures on the neuronal-glial balance was also assessed (Figure **5.8B**) along with neurosphere number and diameter (Davidson, Jamshidi et al. 2007). Although neurosphere size and number remained unchanged, CRALBP expression was stronger in neurospheres treated with 25  $\mu$ M GSK-3 $\beta$  inhibitor, with no apparent difference in  $\beta$ -III tubulin expression, which was expected to be low in neurospheres cultured in NGM for 7 days *in vitro* (See chapter 3, figure 3.6). When Chx10<sup>orJ/orJ</sup> CE-derived cells were treated with the same amount of GSK-3 $\beta$  (Figure **5.8b**), similar results were obtained with an increased CRALBP expression and with no apparent  $\beta$ -III tubulin expression, and no alteration in neurosphere size and number in comparison to standard Chx10<sup>orJ/orJ</sup> CE NGM assay. Finally, when 25  $\mu$ M of GSK-3 $\beta$  inhibitor was included in the culture of dissociated Chx10<sup>orJ/orJ</sup> CNR cells and allowed to form neurospheres for 7 days, no alteration was observed in CRALBP and  $\beta$ -III tubulin expression and there was no difference in neurosphere size and number (Figure **5.8Bc**). In

conclusion, activation of the endogenous Wnt signalling neither promotes proliferation or differentiation in this system.

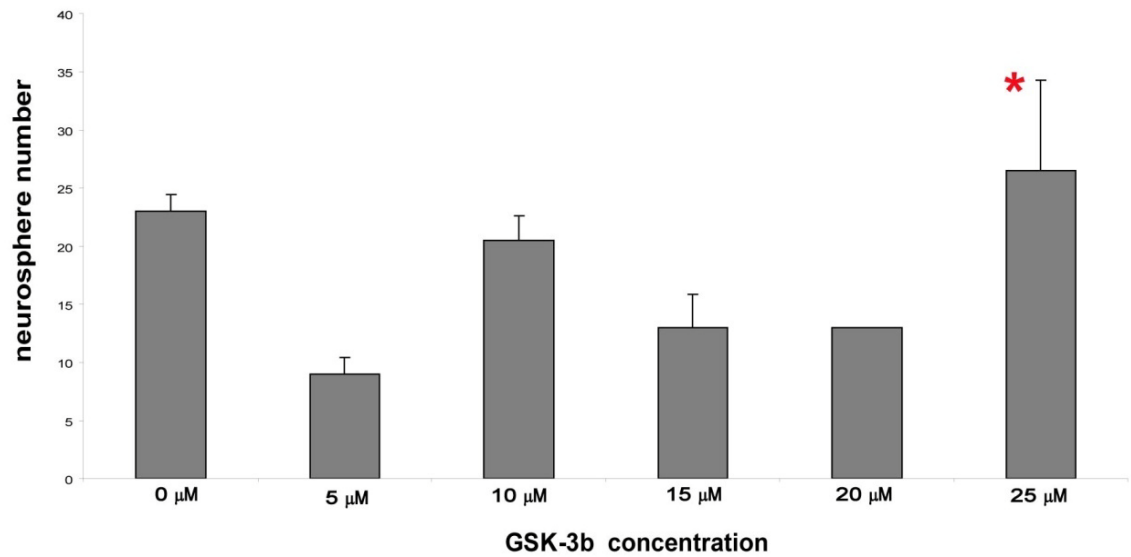
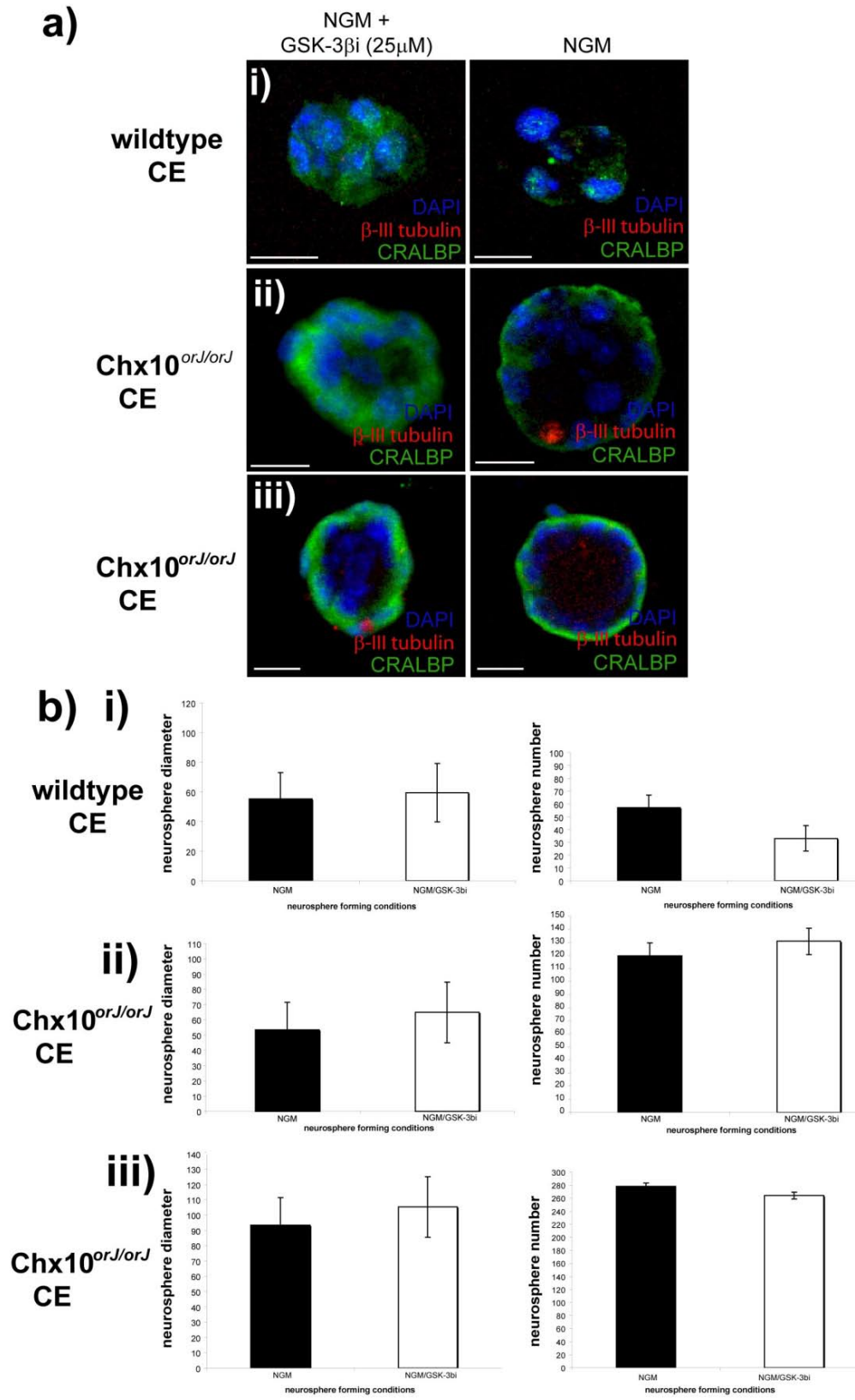


Figure 5.8A Effect of GSK-3b inhibitor in wildtype CE neurosphere formation  
A graph showing the effect of GSK-3b inhibitor in wildtype CE-derived neurosphere number, counted after 7 days in vitro in the presence of NGM with GSK-3b inhibitor in concentrations ranging from 5 – 25 μM. \*Concentration of this inhibitor chosen for further experiments.

N=2 independent experiments, 4 eyes per experiment. Error bars; SD.



**Figure 5.8B Effect of GSK-3 $\beta$  inhibitor in neurosphere formation derived from adult wildtype and Chx10<sup>orJ/orJ</sup> retina**

Cells were cultured for seven days in the presence of NGM with or without GSK-3 $\beta$  (25  $\mu$ M). **(a)** Representative photomicrographs of neurospheres deriving from wildtype CE **(i)**, Chx10<sup>orJ/orJ</sup> CE **(ii)** and Chx10<sup>orJ/orJ</sup> CNR **(iii)** neurospheres co-immunolabelled with antibodies against  $\beta$ -III tubulin (neurons, red) and CRALBP (Müller glia, green).

**(b)** Bar charts representing wildtype CE **(i)**, Chx10<sup>orJ/orJ</sup> CE **(ii)** and Chx10<sup>orJ/orJ</sup> CNR **(iii)** neurosphere diameter and number after 7 days *in vitro*.

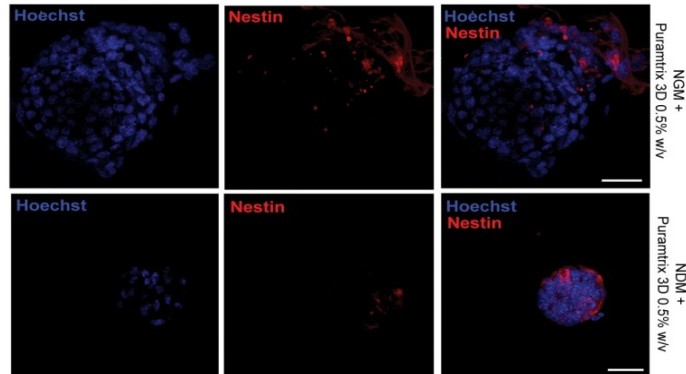
N=2 independent experiments. Cells were counter-stained with Hoechst 33342. Scale bars= 10  $\mu$ m.

The use of scaffold materials in culture systems has been used for maintaining the progenitor state of dividing cells, or to enhance their differentiation potential. I tested a three-dimensional gel (Puramatrix™) which is able to form a matrix sheet where cells can incorporate and either proliferate or differentiate. Under appropriate conditions it assembles into a fibrous structure with an average pore size of 50-200 nm or a gelatin-like cell surface is formed for cells to settle onto or adhere to. In Figure **5.9a** (in appendix - movies 5.9) wildtype CE-derived cells were cultured in the presence of NGM and allowed to form neurospheres in the presence of 0.5% Puramatrix gel. Further, neurospheres grown in NGM but without Puramatrix gel scaffold were then subsequently cultured in a growth factor-free medium in the presence of 0.5% Puramatrix gel for 14 days *in vitro*. An antibody against nestin was used to assess the maintenance of the immature state of CE stem cells. Results showed that 0.5% Puramatrix gel did not alter nestin expression. However, there was an inhibition of neurosphere differentiation as cells did not flatten and migrate away from the neurosphere site as shown in other differentiation cultures. Similar findings were observed when Chx10<sup>orJ/orJ</sup> CE and CNR cells were cultured under the same conditions (Figures **5.9b** and **5.9c**).

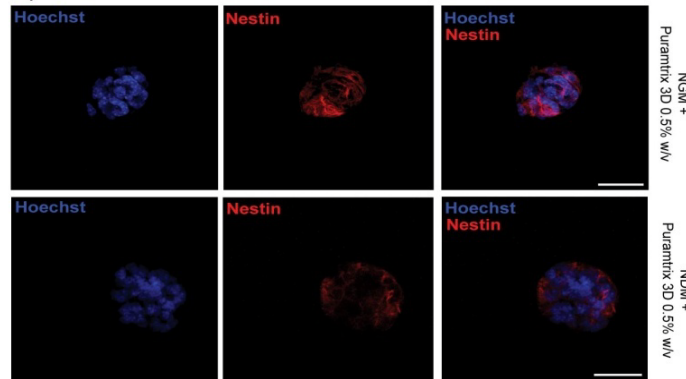
In conclusion, Puramatrix gel appeared to inhibit differentiation and maintain the neurosphere niche with no apparent change in the number of Nestin<sup>pos</sup> neurosphere progenitors.



a) Wildtype CE



b) Chx10<sup>orJ/orJ</sup> CE



c) Chx10<sup>orJ/orJ</sup> CNR

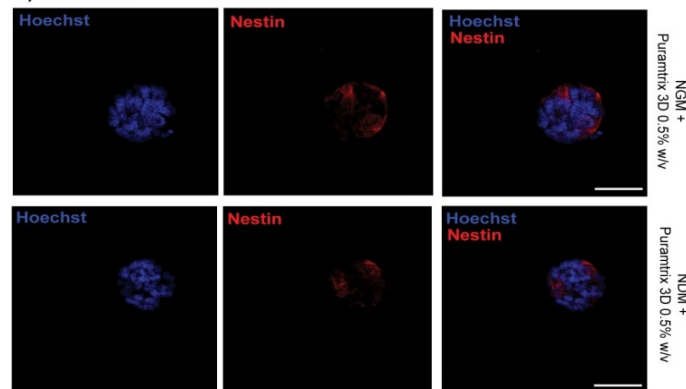


Figure 5.9 Effect of Puramatrix scaffold in the undifferentiated state of neurospheres derived from adult wildtype and Chx10<sup>orJ/orJ</sup> retina. Neurosphere-forming cells were cultured for seven days in the presence of NGM and then were cultured for a further 14 days in the presence of 0.5% w/v Puramatrix 3D in either NDM or NGM. Representative photomicrographs of wildtype CE (a), Chx10<sup>orJ/orJ</sup> CE (b) and Chx10<sup>orJ/orJ</sup> CNR (c) neurospheres immunolabelled with an antibody against nestin (neural stem/progenitors, red) after a total of 21 days in vitro. Cells were counter-stained with Hoechst 33342. Scale bars= 10 mm. N=2 independent experiments.

#### 5.2.4 Wildtype and Chx10-deficient retinal progenitor expansion using the monolayer assay

The adult retinal stem cells found in the pigmented ciliary margin (PCM) of the mammalian eye (Tropepe V 2000) and constitute only ~0.6% of the total population. As well as the need to induce neurosphere cells to differentiate to retinal neuronal types of interest, it is also essential to have a large source of plastic cells for analysis and for retinal transplantation studies. For this reason, I included an additional step in my culture time-line from neurosphere formation to differentiation. This included a 4-day priming step (Figure 5.10a) in low serum conditions along with the presence of growth factors (EGF/ FGF-2) similar to what was previously used (Yin, Feng et al. 2004; Macneil, Pearson et al. 2007). In addition, three different combinations of substrates were used in order to assess their potential for promoting neurogenesis. Cells from wildtype CE and Chx10<sup>orJ/orJ</sup> CE and CNR were cultured for 7 days in NGM and then plated onto PLL/Laminin, or PO/Laminin, or PO/Fibronectin coated glass coverslips in 1% FCS for 4 days, prior to inducing them with NDM. Figure 5.10b illustrates representative photomicrographs of wildtype and Chx10<sup>orJ/orJ</sup> CE as well as Chx10<sup>orJ/orJ</sup> CNR primed cells after 4 days in priming conditions before culture in differentiating conditions. Under those conditions, cells readily migrated away from the spheres and adhered, compared with cells grown in NDM without the priming step (See figure 5.1) which showed less migration.

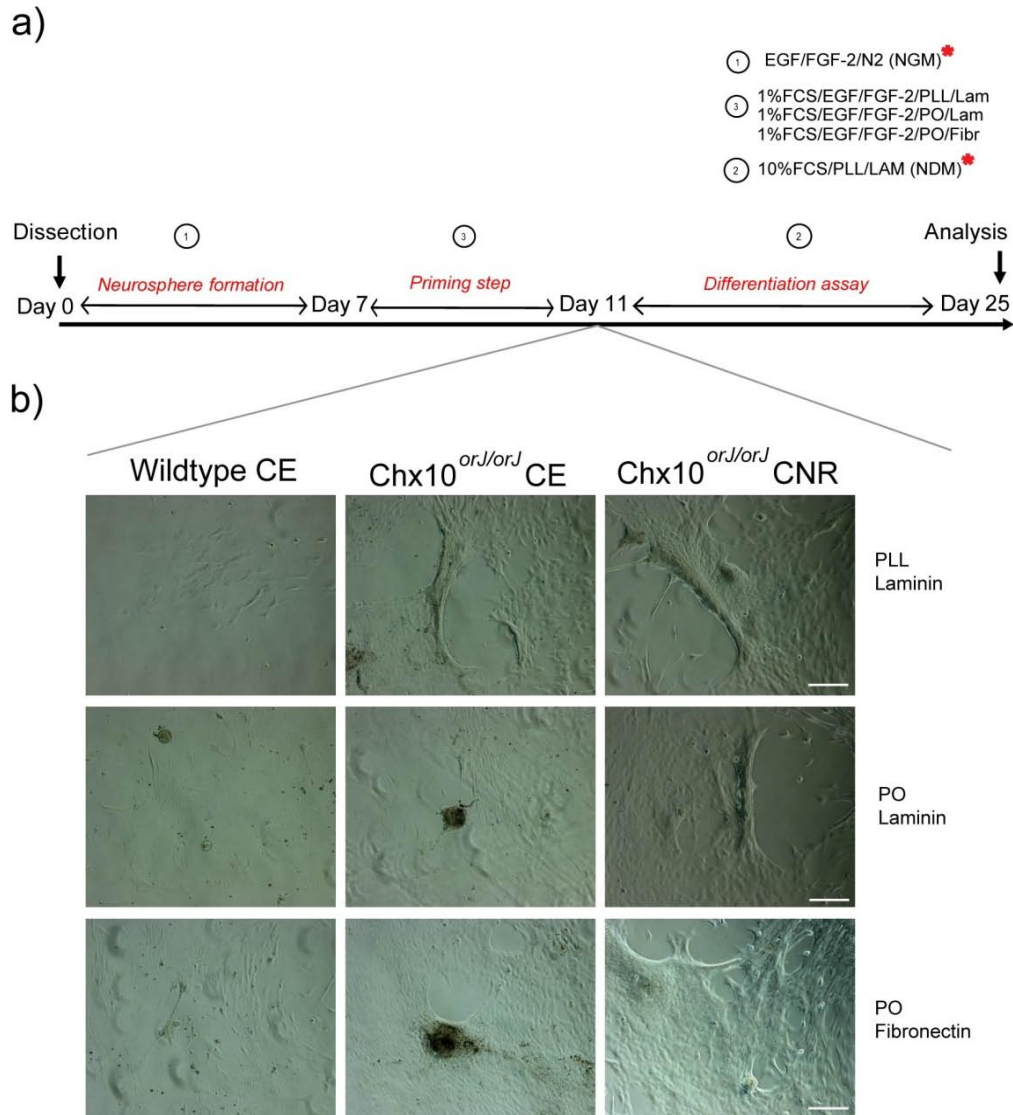


Figure 5.10 Effect of substrates on the differentiation potential of cells derived from adult wildtype and Chx10<sup>orJ/orJ</sup> retinal neurospheres

(a) Timeline of conditions used in culture for obtaining and differentiating wildtype and Chx10-deficient neurospheres. Eyes were dissected on day 0 and after dissociation cells were exposed in \*NGM for 7 days ①. Next, a priming step was preferred in which low serum was introduced with the presence of growth factors for 4 days on different substrates ③, before switching to standard neurosphere differentiation with the withdrawal of growth factors and supplementing with 10% FCS for 14 days in PLL and Laminin coated glass coverslips (\*NDM) ②. Assay analysis was performed at the end of the differentiation assay.

(b) Representative photomicrographs of neurospheres after priming step. Neurospheres lose their typical spherical shape and usually adhere on the bottom of the culture plate with cells migrating away from the neurosphere site. Scale bars = 10 mm.

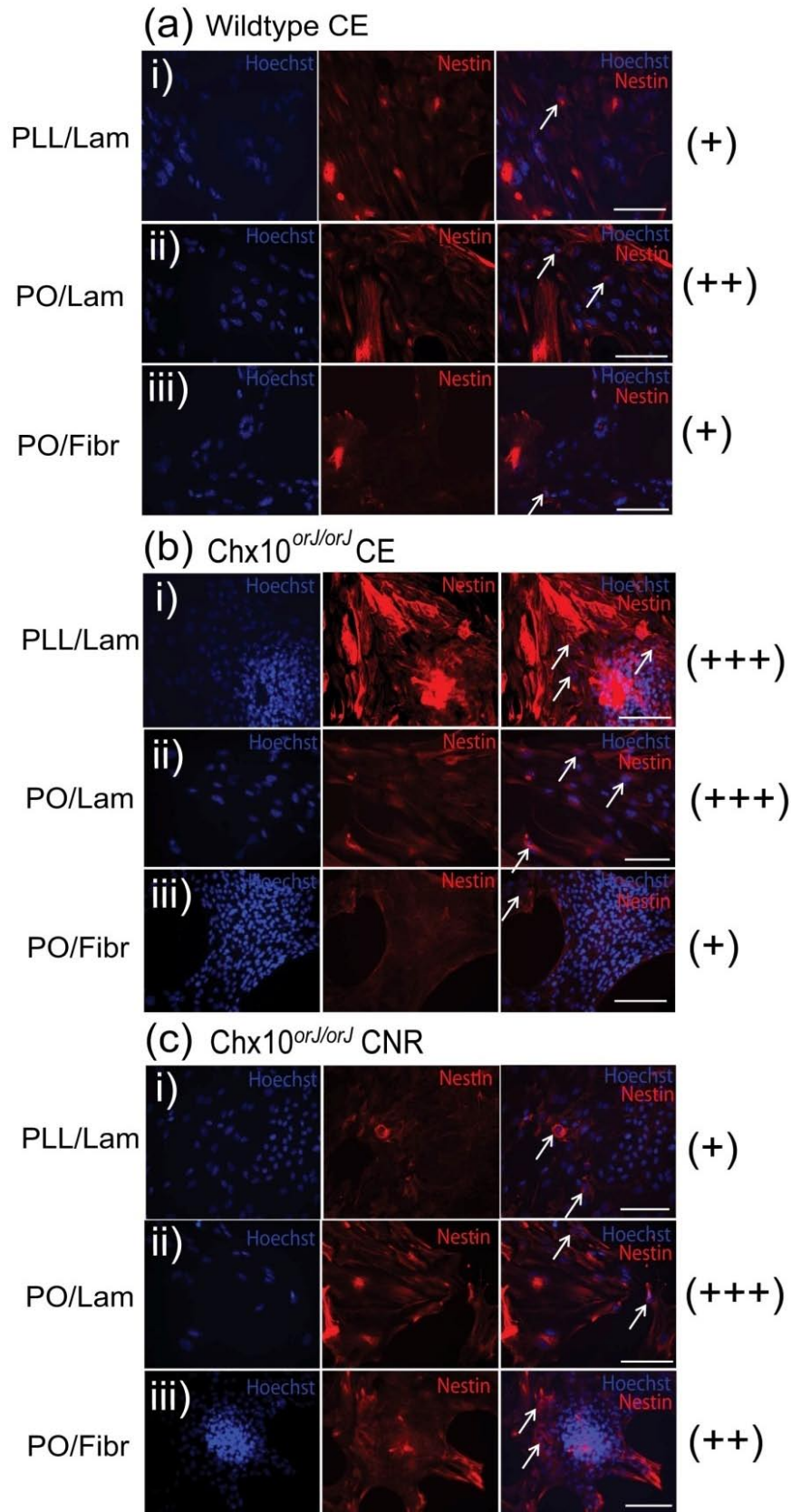
Analysis was performed after the differentiation assay step when cells were investigated for the presence of neural stem/progenitors (Nestin), neurons ( $\beta$ -III tubulin) and Müller glia cells (GS) (Figures **5.11**, **5.12** & **5.13**, respectively). Although a quantitative analysis was not performed, Immunostaining levels were scored based on the level of positive cells in two independent cultures, one slide per antibody. When PLL and Laminin were used as substrates [designated as (i) in the above mentioned figures], in wildtype CE there was a low expression of the progenitor marker Nestin (Figure **5.11a**), with a strong expression of neuronal proteins and absence of glial cells (Figures **5.12a** & **5.13a**, respectively). In contrast,  $Chx10^{orJ/orJ}$  CE cultures showed a very strong nestin expression with a similar expression of  $\beta$ -III tubulin and very low GS expression in comparison to wildtype CE (Figures **5.11b**, **5.12b** & **5.13b**).  $Chx10^{orJ/orJ}$  CNR differentiated cultures showed a similar expression profile as wildtype CE but with lower levels of  $\beta$ -III tubulin (Figures **5.11c**, **5.12c** & **5.13c**).

In the presence of PO and Laminin [designated as (ii) in the aforementioned figures], wildtype CE showed very strong expression of the retinal progenitor marker nestin (Figure **5.11a**), with low expression of neuronal proteins and absence of glial cells (Figures **5.12a** & **5.13a**, respectively). Cells derived from the  $Chx10^{orJ/orJ}$  CE showed a very strong nestin expression with very low expression of  $\beta$ -III tubulin and high GS expression in comparison to wildtype CE (Figures **5.11b**, **5.12b** & **5.13b**, respectively). The immuno-cytochemical profile from differentiated cells from the  $Chx10^{orJ/orJ}$  CNR showed very strong expression of Nestin with a strong and low expression for  $\beta$ -III tubulin and GS, respectively (Figures **5.11c**, **5.12c** & **5.13c**).

When PO and Fibronectin were used as substrates [designated as (iii) in the aforementioned figures], in wildtype CE there was low expression Nestin (Figure **5.11a**), with low expression of neuronal proteins and strong expression of glial cells (Figures **5.12a** & **5.13a**, respectively). Similarly,  $Chx10^{orJ/orJ}$  CE cultures

showed a low nestin expression with similar expression of  $\beta$ -III tubulin and very strong GS expression (Figures **5.11b**, **5.12b** & **5.13b**, respectively). Also, differentiated cells from the Chx10<sup>orJ/orJ</sup> CNR showed a similar expression profile with wildtype and mutant CE but with higher levels of nestin expression and lower for GS (Figures **5.11c**, **5.12c** & **5.13c**, respectively).

In conclusion, the priming step appeared to promote a better migration away from the neurosphere sites and increased cell number although this was not quantified on this occasion. Laminin and PLL were considered the most appropriate substrates for differentiating towards a neuronal fate and away from glial differentiation, while PO and Fibronectin favoured glial differentiation at the expense of neurons. Laminin/PLL was used in the differentiation assays presented in Figures 5.1 & 5.3.



**Figure 5.11 Analysis of the effect of different substrates in the expression of Nestin on cells derived from adult wildtype and Chx10<sup>orJ/orJ</sup> retinal neurospheres**

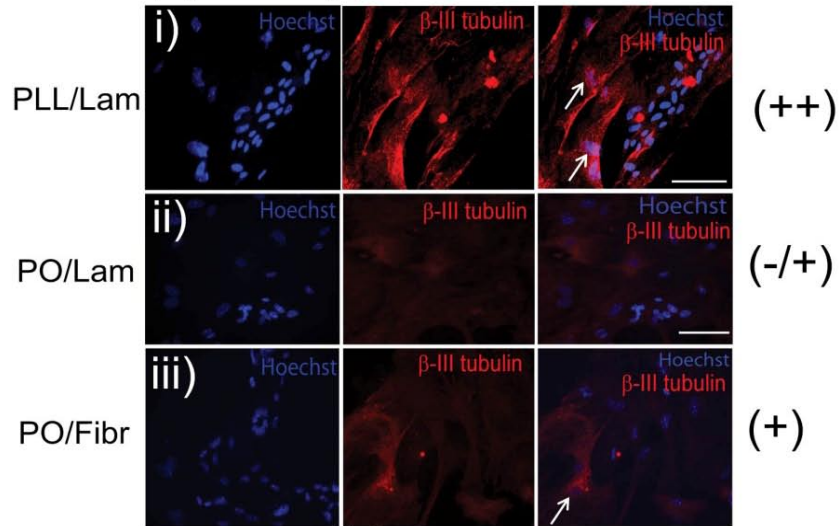
Representative photos of cells migrated away from neurospheres after priming and differentiation steps. Cells from wildtype (a) Chx10<sup>orJ/orJ</sup> CE (b) and Chx10<sup>orJ/orJ</sup> CNR (c) were immunolabelled with antibodies against Nestin (red) for the detection of neural stem/progenitors upon plating onto PLL/Laminin (i), PO/Laminin (ii) and PO/Fibronectin (iii) in the priming step before seeded to NDM for 14 days *in vitro*.

Key: + = low expression, ++ = strong expression, +++ = very strong expression. Scoring system indicates how strong the expression was in total in comparison to wildtype CE.

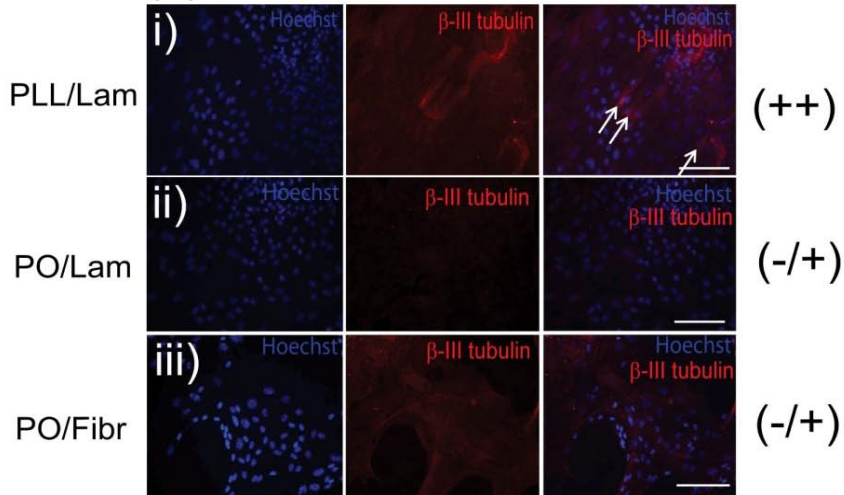
Cells were counter-stained with Hoechst 33342. Scale bars= 10  $\mu$ m. N=2 independent cell cultures per genotype.



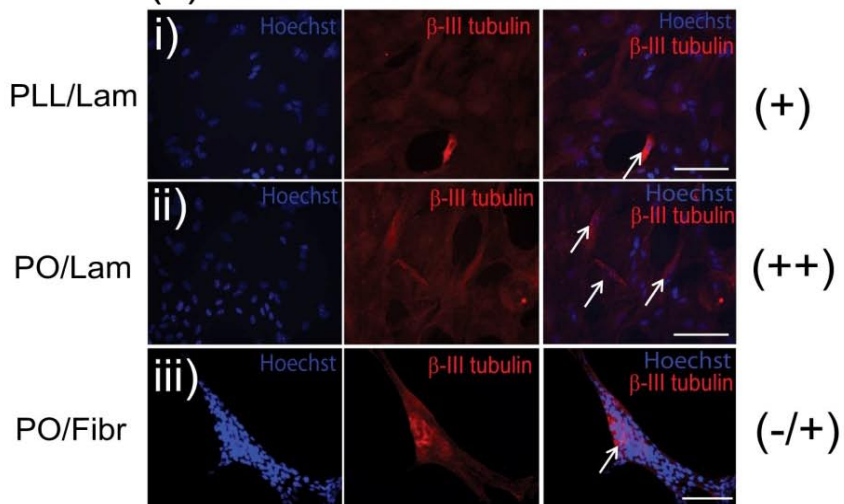
(a) Wildtype CE



(b) *Chx10<sup>or/orJ</sup>* CE



(c) *Chx10<sup>or/orJ</sup>* CNR



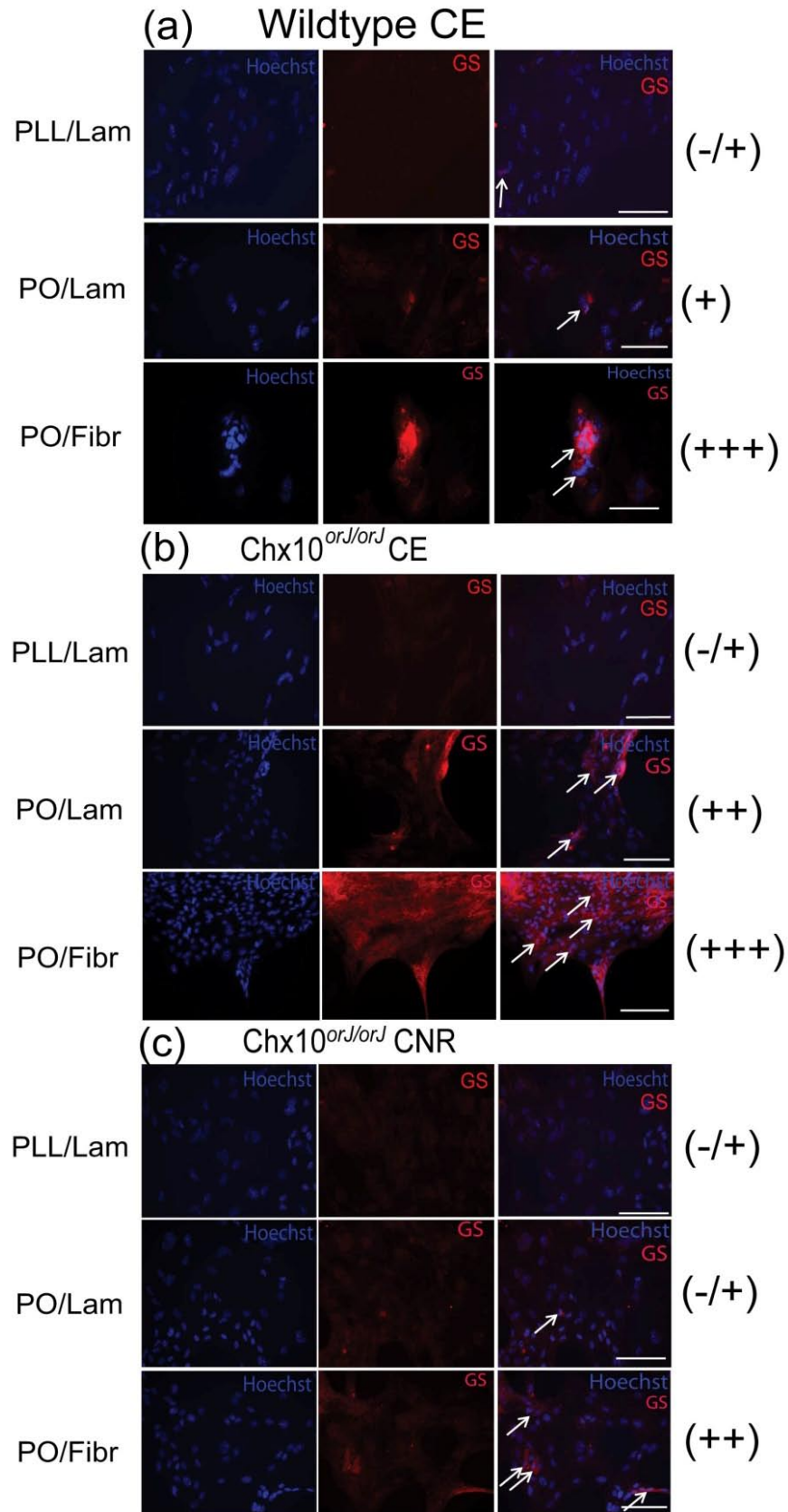


**Figure 5.12 Analysis of the effect of different substrates in the expression of  $\beta$ -III tubulin on cells derived from adult wildtype and  $Chx10^{orJ/orJ}$  retinal neurospheres**

Representative photos of cells migrated away from neurospheres after priming and differentiation steps. Cells from wildtype (a)  $Chx10^{orJ/orJ}$  CE (b) and  $Chx10^{orJ/orJ}$  CNR (c) were immunolabelled with antibodies against  $\beta$ -III tubulin (red) for the presence of early-born neurons upon plating onto PLL/Laminin (i), PO/Laminin (ii) and PO/Fibronectin (iii) in the priming step before seeded to NDM for 14 days *in vitro*.

Key: -/+ = very low or no expression, + = low expression, ++ = strong expression, +++ = very strong expression. Scoring system indicates how strong the expression was in total in comparison to wildtype CE.

Cells were counter-stained with Hoechst 33342. Scale bars= 10  $\mu$ m. N=2 independent cell cultures per genotype.



**Figure 5.13 Analysis of the effect of different substrates in the expression of GS on cells derived from adult wildtype and Chx10<sup>orJ/orJ</sup> retinal neurospheres**

Representative photos of cells migrated away from neurospheres after priming and differentiation steps. Cells from wildtype (a) Chx10<sup>orJ/orJ</sup> CE (b) and Chx10<sup>orJ/orJ</sup> CNR (c) were immunolabelled with antibodies against GS (red) for the presence of Müller glia upon plating onto PLL/Laminin (i), PO/Laminin (ii) and PO/Fibronectin (iii) in the priming step before seeded to NDM for 14 days *in vitro*.

Key: -/+ = very low or no expression, + = low expression, ++ = strong expression, +++ = very strong expression. Scoring system indicates how strong the expression was in total in comparison to wildtype CE.

Cells were counter-stained with Hoechst 33342. Scale bars= 10 µm. N=2 independent cell cultures per genotype.

### 5.2.5 The *Nrl.gfp*<sup>+/+</sup> transgenic mouse model

In order to better facilitate identification of photoreceptor differentiation, a transgenic mouse model was employed for the identification of rod photoreceptor precursors *in vitro*. The transgenic model carries a eukaryotic *Gfp* coding sequence downstream of a 2.5Kb segment of the mouse neural leucine zipper (*Nrl*) gene (Akimoto et al. 2006). This TF promotes rod photoreceptor gene expression and has been shown that cells expressing *Nrl* in these transgenic models, will also express GFP (Mears AJ 2001). I tested this in my system by obtaining *Nrl.gfp*<sup>+/+</sup> adult eyes confirming the presence of GFP in the retinal tissue of the whole eye [Figure 5.14a(i)]. Also, saggital retinal cryosections were immunolabelled with recoverin (photoreceptor marker, red) and showed co-localisation with the endogenous GFP signal in the ONL [Figure 5.14a (ii and iii)]. As negative controls, differentiated cells were also assessed for GFP expression from wildtype SV129 adult CE-derived neurospheres [Figure 5.14b (i-iii)] and saggital retinal sections [Figure 5.14b (IV)], where no GFP expression was observed, as expected.

### 5.2.6 Rod photoreceptor precursor potential of *Nrl.gfp*<sup>+/+</sup> CE neurospheres

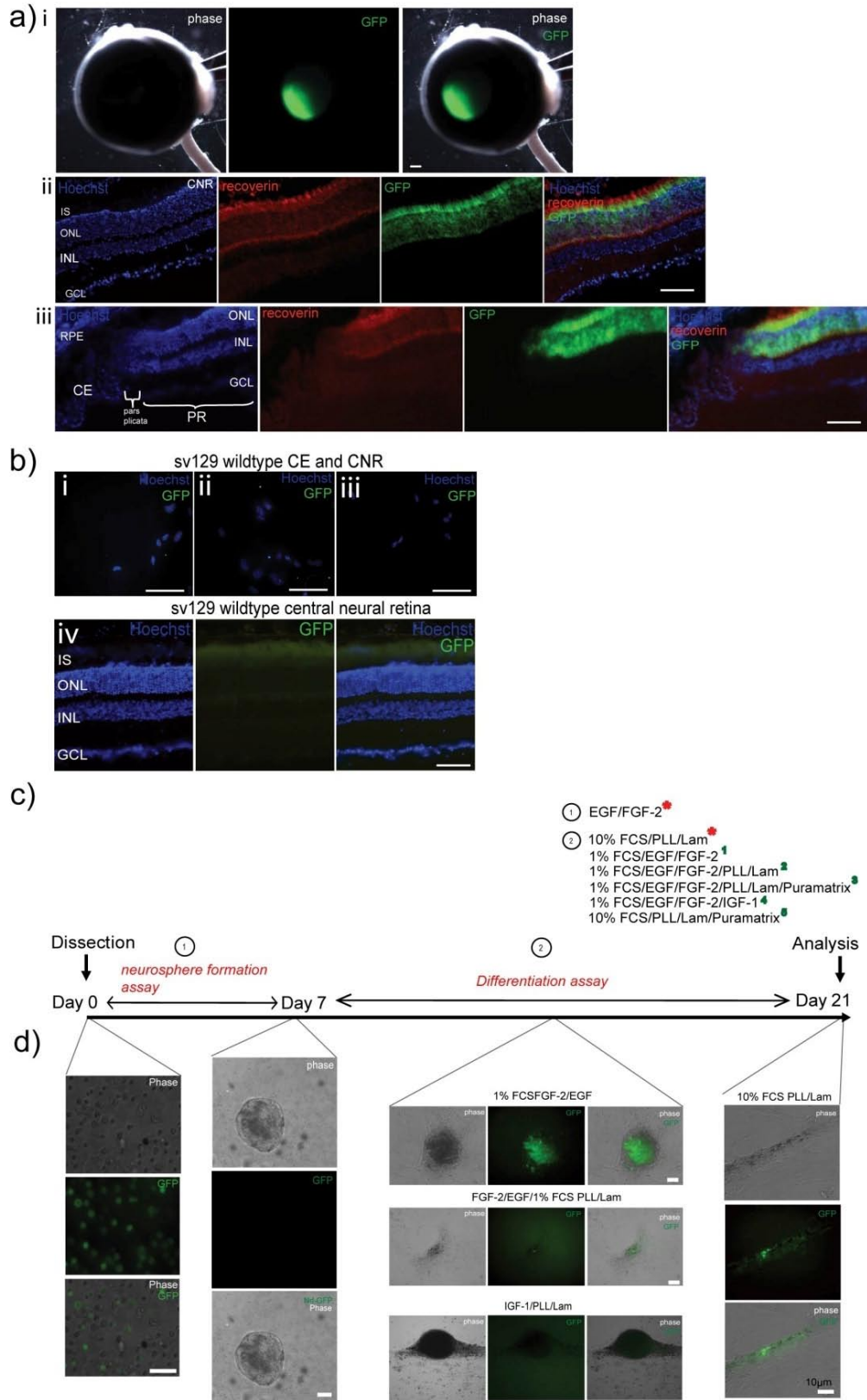
The optimal differentiation culture conditions identified in previous experiments were tested in the *Nrl.gfp* mouse cultures. For this set of experiments, a neurosphere formation step was included in the presence of growth factors prior to a one-step differentiation assay in high or low serum supplement on PLL/Laminin-coated glass coverslips. In addition, Puramatrix<sup>TM</sup> and insulin growth factor-1 (IGF-1) were also tested for their effect on obtaining rod photoreceptor precursors (Figure 5.14c) (Cameron, Hazel et al. 1998; James J 2004). Cells were dissociated from the adult CE of *Nrl.gfp*<sup>+/+</sup> mice on day 0 and

cultured for 7 days in NGM. Representative photomicrographs show the GFP<sup>POS</sup> cells from the acutely dissociated CNR of the transgenic mice (Figure 5.14d), while CE-derived neurospheres prior to differentiation showed no GFP expression. Once placed in differentiating conditions, some cells began to express GFP, an indicator of rod photoreceptor precursor production early in the differentiation assay (from day 4) up to Day 14. No apparent GFP expression was observed in cultures with IGF-1 (20 ng/ ml) in the absence of serum.

Immunocytochemical analysis was performed at the end of the differentiation assay, where cells were co-immunolabelled with recoverin and an antibody against GFP. Several differentiation conditions were tested. In the absence of any adhesive substrate and only in NGM and 1% FCS conditions were not ideal for cell migration away from the neurosphere site but showed a strong expression and co-localisation of recoverin and GFP within the neurosphere (Figure 5.15a). When plated into PLL and Laminin coated glass coverslips, cells migrated away from the neurosphere site with a small proportion shown to express recoverin and GFP (Figure 5.15b). No cells were shown to express one or the other marker. When 0.1% or 0.5% w/v Puramatrix<sup>TM</sup> as a cell surface adherent matrix was used in the presence of growth factors, low amount of serum (1%) and PLL and Laminin, a proportion of cells showed recoverin/GFP co-expression at 0.5% w/v Puramatrix<sup>TM</sup> but with no relative increase in the number of GFP<sup>POS</sup> cells (Figure 5.15c).

Switching to a higher amount of FCS (10%), with PLL and Laminin coated-glass coverslips showed co-localisation of recoverin and GFP with the morphology of some more cells resembling that of neurons (Figure 5.15d). When 0.1% or 0.5% w/v Puramatrix<sup>TM</sup> was employed with 1% FCS, there was a stronger presence of recoverin/GFP-positive cells, especially when used at 0.5% w/v concentration (Figure 5.15e). As a negative control, Puramatrix was used in neurosphere formation assay of *Nrl.gfp*<sup>+/+</sup> cells, and analysed on day 7 by immunolabelling

with anti-GFP. Visualisation under a fluorescence microscope showed no GFP expression (Figure **5.15f**).



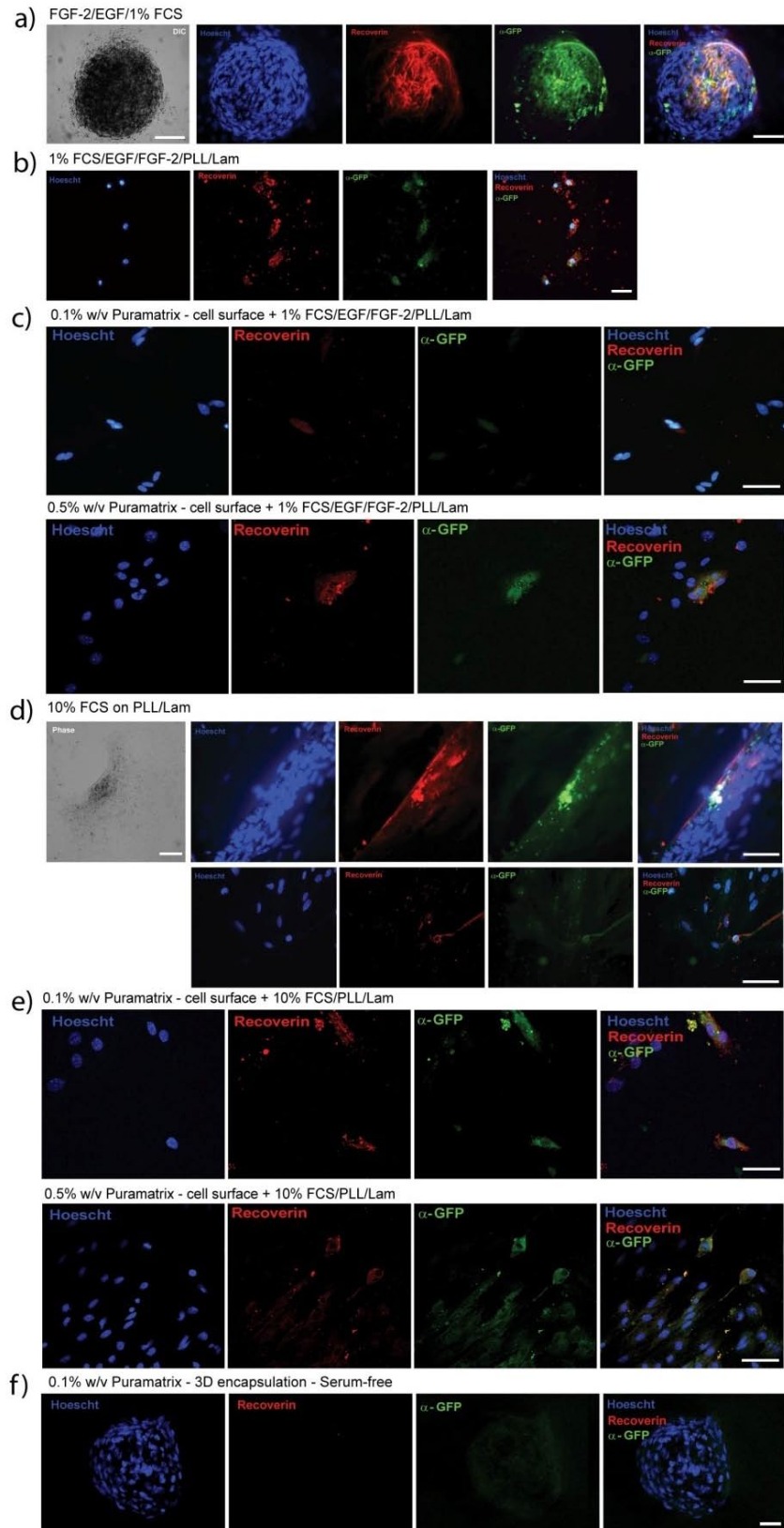
**Figure 5.14 The *Nrl.gfp<sup>+/+</sup>* transgenic mouse model**

**(a)** Phase and fluorescent *gfp* photographs of a dissected whole eye of an adult *Nrl.gfp<sup>+/+</sup>* mouse **(i)**. Saggital cryosections of the central neural retina **(ii)** and peripheral retina **(iii)** of an adult *Nrl.gfp<sup>+/+</sup>* mouse immunolabelled with Recoverin (red) and *Nrl<sup>POS</sup>* cells expressing GFP (green). **(b)** As controls, cells derived from sv129 wildtype dissociated neural retina **(i)**, CE **(ii)**, and CE cells differentiated in NDM after neurosphere formation **(iii)** and saggital adult retinal cryosections were visualised for GFP expression using the same imaging conditions as for **(a)**. **(c)** Timeline of conditions used in culture for obtaining and differentiating *Nrl.gfp<sup>+/+</sup>* cells from CE-derived neurospheres. Eyes were dissected on day 0 and after dissociation cells were exposed in NGM for 7 days. In the differentiation step, low serum or high serum was introduced in the presence of NGM using PLL/Laminin with or without Puramatrix or no substrate with or without IGF-1 (20 ng/ ml), for 14 days. Assay analysis was performed at the end of the differentiation assay. **(d)** Representative photos of neurospheres and cells in different time points during the timeline assay. Day 0; dissociated retinal cells from the *Nrl.gfp<sup>+/+</sup>* central neural retina. Day 7; Live neurosphere phase and GFP imaging of neurospheres from the wildtype CE in NGM. Intermediate day; live neurosphere phase and GFP imaging of neurospheres from the wildtype CE in low-serum conditions in the presence of growth factors with or without substrates or IGF-1 (20 ng/ ml) *in vitro*. Day 25; Live neurosphere phase and GFP imaging of neurospheres from the wildtype CE in 10% FCS in PLL/Laminin-coated glass coverslips. Scale bars; 10  $\mu$ m.

Cells were counter-stained with Hoechst 33342. Scale bars=10  $\mu$ m. N=1 experiment.

Abbreviations: PR=peripheral retina, CNR=central neural retina, CE=ciliary epithelium, RPE=retinal pigment epithelium, ONL=outer nuclear layer, INL=inner nuclear layer, GCL=ganglion cell layer, IS=inner segment





**Figure 5.15 Comparative analysis of the effect of different substrates, serum and growth factors in the production of rod photoreceptor precursors from cells derived from the adult wildtype CE of the *Nrl.gfp<sup>+/+</sup>* mouse model.**

After neurosphere formation in the presence of NGM, neurospheres were allowed to differentiate for 14 days in the presence of low serum and NGM (**a-c**) in PLL/Laminin-coated glass coverslips (**b**) and 0.1% or 0.5% Puramatrix (**c**). Also, growth factors were withdrawn and high presence of FCS was introduced (**d-e**) in the presence of 0.1% or 0.5% Puramatrix in PLL/Laminin-coated glass coverslips. During the neurosphere formation step 0.1% Puramatrix was added. Cells and neurospheres were immunolabelled with recoverin (red) and an antibody against GFP (green) was used to detect any co-localisation. As a negative control, 0.1% Puramatrix in a serum-free medium in the presence of NGM was used (**f**).

Cells were counter-stained with Hoechst 33342. Scale bars=10  $\mu\text{m}$ . N=2 independent cultures.

The detection of GFP<sup>pos</sup> cells under several conditions prompted me to perform a more thorough comparative analysis of cells from different conditions. CE-derived neurospheres were *in vitro* cultured for 14 days in the presence of 1% FCS or 10% FCS in the presence or absence of growth factors on PLL and Laminin-coated glass coverslips or with no adhesive substrates (Figure **5.16a**). It was not possible to visualise GFP without the use of an antibody against GFP. The presence of *Gfp* mRNA was confirmed by quantitative Re-Ti PCR using a gene expression assay for *gfp* detected transcripts in all three culture conditions and in *Nrl.gfp*<sup>+/+</sup> acutely dissociated CNR cells, but signal was absent in *Nrl.gfp*<sup>+/+</sup> CE-derived neurosphere-forming cells grown for 7 days in NGM only (Figure **5.16b**). In conclusion, CE neurosphere-derived cells can switch on *Nrl* transcription upon differentiating conditions only.

In conclusion, while these data show some level of expression of the rod photoreceptor marker *Nrl*, conditions to promote high expression levels in significant number of cells were not identified.

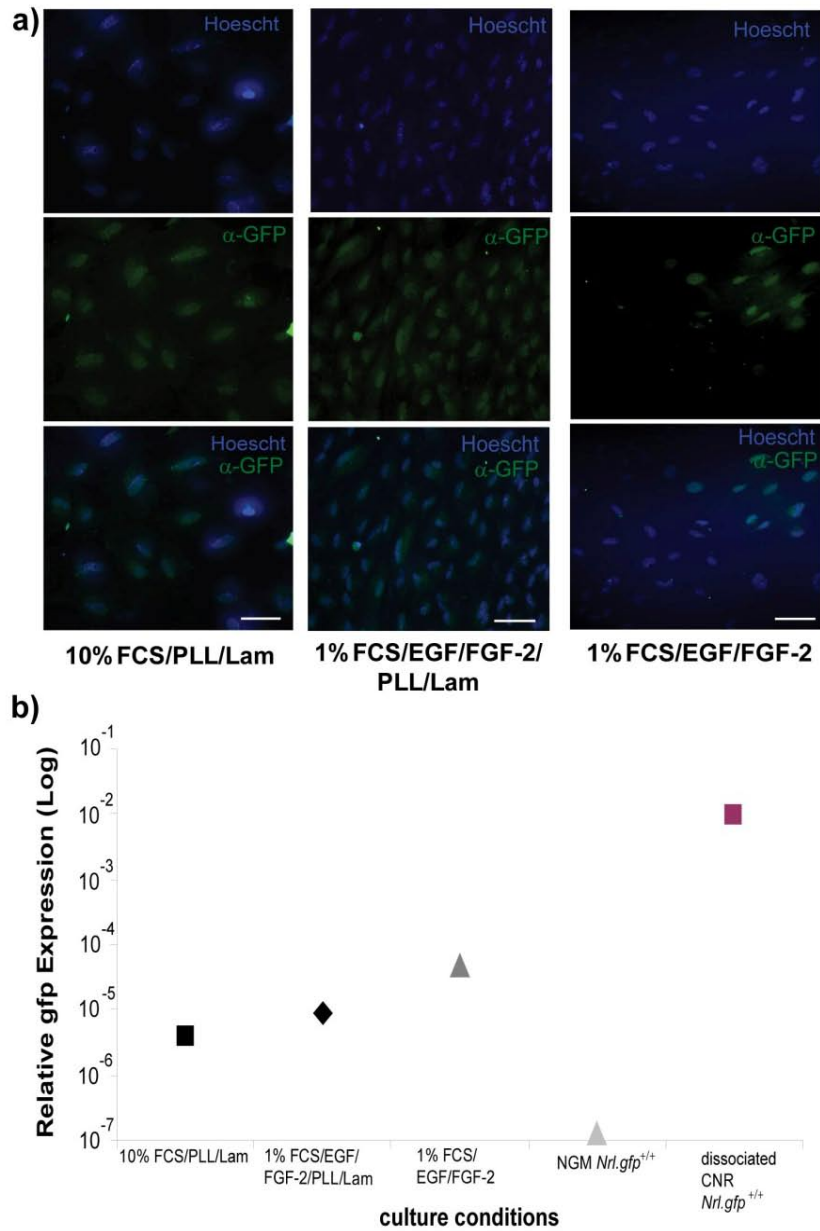


Figure 5.16 Effect of different substrates, serum and growth factors in the production of rod photoreceptor precursors from cells derived from the adult wildtype CE of the *Nrl.gfp*<sup>+/+</sup> mouse model

(a) Representative photographs of differentiated cells. Cells were immunolabelled with an antibody against GFP. Cells were counter-stained with Hoechst 33342. Scale bars=10 mm.

(b) Quantitative Re-Ti PCR analysis of *gfp* gene expression in differentiated CE-neurosphere derived cells cultured in NDM (black square), 1% FCS with growth factors on PLL/Lam (black triangle) and 1% FCS with growth factors in the absence of a substrate (dark grey triangle). Wildtype *Nrl.gfp*<sup>+/+</sup> CE-derived neurospheres and CNR were also tested for GFP gene expression. Relative expression levels were normalized to *Gapdh* (N=1 experiment).

### 5.2.7 The *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* transgenic mouse model

The *Nrl.gfp<sup>+/+</sup>* transgenic mouse model is a useful tool for investigating the potential of neurosphere-forming cells to produce retinal neurons or retinal precursors *in vitro*. The adult *Chx10<sup>orJ/orJ</sup>* CNR was shown to give rise to more and larger neurospheres than the wildtype CE (See figures 3.9–3.11). In addition, quantitative PCR indicated an upregulation of photoreceptor genes in the differentiated cultures of *Chx10<sup>orJ/orJ</sup>* CE cultures (See section 5.2.1). *Chx10<sup>orJ/orJ</sup>* mice were crossed with *Nrl.gfp<sup>+/+</sup>* mice to give rise to an F1 progeny of *Chx10<sup>orJ/+</sup>/Nrl.gfp<sup>+/-</sup>* showing a normal phenotype. The *Nrl.gfp<sup>+/+</sup>* transgene has 6 copies of the gene encoding the GFP. Mice from the F1 progeny were then inter-crossed producing a mixed progeny (F2) for the orJ and GFP alleles, according to the Law of Segregation (Mendel's First Law) and Punnett square (Figure 5.17a). Mice from the F2 progeny that were microphthalmic were genotyped for the GFP transgene by quantitative Re-Ti PCR and the ones positive were used for our experiments (Figure 5.17b). Quantitative PCR did not accurately distinguish *Nrl.gfp<sup>+/+</sup>* and *Nrl.gfp<sup>+/-</sup>* in these experiments, but presence and absence of the transgene was easily detected. Saggital sections of adult retinæ were obtained and examined for their expression of GFP and recoverin via immunolabelling (Figure 5.18). Due to the orJ retina being hypocellular with a very thin ONL, there were rare GFP<sup>pos</sup> cells, as well as few Recoverin<sup>pos</sup> cells with some showing co-expression for those two markers. No expression of GFP or recoverin was observed in the ciliary epithelium of these mice (Figure 5.18a).

I setup a timeline experiment with an overall goal to obtain more *Nrl.gfp<sup>pos</sup>* cells *in vitro* from retinal neurospheres (Figure 5.18b, c). Cells were initially dissociated from the CE and CNR of the *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* eyes in NGM for 7 days to allow neurosphere formation. That was followed by a 4-day priming step in low serum conditions along with NGM with or without IGF-1 (20 ng/ ml). Cells

were then allowed to differentiate in NDM for 14 days. Analysis was performed after the end of the priming and differentiation steps.

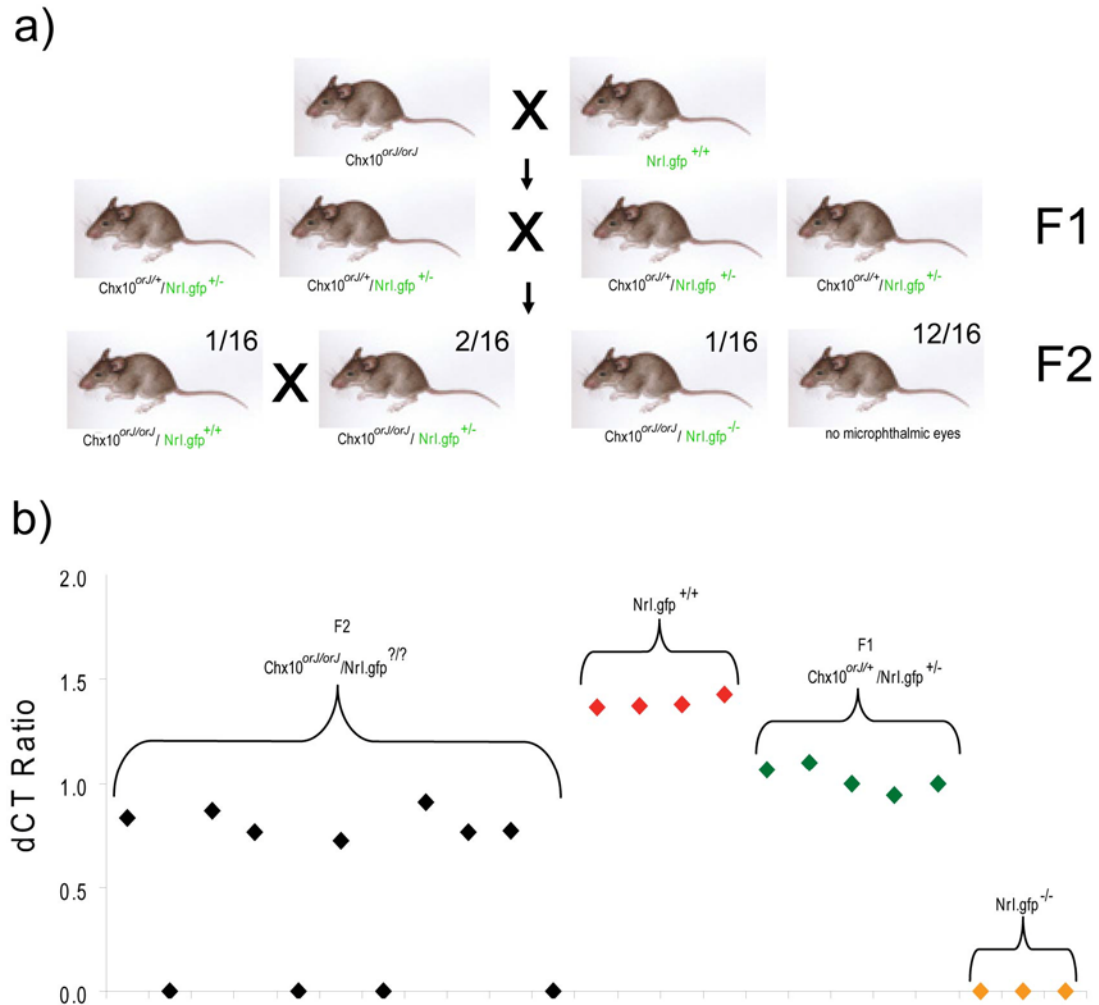
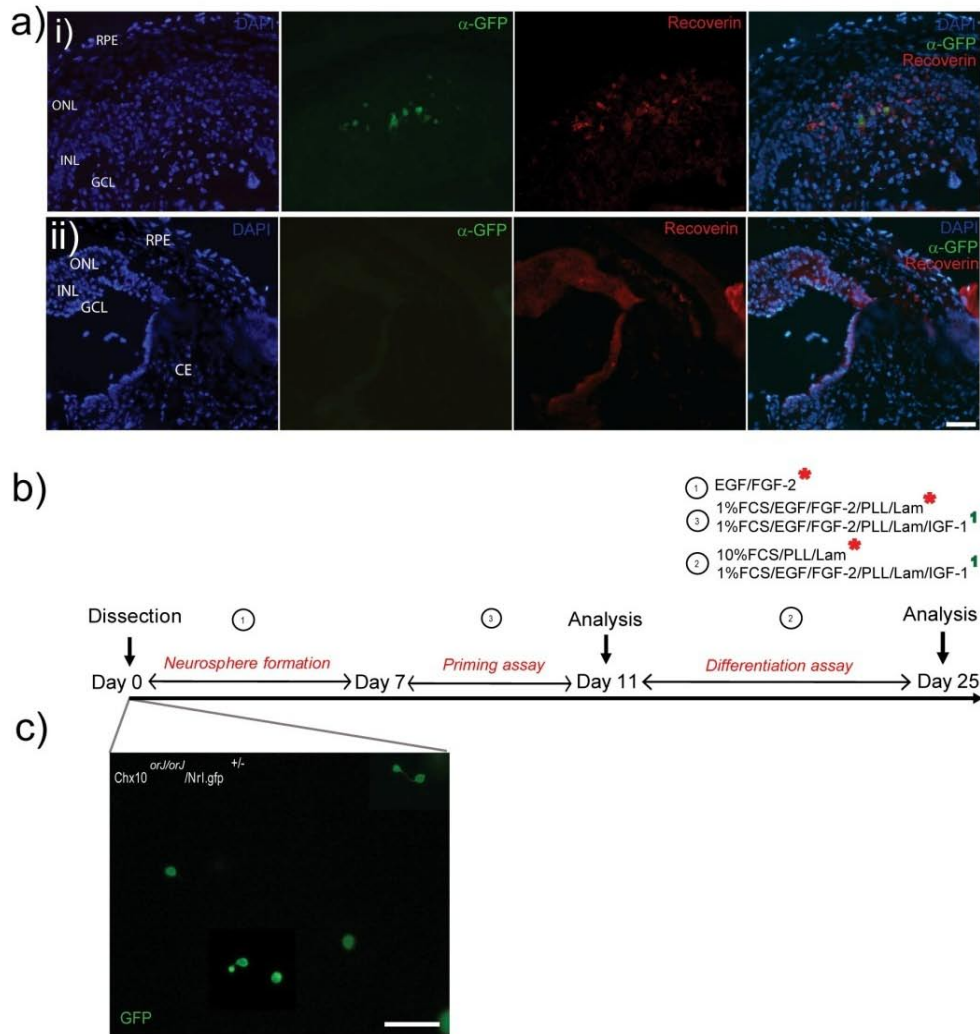


Figure 5.17 Generation of the  $Chx10^{orJ/orJ}/Nrl.gfp$  transgenic mouse model  
**(a)** Generation of the  $Chx10^{orJ/orJ}/Nrl.gfp$  mouse model was obtained through crossing  $Chx10^{orJ/orJ}$  and  $Nrl.gfp^{+/+}$  mice. F1 progeny inter-crossed giving rise to the F2 generation. From these, mice having a microphthalmic phenotype were screened for Gfp expression and used in analysis.  
**(b)** Quantitative Re-Ti PCR analysis of genomic DNA GFP to determine copy number in the F1 and F2 generations. F1:  $Chx10^{orJ/+}/Nrl.gfp^{+/-}$  (green diamonds) F2: black diamonds, wildtype  $Nrl.gfp^{+/+}$  (red diamonds), and wildtype sv129  $Nrl.gfp^{-/-}$  mice (yellow diamonds). Relative expression levels were normalized to Gapdh.





**Figure 5.18** Differentiation of  $Chx10^{orJ/orJ} /Nr1.gfp$  CE- and CNR- neurospheres (a) Coronal cryosections of the central neural retina (i) and peripheral retina (ii) of an adult  $Chx10^{orJ/orJ} /Nr1.gfp$  mouse immunolabelled with Recoverin (red) and an antibody against GFP (green).

(b) Timeline of conditions used in culture for obtaining and differentiating cells from  $Chx10^{orJ/orJ} /Nr1.gfp$  CE- and CNR-derived neurospheres. Eyes were dissected on day 0 and after dissociation cells were exposed in NGM for 7 days. In the priming step, low serum or high serum was introduced in the presence of NGM using PLL/Laminin with or without IGF-1 (20ng/ ml) for 4 days, followed by differentiation induction in NDM or 1% FCS/ NGM and IGF-1 for 14 days in vitro. Assay analysis was performed at the end of the priming and differentiation assays.

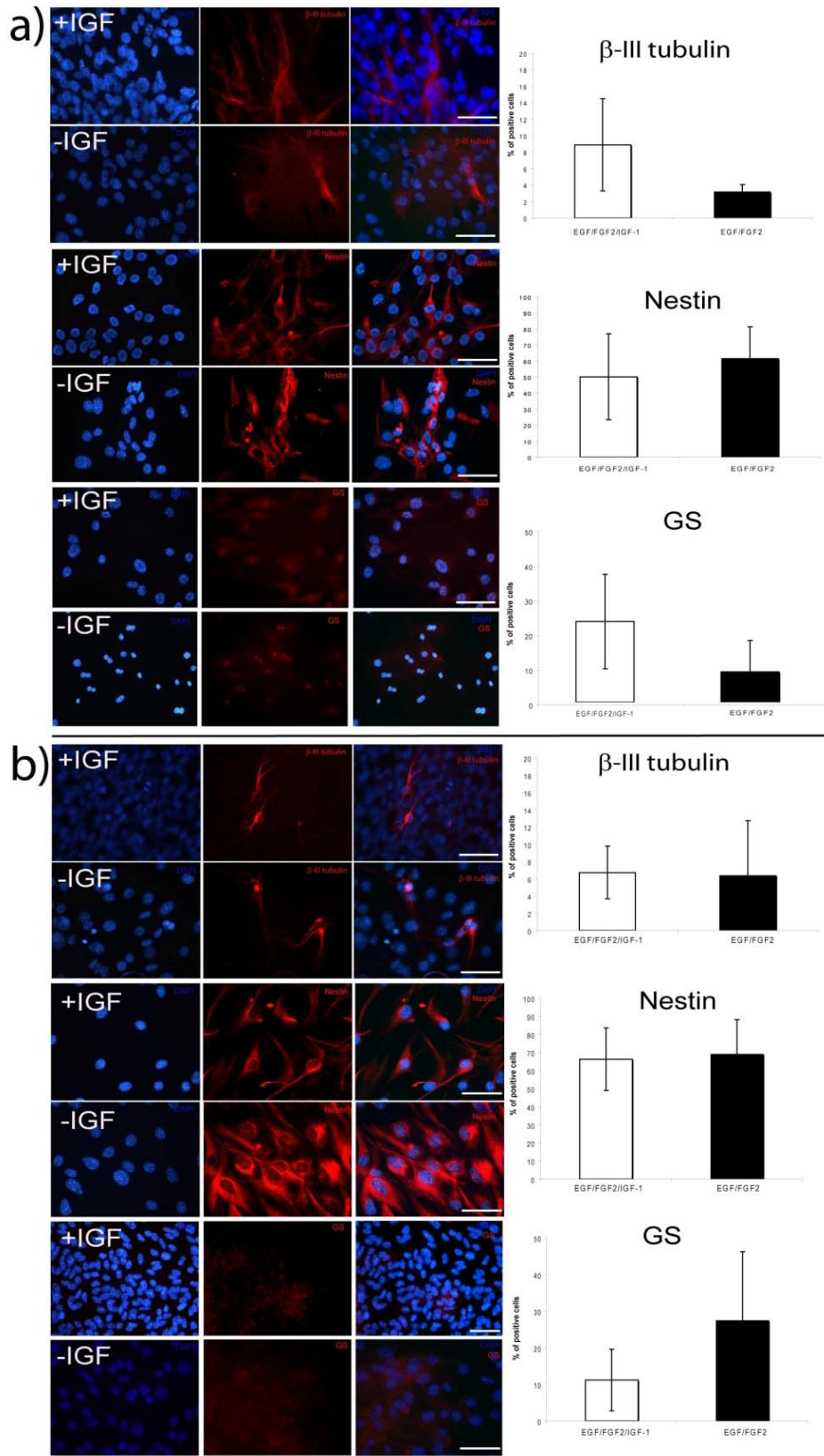
(c) Representative photo of dissociated central neural retinal cells from  $Chx10^{orJ/orJ} /Nr1.gfp$  adult mice, expressing GFP.

Abbreviations: PR=peripheral retina, CNR=central neural retina, CE=ciliary epithelium, RPE=retinal pigment epithelium, ONL=outer nuclear layer, INL=inner nuclear layer, GCL=ganglion cell layer. Scale bars = 10  $\mu$ m.



Cell cultures treated with NGM, plus IGF-1 at the priming step, were compared to cultures treated with NGM only, for their progenitor, neuronal and glial potential after the end of the priming and differentiation steps. Although immature rod photoreceptor precursors from the developing *Nrl.gfp<sup>+/+</sup>* retina express GFP at levels readily detectable by fluorescent microscopy, in the *in vitro* CE cultures no GFP was detected (MacLaren, Pearson et al. 2006). For this reason immunocytochemistry was performed to assess cell phenotype (Figure 5.19). *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* CE derived cells, upon IGF-1 treatment showed no alteration in the percentage of cells positive for progenitor (Nestin), neuronal ( $\beta$ -III tubulin) and glial (GS) protein markers judging from immunocytochemistry after the priming step. Similarly, after 14 days in high serum, cells did not alter their potential significantly towards a neuronal fate, although there was a trend for decreased GS expression (Glia) [Figure 5.19A (a)]. In addition, the proportion of cells positive for these markers was not altered significantly between priming and differentiation steps [Figure 5.19A (b)].

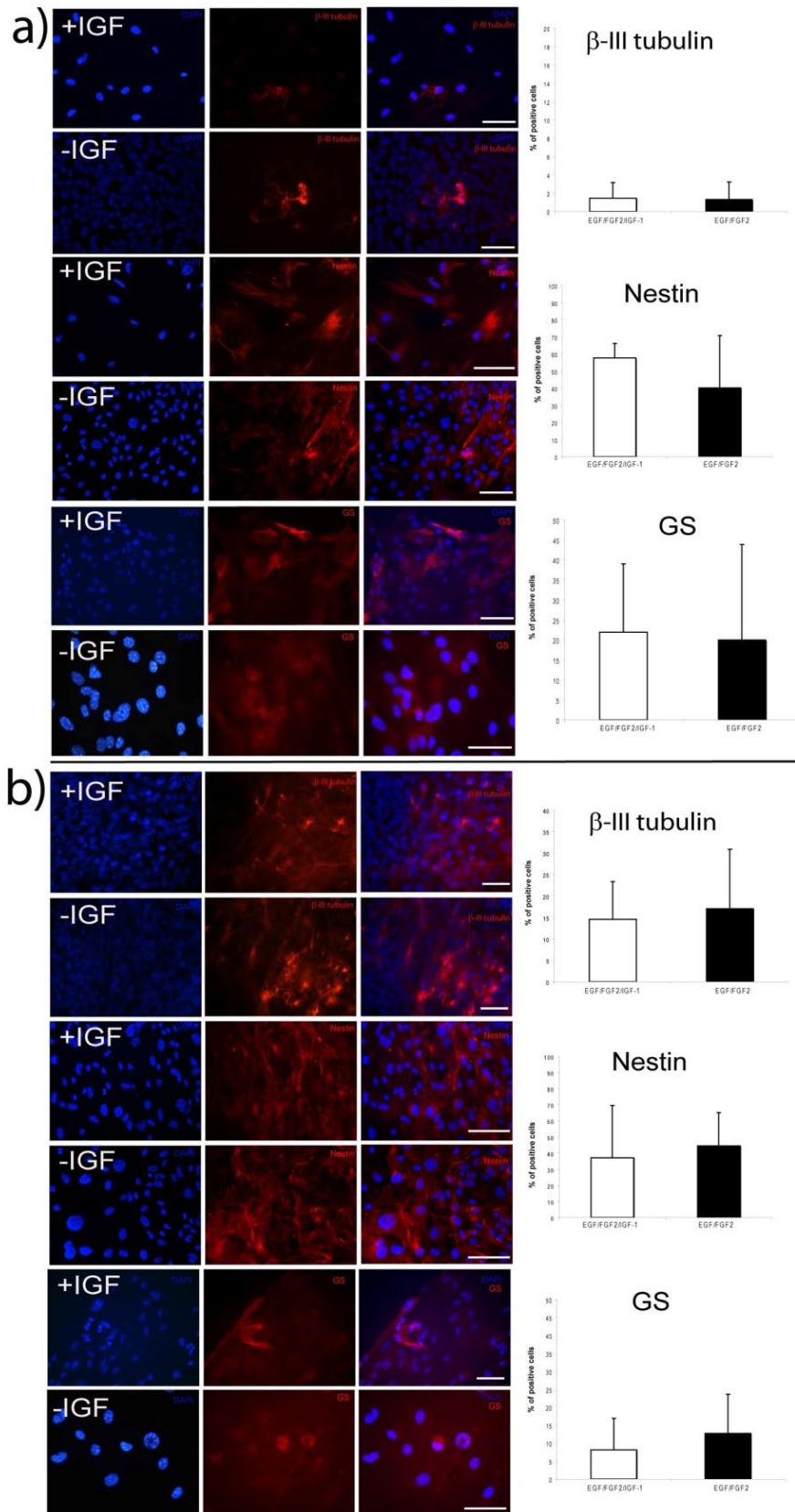
Similarly, *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* CNR derived cells, upon IGF-1 treatment had the same proportion of cells positive for progenitor (Nestin), neuronal ( $\beta$ -III tubulin) and glial (GS) protein markers as their EGF/FGF-2 treated counterparts after the priming step [Figure 5.19B (a)]. After 14 days in high serum, cells did not alter their potential significantly towards a neuronal fate [Figure 5.19B (b)]. Finally, the percentage of cells positive for progenitor and glia markers was not altered significantly between priming and differentiation steps, but there were more  $\beta$ -III tubulin<sup>pos</sup> cells after priming implying an increase in the neuronal cell population.



**Figure 5.19A Comparative analysis of the effect of IGF-1 in the priming and differentiation steps towards the potential of cells derived from adult Chx10<sup>orJ/orJ</sup>/Nrl.gfp CE neurospheres**

The effect of IGF-1 (20 ng/ ml) in the priming step (a) and in the differentiation step (b) of mutant CE-derived neurospheres after 7 days *in vitro* is shown by the comparative expression of Nestin (neural stem/progenitors, red)  $\beta$ -III tubulin (neurons, red) and GS (Müller glia, red). Graphs show the percentage of cells found positive for the above markers in the presence or absence of IGF-1.

Cells were counter-stained with DAPI. Scale bars=10  $\mu$ m. N=3 independent cultures. Two representative plates for each.



**Figure 5.19B Comparative analysis of the effect of IGF-1 in the priming and differentiation steps towards the potential of cells derived from adult Chx10<sup>orJ/orJ</sup>/Nrl.gfp CNR neurospheres**

The effect of IGF-1 (20 ng/ ml) in the priming step (a) and in the differentiation step (b) of mutant CNR-derived neurospheres after 7 days *in vitro* is shown by the comparative expression of Nestin (neural stem/progenitors, red)  $\beta$ -III tubulin (neurons, red) and GS (Müller glia, red). Graphs show the percentage of cells found positive for the above markers in the presence or absence of IGF-1.

Cells were counter-stained with DAPI. Scale bars=10  $\mu$ m. N=3 independent cultures. Two representative plates for each condition.

Since there were prior indications that *Nrl.gfp*<sup>+/+</sup> CE-derived neurospheres showed expression of GFP (See Figure 5.16), a comparative analysis was performed on cells treated with different conditions in the *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp* mouse model and then immuno-stained for GFP expression. Mutant CE-derived neurospheres were *in vitro* cultured for 14 days in the presence of 1% or 10% FCS in the presence or absence of growth factors onto PLL and Laminin-coated glass coverslips or with no adhesive substrates (Figure **5.20a**). Mutant CNR-derived neurospheres were also *in vitro* cultured for 14 days in the presence of 1% FCS or 10% FCS in the presence or absence of growth factors on PLL and Laminin-coated glass coverslips or with no adhesive substrates (Figure **5.20b**). Detection of GFP<sup>pos</sup> cells was observed but not conclusive to whether there was a difference in comparison to wildtype. By immuno-staining, low levels of GFP expression were detected suggesting some activation of the *Nrl.gfp* transgene was occurring in those culture conditions. This is consistent with the *Recoverin* and *Crx* detection detected in figures 5.3 and 5.6.

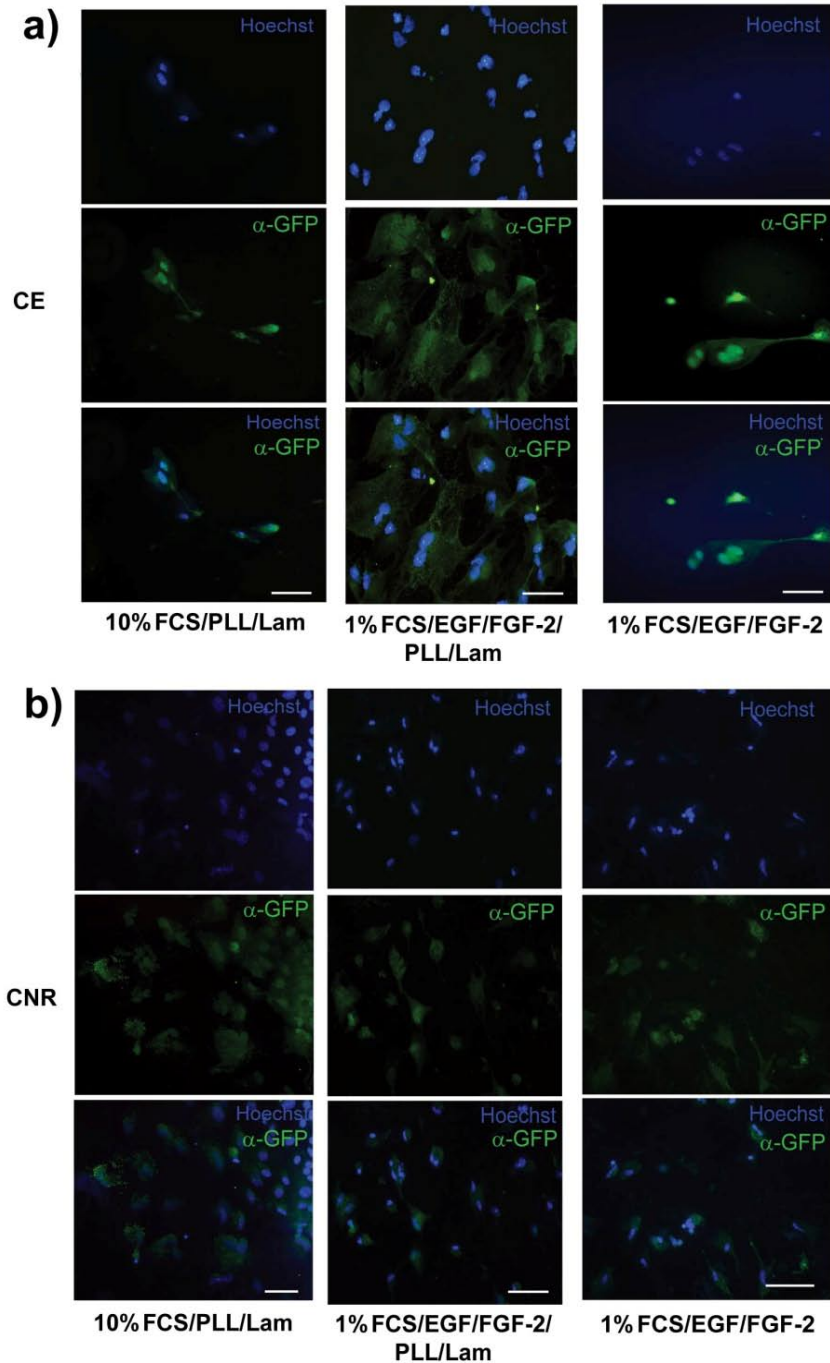


Figure 5.20 Effect of different substrate, serum and growth factors in the production of rod photoreceptor precursors from cells derived from adult *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* CE and CNR retinal neurospheres. Representative photographs of differentiated CE-derived (a) and CNR-derived (b) cells in each condition illustrated on graph.

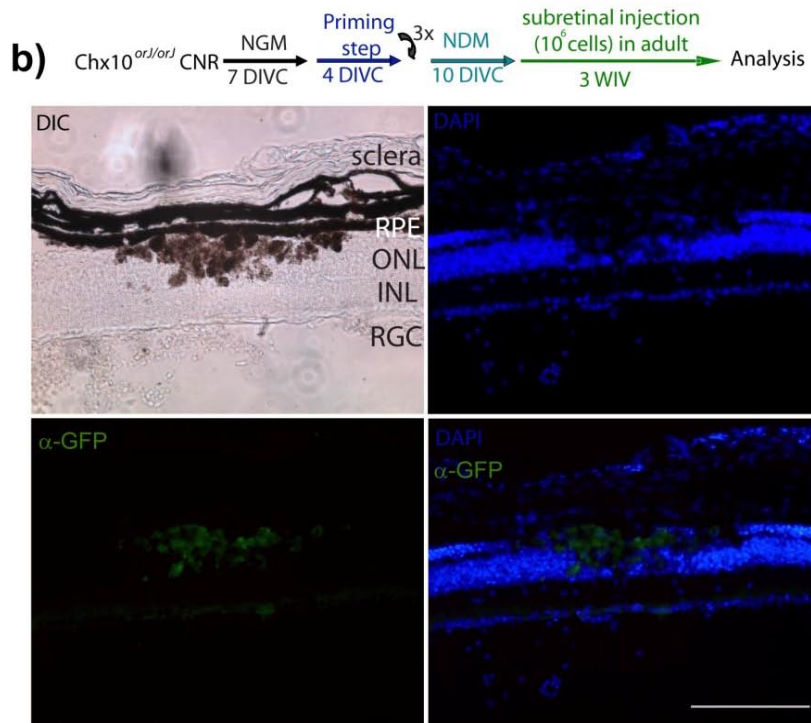
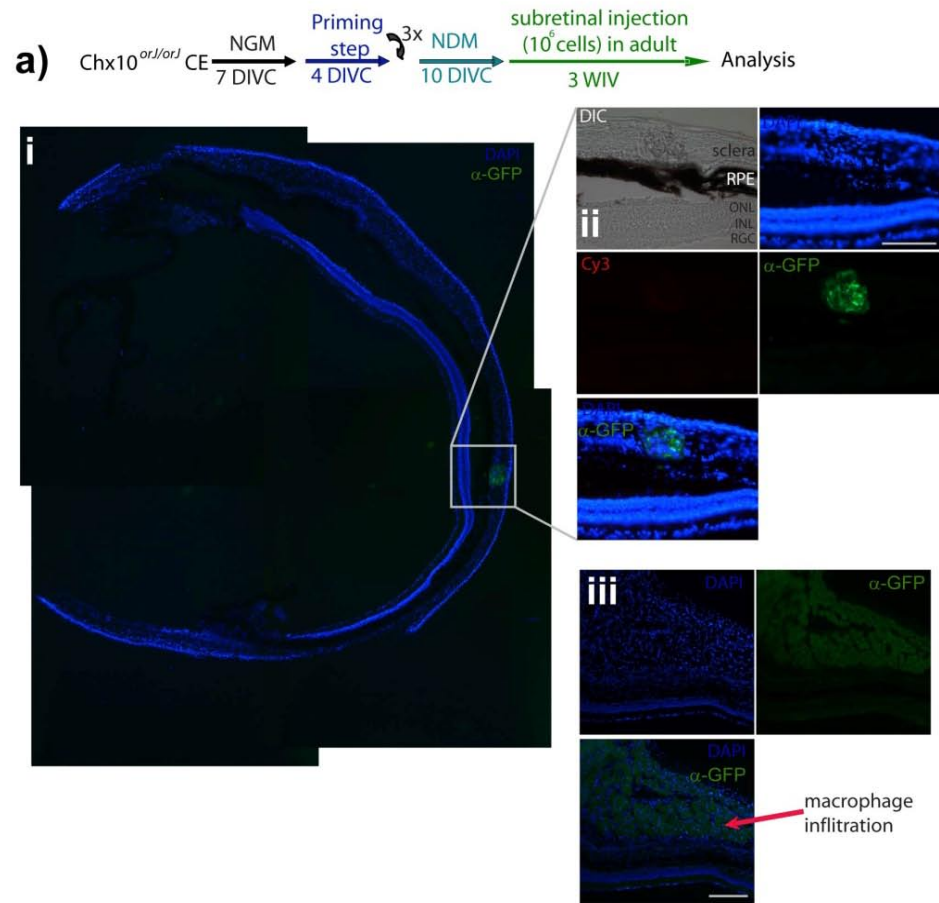
Cells were immunolabelled with an antibody against GFP and counter-stained with Hoechst 33342. Scale bars=10  $\mu$ m.

### 5.2.8 Allogeneic transplantation of $Chx10^{orJ/orJ}/Nrl.gfp^{+/-}$ differentiated neurosphere-derived cells

In parallel with trying to obtain rod photoreceptor precursors *in vitro*, I asked if adult derived progenitors had the ability to migrate, differentiate and integrate into the adult murine retina as has been shown previously for P1 cells (MacLaren, Pearson et al. 2006). Cells from the  $Chx10^{orJ/orJ}/Nrl.gfp$  CE and CNR were dissociated and cultured for 7 days in NGM prior to the priming step with low serum (1%) on PLL and Fibronectin glass coverslips (Figure 5.21). To expand cell number sufficiently to perform at least four sub-retinal transplantations, cells were *passaged* 3 times before induced to differentiate in 10% FCS onto PLL and Laminin for 14 days. Sub-retinal injections of one million cells per injection were performed in the adult murine retina in the retinal dorsal region closer to the optic nerve exit as previously described (MacLaren, Pearson et al. 2006). Recipients were sacrificed 3 weeks after transplantation and adult sagittal cryosections were obtained. Out of the 4 eyes examined, for CE-transplantation injections, one showed a cluster of  $Chx10^{orJ/orJ}/Nrl.gfp$  CE-derived cells to label positive for an antibody against GFP [Figure 5.21a(i)]. These cells were not located in the ONL or the sub-retinal space, but rather were found outside the RPE, in the scleral region [Figure 5.21a (ii)]. Examination at the 568 nm fluorescence emission wavelength showed no artefact confirming that the GFP fluorescence is viable. In other sections, cells that might also show expression for GFP are infiltrating white blood cells, such as macrophages, that possibly phagocytose transplanted cells and while enucleated for progressive degradation proteins are found within those cells giving the impression that are cells expressing GFP [Figure 5.21a(iii)]. It is possible that macrophage infiltration possibly did not allow for cells to differentiate or migrate properly.



Cells from the *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* CNR-derived neurospheres were similarly transplanted into the sub-retinal space of 4 eyes. GFP<sup>pos</sup> cells were found in only one of the 4 eyes and these were located in the ONL and in bright-field appeared to be pigmented.



**Figure 5.21 Transplantation of differentiated neurosphere-derived cells from the adult *Cxh10<sup>orJ/orJ</sup>/Nrl.gfp* CE and CNR to the adult murine retina**

Cells were dissociated from the CE (a) and CNR (b) of adult *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* mice and allowed to form neurospheres in NGM for 7 days. Neurospheres were then induced to form monolayers during the priming step for 4 days and after 3 passages cultured in NDM for 10 days following sub-retinal injection of  $10^6$  cells into adult wildtype retina, where they were allowed 3 weeks to migrate, integrate and differentiate. Montage of a sagittal section through a wildtype eye (lens removed) (i). Sections show GFP images in which nuclei are counter-stained with DAPI. *Inserts*, region where GFP positive cells were observed (ii). Photomicrographs of retinal cryosections immunolabelled with anti-GFP and viewed on both 468 and 514 nm fluorescence channels. Macrophage infiltration was frequent at the site of transplantation (iii).

(b) Cells were dissociated from the CNR of adult *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* mice the same protocol was followed as for CE-derived cells. Photomicrographs of retinal sagittal cryosections immuno-labelled with an antibody against GFP.

Cells were counter-stained with DAPI. Scale bars=10  $\mu$ m. Macrophage infiltration was frequent at the site of transplantation.

### **5.3. Discussion**

#### **5.3.1 Summary of findings**

In this chapter I described setting up an *in vitro* system for differentiating neurosphere-derived cells obtained from the adult  $Chx10^{orJ/orJ}$  CE and CNR. In addition, the  $Nrl.gfp^{+/+}$  and  $Chx10^{orJ/orJ}/Nrl.gfp$  transgenic mice were employed in order to assess if there is potential for production of rod photoreceptor precursors from these cultures, which have shown the highest efficiency for successful retinal transplantation in adult recipients (MacLaren, Pearson et al. 2006).

From this study the following conclusions can be drawn;

- i. Neurospheres from the adult  $Chx10^{orJ/orJ}$  CNR and CE show similar differentiation potential to their wildtype CE counterparts, with the exception of bipolar cell formation.
- ii. Differentiated  $Chx10^{orJ/orJ}$  CE and CNR neurosphere-derived cells express higher levels of photoreceptor genes than their wildtype CE counterparts.
- iii. Differentiated  $Chx10^{orJ/orJ}$  CNR and CE neurosphere-derived cells could not efficiently integrate or migrate to the adult retina.

#### **5.3.2 Adult $Chx10^{orJ/orJ}$ central neural retina neurosphere-derived cells possess differentiation potential**

Upon differentiation, cells derived from neurospheres from all three regions (CE from both genotypes, and CNR from  $Chx10^{orJ/orJ}$ ) expressed markers specific to a variety of retinal cell types including rod photoreceptors, ganglion and

amacrine cells as well as quiescent and reactive Müller glia. It is important to note that while these cells express retinal cell markers, as observed via immunohistochemistry, when grown on a PLL/Laminin substrate they did not adopt recognisable retinal cell morphologies, and this is also typical of previous reports (Tropepe V 2000; Giordano et al. 2007; Jomary and Jones 2008). It is suggested that this may reflect an immature state of differentiation as seen from the PH3/ p27<sup>kip1</sup> experiments showing some cells are still dividing in the cultures. No differentiated cells expressed the Sox2 protein, indicating a degree of differentiation. It is important to exercise caution when inferring cell identity from expression of a small number of markers. Demonstration of the true generation of new retinal cells, including photoreceptors, will require functional analysis. The elevated *Gfap* expression can be explained by the possibility of the GFAP<sup>pos</sup> starting population which possibly did not switch off its Müller/progenitor phenotype proceeding to a more differentiated state.

By comparing the gene expression levels of *β-III tubulin*, a gene expressed in immature and mature neurons including retinal ganglion cells (Sharma and Netland 2007), as well as Growth-associated protein 43 (*Gap43*) (Ivanov, Dvorianchikova et al. 2006), no statistical difference was observed in these gene expression levels between differentiated cultures from all genotypes and regions. *Gap43* is a neuronal growth protein known to be expressed in the axons of ganglion cells and in cells in the IPL (McIntosh H 1991). Also, it has been noted that increased *Gap43* protein expression is accompanied by neuronal regeneration in the brain after an injury event in rats (Doster SK 1991). On my quantitative Re-Ti PCR data set, this mRNA transcript was detected in both differentiated and non-differentiated neurosphere-derived retinal cells in wild-type and *Chx10<sup>orJ/orJ</sup>* CE as well as *Chx10<sup>orJ/orJ</sup>* CNR cells, *Gap43* showed a trend of increased gene expression levels in differentiated cultures but was not statistically significant.

Alongside Gap43,  $\beta$ -III tubulin protein is expressed in young neurons in the central and peripheral nervous system. In adult rats it has been detected in CB- and RPE-derived cells (Engelhardt M 2005). Immunocytochemical analysis showed low expression of this protein in cells within the primitive neurosphere in both mutant and neurospheres deriving from both CE and CNR regions consistent with a previous report (Engelhardt M 2004). In the mouse CE cultures in differentiating conditions, cells readily express this protein on their processes and their morphology resembles that of neurons. However, quantitative Re-Ti PCR analysis showed mRNA expression for this neuronal protein in both undifferentiated and differentiated retinal cells.

I aimed to use genes that would show differences between retinal progenitors and post-mitotic retinal cells. Sox2 has been shown to be expressed only in neural retinal stem/progenitors and a subpopulation of amacrine cells in the adult mouse retina (Le Rouédec D 2002; Ellis P 2004; Hagstrom SA 2005; Taranova OV 2006). Sox2 is a TF essential for neural development and its absence shows defects in ocular development such as anophthalmia (Fantes J 2003) (Kelberman et al. 2006). Sox2 gene expression was predicted to be readily expressed in undifferentiated cells (retinal progenitors) and then to decrease substantially in differentiated retinal cells. However, expression levels for this gene remained unaltered between differentiated and undifferentiated cultures for all genotypes and regions investigated, but with a trend for decreased gene expression in differentiated cultures. This is probably due to the fact that Sox2 is expressed in both retinal progenitors and amacrine and horizontal cells (Taranova OV 2006). Interestingly, *Chx10<sup>orJ/orJ</sup>* CE and CNR undifferentiated cells had a trend of increased Sox2 gene expression in comparison to wildtype CE cells. Immunocytochemical analysis confirmed that Sox2 is expressed readily in neurospheres and could be detected in wild-type neural-retina.

It is essential to note that post-translational modification of protein may be important. There have been reports for this kind of modifications for Gap43 (Doster SK 1991; Taniguchi H 1994) and Sox2 (Le Rouëdec D 2002).. Conclusively, Gap43 gene expression remains unaltered in wildtype and *Chx10<sup>orJ/orJ</sup>* CE as well as in *Chx10<sup>orJ/orJ</sup>* CNR neurosphere-derived cells. However, the level of functional protein was not assessed. A suggestion could be to use Western blotting analysis to assess the amount of protein present. Similarly,  $\beta$ -III tubulin undergoes post-translational modification (Lee MK 1990). Literature indicates that the  $\beta$ -III tubulin polypeptide alters its charge within its extreme C-region and that modifications increase from E14 to adult (P30). Specifically, in rat embryos, only one charge-variant of this protein was observed by isoelectric focusing 2-D poly-acrylamide gel electrophoresis, and reached seven isoforms in adulthood. In Neurospheres in non-differentiating conditions maybe gene expression of this neuronal protein occurs but does not necessarily lead to neuronal protein production, In conclusion,  $\beta$ -III tubulin mRNA transcripts and protein are detected in differentiated neurosphere-derived cells of both wildtype and *Chx10*-deficient retinas. *Chx10* does not alter the neurogenic potential of retinal progenitors.

Throughout the experiments conducted here, the differentiation potential showed differences between neurospheres deriving from the same region, genotype and dissection sessions. This implies that there is heterogeneity in the starting population (i.e a mixture of stem progenitor and differentiated cells) that might leads to variation in differentiation behaviour *in vitro*. The representative photomicrographs in figure 5.1 show how some neurospheres differ in terms of some will not flatten as much as others or that only one side of the neurosphere will show cell migration.

### 5.3.3 Lack of Chx10 leads to an increased photoreceptor differentiation potential

Along with investigating whether neurospheres have the potential to give rise to neuron and glial cells, I sought to explore the possibility of obtaining cells with rod and cone photoreceptor characteristics. Since *Chx10* clearly affects photoreceptor development, either directly or indirectly, whether lack of *Chx10* had an effect on the *in vitro* differentiation of photoreceptor cell arising from adult-derived progenitor cells was investigated. Interestingly, we found that neurosphere cultures derived from the *Chx10<sup>orJ/orJ</sup>* retina (CE and CNR), and therefore lacking *Chx10* activity, showed a higher level of expression of photoreceptor genes upon differentiation. Two consistent differences were observed, in the expression profile of the two genotypes; a lack of cells immunoreactive for PKC, a distinct bipolar expressing protein as well as an increase in rod and cone photoreceptor gene expression (*Recoverin* and *Crx*) with an absence of the cone photoreceptor-specific mRNA transcripts for *Arrestin 3* in the differentiated cultures of wildtype and mutant CE as well as mutant CNR. Recoverin is a 23-kDa calcium-binding protein that activates retinal rod guanylate cyclase (Dizhoor AM 1991; Lambrecht HG 1992) and is expressed in rod and cone photoreceptors and midget cone bipolar cells (Milam AH 1993; McGinnis JF 1997), although the latter cell type residing in the INL was not apparent in my immuno-histochemical analysis. This protein is essential for photoreceptor function as it binds to rhodopsin and phosducin proteins. By obtaining mRNA from pools of cells deriving directly from undifferentiated and differentiated neurospheres, cDNA was used for performing quantitative Re-Ti PCR that showed a remarkable increase in recoverin mRNA transcripts upon differentiation of neurosphere-derived *Chx10*-deficient CE cells. Immunocytochemical analysis, using a polyclonal antibody against recoverin showed that differentiated cells expressed this protein, with strongest expression



detected in *Chx10*-deficient CE-derived cells, consistent with the real-time PCR data.

In addition, our data are consistent with recent literature indicating *Chx10* as a photoreceptor repressor acting through negative regulation of *Crx* expression (Dorval KM 2005; Livne-Bar I 2006). By contrast, the P14 *Chx10<sup>orJ/orJ</sup>* retinae showed fewer photoreceptors present than in wildtype, and *Crx* was delayed in *Chx10*-deficient retina (Rutherford AD 2004) although that might be a secondary effect due to reduced cell number (Rowan S 2004). *Chx10<sup>orJ/orJ</sup>* CNR-derived differentiated cells did not show a statistically significant increase in these genes but there was a trend for increased expression in comparison to wildtype. The highest level of expression was observed in cultures of *Chx10<sup>orJ/orJ</sup>* CE-derived cells. These observations suggest that repression of *Chx10* activity may be a useful tool for promoting photoreceptor development in stem cell cultures.

Ectopic *Chx10* expression promoted bipolar instead of rod differentiation in newborn retina (Livne-Bar I 2006) and gel shift assays and chromatin immunoprecipitation studies have shown that *Chx10* binds to photoreceptor gene regulatory sequences and may play a role in the inhibition of photoreceptor gene expression (Dorval KM 2006). In the *Chx10<sup>orJ/orJ</sup>* retina, photoreceptor differentiation is abnormal as indicated by the altered profile of photoreceptor gene expression and the truncated outer segment appearance (Rutherford AD 2004).

The absence of bipolar cell marker (PKC) can be predicted if adult progenitor cell differentiation is regulated by mechanisms similar to developmental retinal histogenesis. That *Chx10* is required for bipolar cell differentiation during development has been consistently observed firstly documented by Burmeister *et Al.* (Burmeister M 1996; Rowan S 2004) and the continued expression of

*Chx10* in mature bipolar cells in the adult retina suggest it has different roles in RPC and bipolars.

#### **5.3.4 Priming of retinal progenitors from the wildtype and *Chx10*<sup>orJ/orJ</sup> retina**

The effect of FCS in retinal cultures promotes differentiation away from an immature state with downregulation of progenitor markers and the upregulation of post-mitotic markers (Ahmad I 1999). Researchers have used in their culture system, low amounts of FCS (in the range of 1-10%) for differentiation induction (Klassen HJ 2004), while others have reported the effect of low amounts of FCS (1-5 %) not to induce differentiation but to keep progenitors in an immature state, when growth factors were still present (Ahmad I 1999). Taking these into consideration, I investigated the potential of cells primed with a low serum step and in the presence of substrates, to induce progenitor expansion as shown recently in porcine CE cultures (Macneil, Pearson et al. 2007), before the standard differentiation (NDM) step. My data indicated that neurosphere-derived retinal progenitors behave differently when exposed to different substrates after the priming step; when Laminin was used, progenitors acquired a neuronal fate, while Fibronectin induced a glial fate.

Interestingly, the majority of cells in differentiating conditions continued to express nestin, suggesting the presence of progenitors. Although, this marker has recently been reported not to explicitly label neural progenitors *in vitro* (Qiu et al. 2007) as shown in differentiated adult brain cells, it still remains a marker for multipotentiality (Wiese et al. 2004). On balance, the presence of Nestin<sup>pos</sup> is an indicator of a non-fully differentiated cell culture.

### 5.3.5 Rod photoreceptor precursors from the wildtype *Nrl.gfp*<sup>+/+</sup> CE

Retinal progenitors have been isolated from the CE of adult mice and humans (Ahmad I 2000; Tropepe V 2000; Mayer EJ 2005; Carter et al. 2007). These cells behave as neural stem/progenitors in culture by forming neurospheres, and upon differentiation, express a variety of retinal neuronal and glial markers, although the numbers of differentiated photoreceptors obtained are small (<4%). CE-derived progenitors are an encouraging candidate for cell replacement therapies but in order to reach that stage, cells need to be efficiently manipulated in an *ex vivo* system becoming cells optimal for retinal transplantation. To date, effective and functional transplantation has come from rod photoreceptor precursors (MacLaren, Pearson et al. 2006). Using the *Nrl.gfp*<sup>+/+</sup> transgenic mouse model, I investigated the potential of CE-derived neurosphere-forming cells to give rise to rod photoreceptor precursors.

Primary neurospheres did not express GFP indicating that immature CE-derived progenitors do not express *Nrl*. When a minimal amount of serum is added, there was an uneven and weak expression of GFP in CE-derived differentiated cells, which was highly variable and transiently expressed. The presence or absence of EGF and FGF-2 did not influence the activation of GFP protein expression. In a similar fashion, Laminin and Puramatrix™ did not alter GFP protein expression. Interestingly, by treating CE-derived neurosphere-forming cells with low and high amount of serum as well as in the absence or presence of Laminin, and immunolabelling with an antibody against GFP, no difference was observed in the percentage of cells positive for this marker. By contrast, quantitative Re-Ti PCR analysis showed an elevation of GFP mRNA transcripts, when no Laminin was used with low FCS amount in the presence of growth factors in comparison to standard conditions using substrate. In conclusion, the cell culture conditions tested were found to be not ideal for proper rod photoreceptor development although transient GFP expression was observed

suggesting that these cells have the potential to differentiate to rod photoreceptor precursors. Furthermore, *Gfp* transcripts were present in the differentiated cultures but the increase was not detectable without GFP immunocytochemistry.

### 5.3.6 Rod photoreceptor precursors from the *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* CE and CNR

*Chx10<sup>orJ/orJ</sup>* adult CNR cells possess a population of progenitor cells behaving similar to neural progenitors in culture judged by their ability to form neurospheres, self-renew and multipotentiality. Furthermore, adult *Chx10<sup>orJ/orJ</sup>* CE-derived differentiated cells express high levels of photoreceptor genes, making them a feasible rod photoreceptor precursor source than wildtype CE-derived differentiated cells.

Exploiting the increased photoreceptor potential of *Chx10<sup>orJ/orJ</sup>* CE and CNR derived cells, this microphthalmic mouse model was crossed with the *Nrl.gfp<sup>+/+</sup>* transgenic mouse to obtain microphthalmic mice carrying at least one allele of *Nrl.gfp*. *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* sagittal retinal cryosections were immunolabelled for recoverin and GFP and it was shown that adult mice have a degenerated ONL with a few rod photoreceptors judged by the recoverin and GFP expression and co-expression results.

A timeline differentiation experiment protocol was performed comprising of a neurosphere formation step in NGM followed by a priming step in a low amount of serum in the presence of growth factors with or without IGF-1 (20 ng/ ml), prior to differentiating cells for 14 days in the presence of 10% FCS to assess the neurogenic potential of our mutant *Nrl.GFP* mouse model. *Chx10<sup>orJ/orJ</sup>/Nrl.gfp* cells from both the CE and CNR showed no alteration in their

neurogenic potential upon IGF-1 administration. Also, no alteration was observed between the priming and differentiation steps in terms of  $\beta$ -III tubulin, Nestin and GS expression.

Similar to *Nrl.gfp*<sup>+/+</sup> CE-derived cells, by treating *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp* CE-derived neurosphere-forming cells with low and high amount of serum as well as in the absence or presence of Laminin, and immunolabelling with an antibody against GFP, a transient expression of GFP was observed. In these cultures it is essential to note that although expression of GFP *in vitro* was evident these cells did not show morphology and characteristics of rod photoreceptor precursors. The indication of Chx10-deficient GFP<sup>POS</sup> cells shows that absence of Chx10 does not alter the potential of these cells to differentiate towards a rod photoreceptor lineage. Therefore, as with previous reports (MacLaren, Pearson et al. 2006) the ontogenetic timing as well as microenvironment, need to be properly tuned to give rise to rod photoreceptor precursors, at least *in vitro*.

### **5.3.7 Allogeneic transplantation of differentiated cells deriving from the *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp*<sup>+/-</sup> CE and CNR**

Foetal allografts have shown good proliferative and differentiation capacities, but lack in integration capabilities (Aramant and Seiler 2002). In contrast, NSC cells from the DG of adult rats, when injected intravitreally, demonstrated a high degree of integration in neonates but not in adults (Takahashi M 1998). These results show that the age of the host retina seems to play a key role in the allo- and xeno- transplantation. ES cells have also been explored for their ability to generate high numbers of cell-specific lineages *in vitro* introduced by ectopic gene expression (Meyer JS 2004; Tabata Y 2004). Finally, HSC have been used and shown to prevent retinal vascular degeneration in rd1 and rd10 mouse models (Otani A 2004).

Recently, our lab has showed that the adult murine retina is receptive for transplantation of cells that are post-mitotic but not fully differentiated (MacLaren, Pearson et al. 2006). The cells that successfully transplanted derived from P1 and P4 murine retinæ comprised of a mixture of progenitors, post-mitotic precursors and differentiated cells with the newly post-mitotic cells not expressing markers indicative of mature photoreceptors. With the use of Fluorescence-Activated Cell Sorter (FACS) and the *Nrl.gfp*<sup>+/+</sup> transgenic mouse model, it was demonstrated that the adult murine retina can incorporate *Nrl*-expressing rod photoreceptor precursors. This indicates that the ontogenetic stage of the transplanted cells is crucial for successful transplantation.

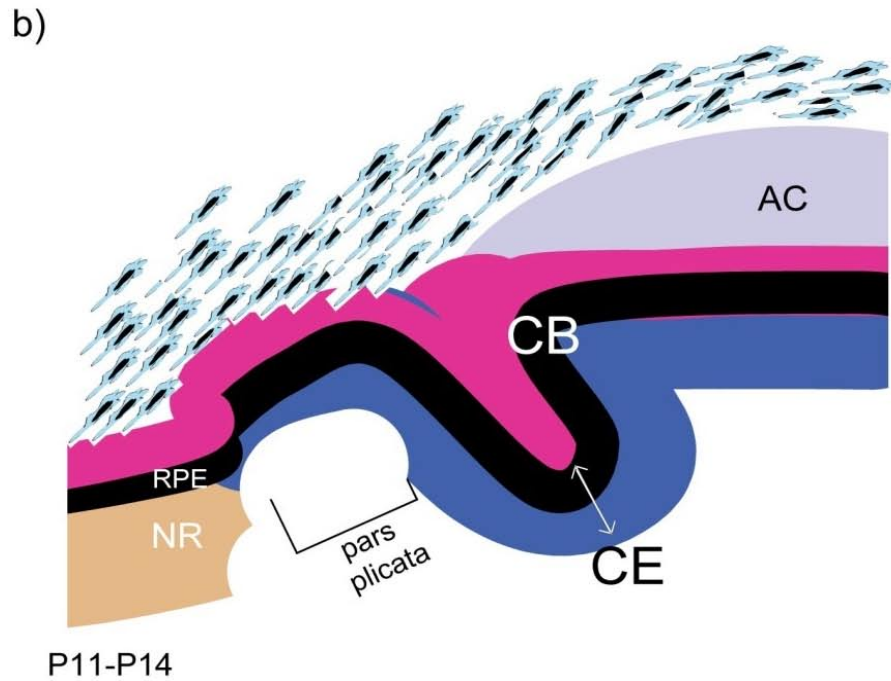
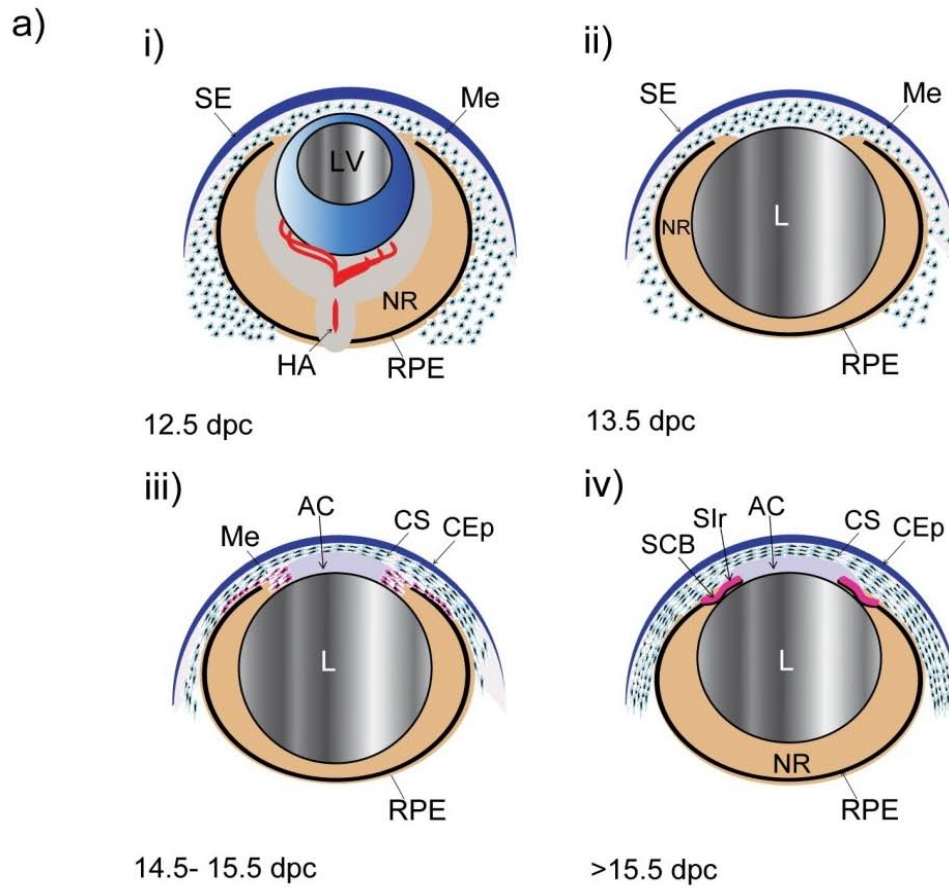
Considering these findings, it was attempted to transplant cells deriving from neurospheres obtained from the CE and CNR of *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp* adult mice. Sub-retinal injections were performed of  $1 \times 10^6$  neurosphere-derived cells that were subjected to differentiation in 10% FCS in the presence of Laminin, after monolayer expansion and *passaging*. Strikingly, a few cells from the CE switched on GFP but did not efficiently incorporate into the retina; rather it was sufficient to have some contact with the RPE. Interestingly, CNR-derived cells show neither incorporation nor GFP expression, indicating that maybe these cells are still in a highly immature state. On both cases, macrophage infiltration was present which might have hindered normal cell behaviour of the transplanted cells. In general, these experiments indicated that the developmental state of the adult-derived progenitor cells prior to transplantation was not optimal.

## **Chapter 6 – Origin of Retinal Stem Cells**

## **6.1. Introduction**

During ocular development, the retina and its auxiliary tissues are formed by distinct developmental processes. After the lens vesicle has detached from the surface ectoderm, a wave of neural crest derived mesenchymal cells migrate into the space between the surface ectoderm and lens vesicle [E12.5, Figure **6.1A (i)**]. This migratory pattern is essential for appropriate anterior segment development (Reneker et al. 2000). The population of mesenchymal cells condense and cells acquire a flat morphology [E13.5, Figure **6.1A (ii)**]. In parallel, the lens has formed and filled with primary lens fibre, while the neuroblastic layer and the retinal pigment epithelium are expanding. The mesenchymal cells will eventually form the corneal endothelium while the surface ectoderm will be the corneal epithelium, and a fluid-filled cavity is formed in the anterior chamber between the lens and the cornea [E14.5, Figure **1A (iii)**]. At this point there is a second wave of mesenchymal cell migration, preferentially found in the edge of the optic cup. While the corneal endothelium differentiates, the margins of the optic cup extend into the anterior chamber forming the CB and iris [E15.5, Figure **6.1A (IV)**]. The mesenchyme cells migrate along those structures and differentiate forming the iris and ciliary body stromas. After birth, the CB is formed and by P11 it has also produced ciliary processes and shows a distinct bilayered ciliary epithelial region comprising of a non-pigmented and a pigmented layer, the former found closest to the lens. The CB also consists of differentiated mesenchyme cells of undetermined origin but probably arising from the cranial paraxial mesoderm and/or neural crest (Johnston et al. 1979; Trainor and Tam 1995) (Figure **6.1B**).





**Figure 6.1 Development of anterior eye and ocular mesenchyme**

**(a)** Schematic diagram of ocular mesenchyme development in the murine eye between embryonic days 12.5 and 15.5. At the end of E12.5 the lens vehicle has detached from the surface ectoderm, invaginating into the optic cup. The first wave of mesenchymal cells (blue cells) has started to migrate between the surface ectoderm and the anterior epithelium. The presumptive neural retina and retinal pigment epithelium are found within the optic cup **(i)**. At the end of E13.5, migrated mesenchymal cells have flattened and are connected to each other by fibrous extracellular matrix **(ii)**. At the beginning of E14.5 the mesenchyme closest to the lens flattens, forming an endothelial monolayer, eventually becoming the corneal endothelium, while the corneal epithelium will derive from the mesenchymal cells closet to the anterior part. At the same time, the lens will detach from the cornea, forming the anterior chamber. In parallel, a second wave of mesenchymal cells (pink cells) migrates at the anterior edge of the optic cup **(iii)**. At the beginning of E15.5 the optic cup is enlarged and elongated with the appearance of the presumptive ciliary body and iris at the anterior edges. The second wave of mesenchymal cells differentiates into stroma of the iris and ciliary body (pink region), structures of the irido-corneal angle.

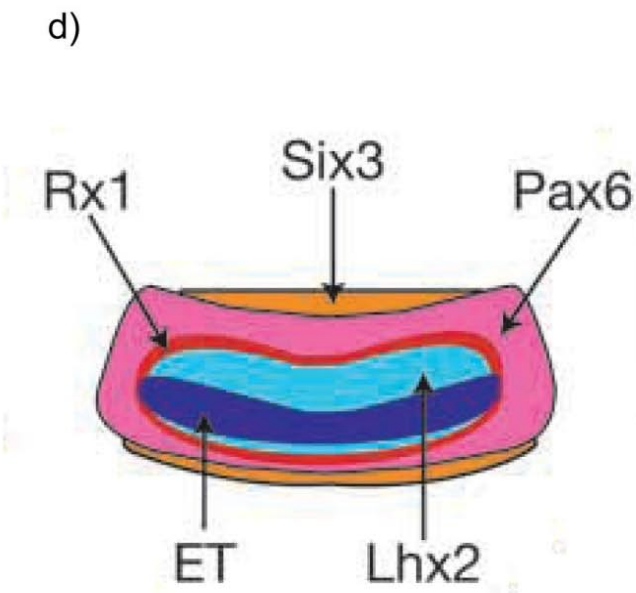
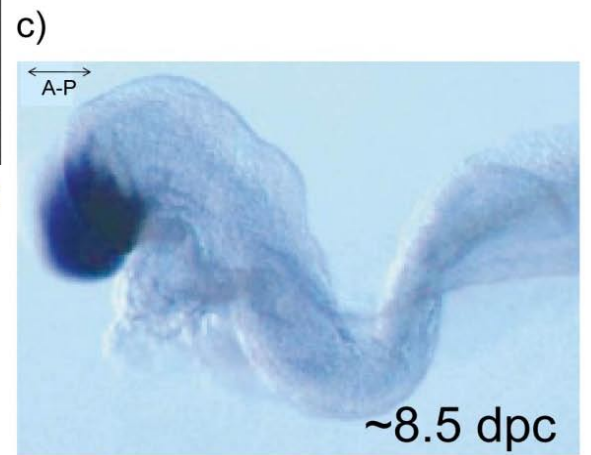
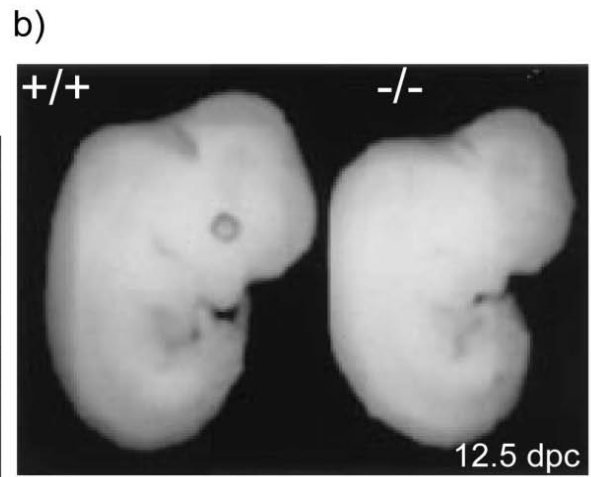
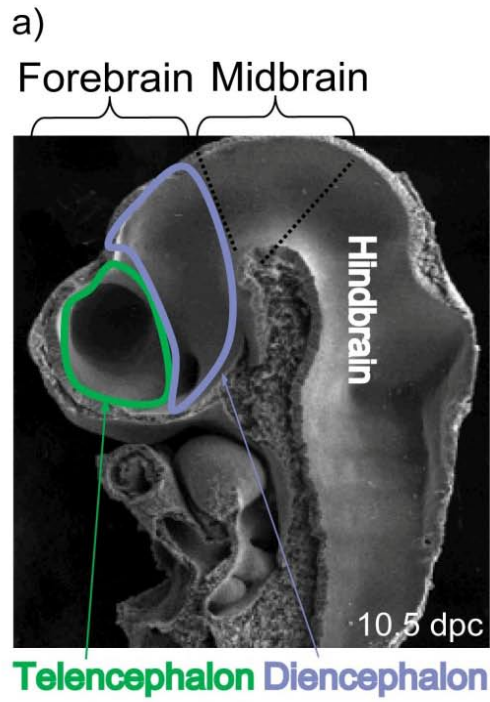
**(b)** Schematic diagram of the ciliary body after postnatal day 11. At this stage the ciliary body possess ciliary processes and bilayered ciliary epithelium consisting of a pigmented region (black) and a non-pigmented one (dark blue), the latter attached to the lens. Mesenchymal cells are also present within the ciliary body.

Abbreviations: SE=surface ectoderm, Me=mesenchyme, NR=Neural retina, HA=hyaloids artery, RPE=retinal pigment epithelium, AC=anterior chamber, CS=corneal surface, CEp=corneal epithelium, SCB=stroma of ciliary body, Sir=stroma of Iris, CE=ciliary epithelium.

Adult retinal stem/progenitor cells are believed to reside in the ciliary body of the adult mouse and it has been suggested that they exist in the pigmented layer of the ciliary epithelium (which is continuous with the RPE) (Tropepe V 2000). By using BrdU labelling to tag slowly dividing cells *in vivo*, we identified a population of dividing cells mainly in the non-pigmented layer of the CE (Dhomen NS 2006). As neurosphere cultures are set up from micro-dissected and dissociated CB tissue, it is not possible to confidently define the embryonic origin, or type of cell that gives rise to a neurosphere *in vitro*. While the ciliary epithelium derives from the periphery of the optic cup, and thus is of neuroepithelial origin, other cells types are also present in this region, specifically ciliary muscle, ciliary body stroma which is of mesenchymal cell origin, neural crest derived melanocytes, and ciliary body vasculature. During micro-dissection, it is not possible to exclude the possibility of contamination by other cell sources.

By genetically labelling cells of anterior ectodermal origin including the eye field region, it is possible to test if embryonic eye field cells give rise to the adult retinal stem cells in the CE. The homeobox gene expressed in ES cells (*Hesx1*), also known as *Rpx* (Rathke's pouch homeobox), is initially observed in anterior primitive cells of the endoderm (Hermesz et al. 1996). As gastrulation progresses, the anterior neural ectodermal *Hesx1* expression is dependent on inductive signals from the visceral endoderm, with its absence causing septo-optic dysplasia in humans and mice (Dattani, Martinez-Barbera et al. 1998; Dattani MT 1999; Dattani et al. 2000; Martinez-Barbera JP 2000; Andoniadou, Signore et al. 2007). *Hesx1* transcripts appear in the adjacent ectoderm which is destined to give rise to the ventral prosencephalon (Thomas and Beddington 1996). Subsequently, *Hesx1* is expressed at the anterior extreme of the rostral neural folds, finally resolving to the ventral diencephalon at E9.0 (See Figure 6.2). *Hesx1*-expressing cells will eventually give rise to the ventral telencephalon. The optic pit is located in the anterior closing neural tube, in the

telencephalic region, next to the diencephalon the other part of the forebrain (Figure **6.2a,b**). *Hesx1*, a homeobox gene, is expressed at the forebrain region, including the presumptive optic vesicle, and targeted inactivation of this gene leads to anophthalmia and reduced telencephalic area (Figure **6.2c**). *Hesx1* transcripts are found in the forebrain principally restricted to the telencephalic area at E8.5. At E9.5 *Hesx1* transcripts are restricted within the ventral diencephalon and on later stages no *Hesx1* transcripts are found in the forebrain.



**Figure 6.2 Murine Brain development and Hesx1 expression**

**(a)** Electron microscopy microphotograph of a saggital whole mount section of a murine E10.5 embryo. The closed cranial neural tube illustrates the prosencephalon or forebrain, mesencephalon or midbrain, and rhombencephalon or hindbrain. The prosencephalon is subdivided into two regions, the diencephalon (purple) and the telencephalon (green). The later is the area where the optic cup will form along with other structures. Photo derived online from [http://www.med.unc.edu/embryo\\_images](http://www.med.unc.edu/embryo_images) and modified.

**(b)** Targeted inactivation of Hesx1 induces a phenotypic effect on embryonic mice. Lateral views of Hesx1<sup>+/+</sup> and Hesx1<sup>-/-</sup> indicate the absence of eyes as well as reduced telencephalon. Image was obtained from Dattani MT *et al.* (Dattani MT 1999).

**(c)** Whole mount expression of Hesx1 in a mouse E8.5 embryo using *in situ* hybridisation. Image was obtained from Thomas P and Beddington R. (Thomas and Beddington 1996).

**(d)** The overlapping expression patterns of the eye field transcription factors at E12.5 in the forebrain of the vertebrate eye. Concentric rings of expression in domains of decreasing size – *Six3* > *Pax6* > *Rx1* > *Lhx2* > *ET*

Abbreviations: paired box 6 = Pax6, LIM homeobox 2 = Lhx2, SIX homeobox 3 = SIX3, *Xenopus* Rax = Rx1, ET = T-Box transcription factor 3.

Hesx1 expression is restricted to the developing anterior pituitary until E11.5 in mice, while its telencephalic expression is undetectable after E.9.5 in mice (Andoniadou, Signore et al. 2007). By the use of a *Hex1*<sup>Cre/+</sup>.*Rosa26*<sup>LacZ/+</sup> mouse model, in this chapter I am attempting to identify the developmental origin of the cells that give rise to neurospheres *in vitro*, and furthermore to investigate the developmental origin of the murine CE and neural retina. In addition, BrdU labelling was used to identify sphere-forming cells *in vivo*.

## **6.2. Results**

### **6.2.1 Expression of Hesx1<sup>GFP/+</sup> in the adult murine retina**

Previously, whole embryos were stained with X-Gal; descendants of Hesx1-expressing cells were located in the telencephalic region as well as in the optic cup at E9.5 (Figure **6.3a**, provided by Cynthia Antoniadou). Paraffin saggital head sections were obtained from *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* E9.5 mice, and after X-Gal staining, descendants of Hesx1-expressing cells could be detected throughout the presumptive neural retina but in a non-uniformly patterned way, especially in the peripheral neuroblastic layer (Figure **6.3b**, provided by Cynthia Antoniadou).



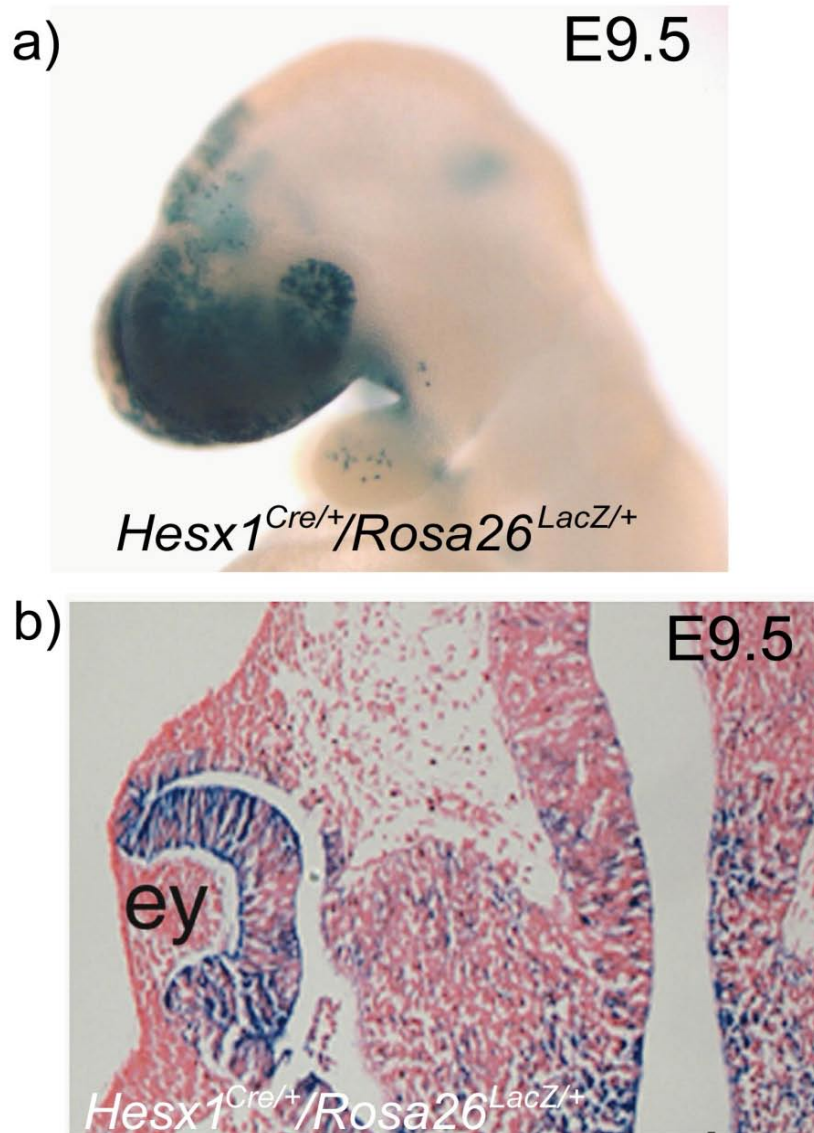


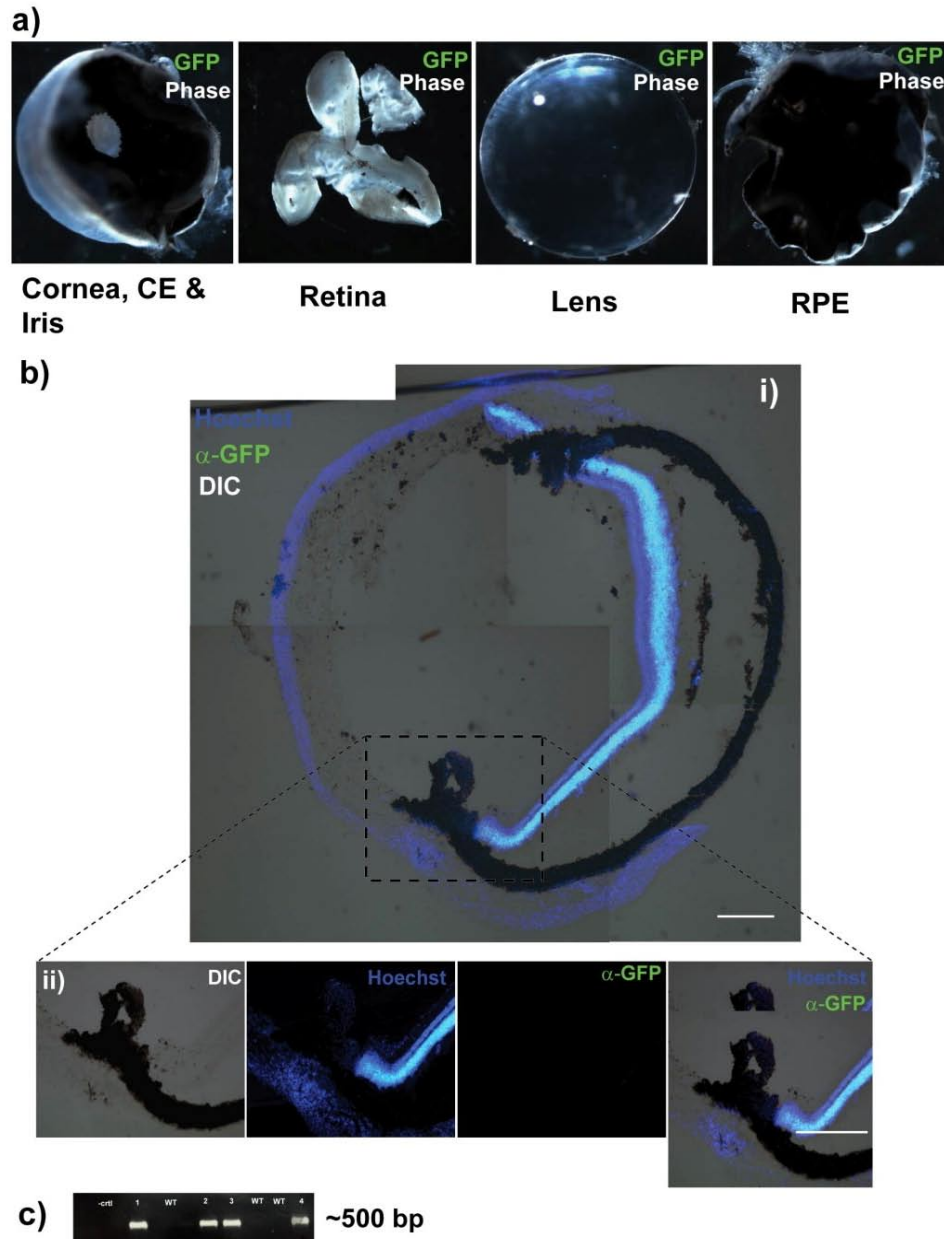
Figure 6.3 Descendants of Hesx1-expressing cells in early mouse embryogenesis

(a) Descendants of Hesx1-expressing cells in the mouse telencephalon. The Hesx1 homeobox gene is expressed in the forebrain neuroepithelium that contributes to the optic vesicle formation between E7 and E9, and thereafter is not expressed in any ocular tissue. X-Gal stained embryos were investigated for blue-forming colonies. The prosencephalon was stained blue with stronger expression in the telencephalic area and the optic grooves. Image was modified from Andoniadou *et al.*, 2007.

(b) Descendants of Hesx1-expressing cells in the mouse optic cup. Embryos were stained with X-Gal and head saggital paraffin sections were obtained showing cells deriving from Hesx1-expressing cells. Image was modified from Andoniadou *et al.*, 2007.

Abbreviations: A-P=Anterior-Posterior

Although the above-mentioned and other studies had shown that Hesx1 expression is turned off in all tissue except the pituitary before optic cup formation, it was tested whether any Hesx1 was expressed in the mature retina (Dattani, Martinez-Barbera et al. 1998; Dattani MT 1999; Martinez-Barbera JP 2000; Andoniadou, Signore et al. 2007). To assess if Hesx1 was actively expressed in the adult murine retina a mouse model was employed where the Hesx1 coding region was replaced with *GFP-dta* (Andoniadou, Signore et al. 2007). Adult *Hesx1<sup>GFP-dta/+</sup>* mice were sacrificed and eyes were investigated for GFP expression. No GFP expression was observed in any of the ocular structures (Figure **6.4a**). In addition, adult retinal saggital cryosections immunolabelled with an antibody against GFP showed no expression of this protein in any part of the retina or CE [Figure **6.4b (i, ii)**]. Therefore, any X-Gal expression observed in the *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* eye tissue is of forebrain epithelium cell origin, rather than cells newly expressing Hesx1.



**Figure 6.4** Hesx1 expression in the adult murine eye  
**(a)** Representative images viewed under excitation wavelength at 468 nm of ocular regions derived from a *Hesx1<sup>GFP-dta/+</sup>* adult mouse. The Cornea, Iridal, CE, Lens, Retinal and RPE tissues were investigated for any GFP expression.  
**(b)** Light microscope montage of a sagittal retinal section of a *Hesx1<sup>GFP-dta/+</sup>* adult murine eye. Sections show phase microphotographs of retinal sections immunolabelled with  $\alpha$ -GFP (i). Inserts show images of higher magnification for the CE and PR regions (ii). **(c)** GFP DNA was confirmed by PCR in 4 different samples.

N=2 independent experiments. Cells were counter-stained with Hoechst 33342. Scale bars= 5 mm.

### 6.2.2 Developmental origin of the murine CE cells

I attempted to identify the developmental origin of the adult retinal stem/progenitor cells located in the CB of the mouse. A *Hesx1*<sup>Cre/+</sup> mouse line was used in this study [provided by JP Martinez-Barbera (Martinez-Barbera JP 2000; Andoniadou, Signore et al. 2007)] in which the *Hesx1* coding region was replaced with *Cre recombinase*, by correctly targeted ES cell clones injected into blastocysts of wildtype mice. *Hesx1*<sup>Cre/Cre</sup> showed a similar phenotype to *Hesx1*<sup>-/-</sup> knock-out mouse model. *Hesx1*<sup>Cre/+</sup> mice were crossed with *Rosa26*<sup>LacZ/LacZ</sup> mice to obtain *Hesx1*<sup>Cre/+</sup>/*Rosa26*<sup>LacZ/+</sup> mice.

The adult CB was examined for LacZ expression to investigate the developmental origin of CE cells. Retinal saggital cryosections were obtained from *Hesx1*<sup>Cre/+</sup>/*Rosa26*<sup>LacZ/+</sup> adult mice and stained with X-Gal (Figure 6.5). Upon visualisation LacZ-expressing cells were occasionally observed in the non-pigmented CE, but not in the pigmented layer of the CE (Figure 6.5a, b).

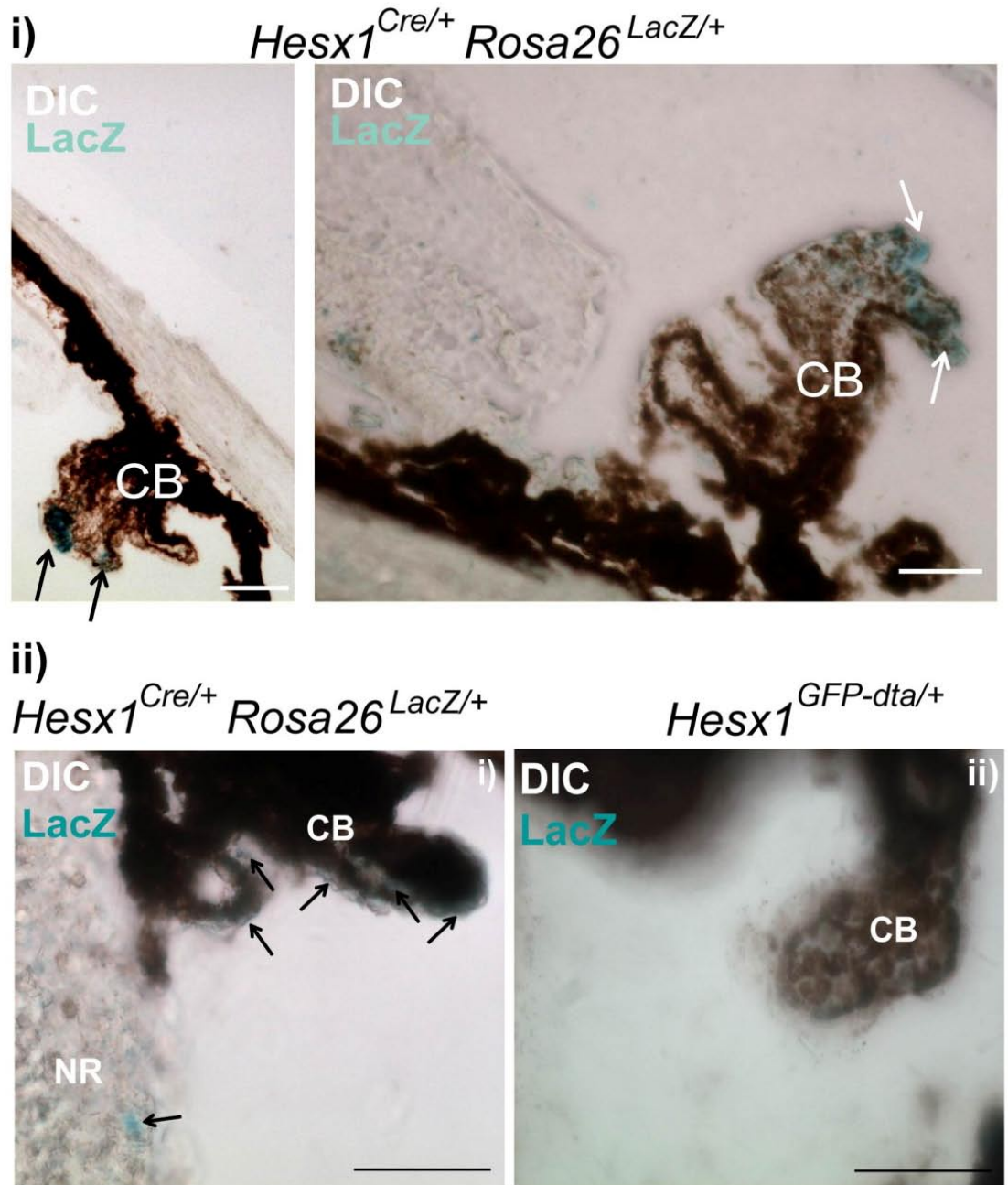


Figure 6.5 Origin of ciliary epithelium cells

(i) Light microscope microphotographs of sagittal CB sections of  $Hesx1^{Cre/+}/Rosa26^{LacZ/+}$  adult murine eyes after X-Gal staining.

(ii) High magnification light microscope images of  $Hesx1^{Cre/+}/Rosa26^{LacZ/+}$  (i) and  $Hesx1^{GFP-dta/+}$  (ii) adult murine CE after staining with X-Gal.

Scale bars= 20  $\mu$ m. N=5 different animals were examined. Arrows indicate  $LacZ^{POS}$  cells.

### 6.2.3 Origin of proliferative adult retinal neurosphere-forming cells

By staining for X-Gal, descendants of *Hesx1*-expressing cells can be detected at later stages of development. The *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* mouse model allowed me to perform a lineage-trace analysis of the cells that form neurospheres *in vitro*.

Specifically, the use of this transgenic mouse allowed me to address the question of whether CB-derived neurospheres generating cells are descendants of cells of the embryonic forebrain neuroepithelium. Adult CE from *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* mice was obtained and cells dissociated prior to culturing for 7 days in NGM allowing the formation of neurospheres. After staining with X-Gal, all of the neurospheres examined showed cells expressing LacZ [Figure 6.6a (i, ii)], although in some regions of the sphere pigment masked LacZ visualisation. When neurospheres from *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* mice were immunolabelled for Nestin and  $\beta$ -III tubulin, they showed strong expression of the former with very faint or absent expression of the latter, confirming their progenitor state [Figure 6.6b (i, ii)]. These data indicate that descendants of forebrain-derived *Hesx1*-expressing progenitors give rise to neurosphere-forming cells in the adult CB.



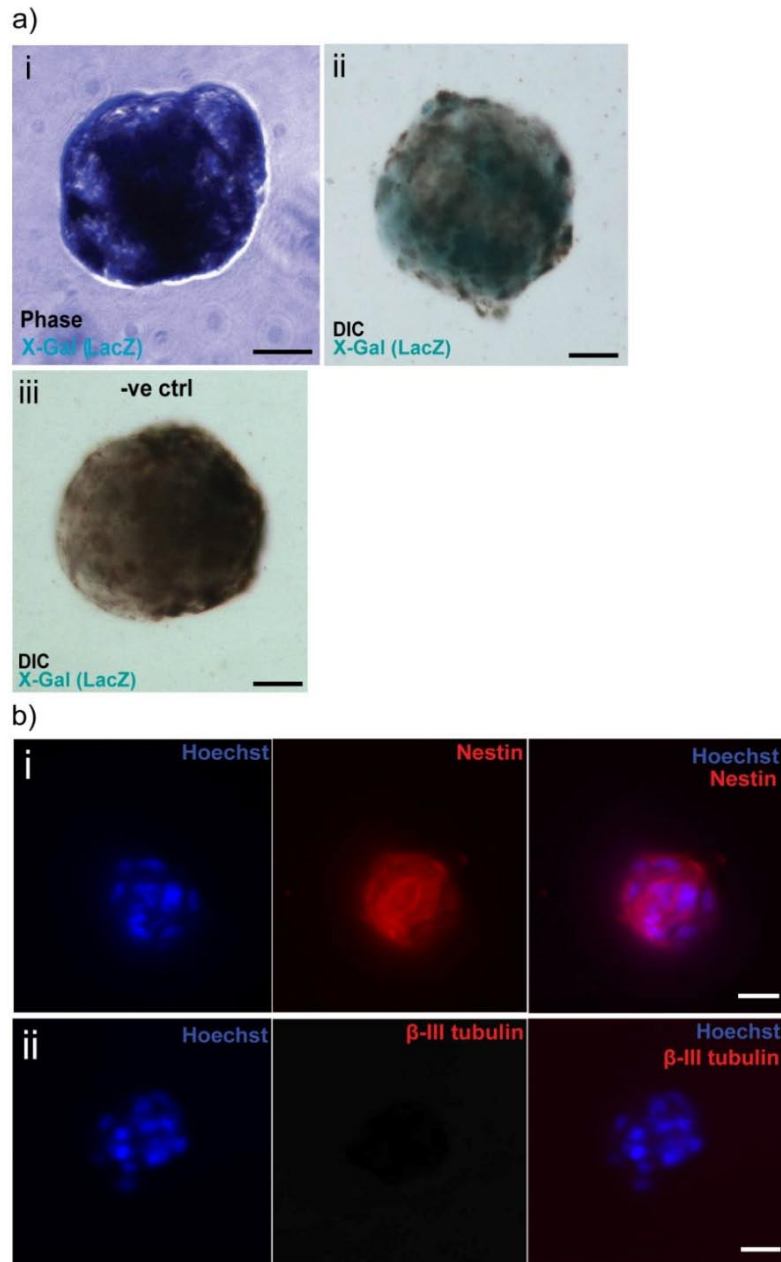


Figure 6.6 Origin of CE-derived neurosphere-forming cells

(a) Representative microphotographs of CE-derived neurospheres after cultured for 7 days *in vitro* in the presence of NGM. Neurospheres were fixed in 4% PFA and then X-Gal stained. Images were either viewed under inverted (i) and non-inverted (ii) light microscopes. As a control, CE-derived cells from SV129 mice were cultured in the presence of NGM for 7 days prior to X-Gal staining.

(b) Neurospheres deriving from adult murine CE were immunolabelled with Nestin (red) (i) and  $\beta$ -III tubulin (red) (ii) for investigating the presence of progenitors and early-born neurons, respectively.

N=3 independent experiments. Cells were counter-stained with Hoechst 33342. Scale bars = 10  $\mu$ m.

I sought to determine if the proliferating cells residing in the CB of the adult mouse are the ones that form neurospheres *in vitro* (Figure 6.7). The analysis involved injecting wildtype adult mice with BrdU for 5 consecutive days before culling. Dissected eyes were separated into two groups. Dissociated cells of the CE (part of the CB) were cultured for 3 days in the presence of NGM, prior to fixation. When CB-derived tissue was dissociated and cultured in the presence of NGM, neurospheres formed after 3 days in culture (Figure 6.7a). Subsequently, upon immunolabelling with anti-BrdU, a small number of cells per neurosphere showed a strong expression with the rest of the cells within the neurospheres being negative for BrdU (Figure 6.7b).

Eyeballs from the second group were fixed and saggital cryosections were obtained (Figure 6.8). Cell cultures and retinal sections were immunolabelled with  $\alpha$ -BrdU and counterstained with Hoechst nuclear dye (Figure 6.8a). Adult retinal saggital sections revealed the presence of BrdU<sup>pos</sup> cells only in the CE but not in the CNR, with proliferating cells present in both pigmented and non-pigmented regions of the CB with some cells rarely labelled positive for BrdU in the stromal CB region (Figure 6.8b).



**a)**

BrdU injections in adult mouse for 5 days, and then sacrifice animal



dissect CB, culture CE-dissociated cells in NGM for 3 days *in vitro*



fix neurospheres in 4% PFA and immunolabel with rat  $\alpha$ -BrdU and Hoechst nuclear dye

**b)**

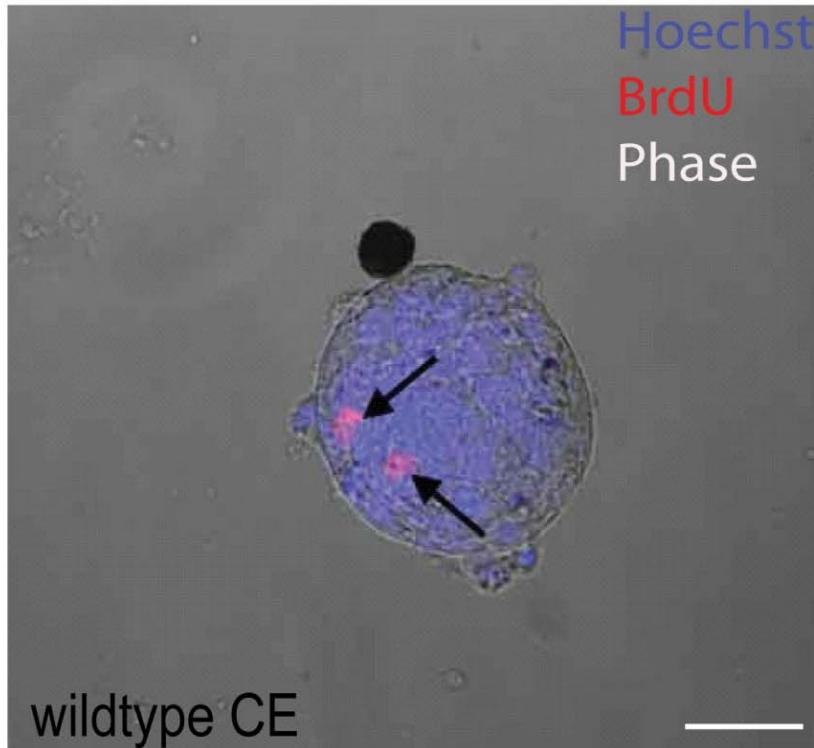


Figure 6.7 Origin of proliferative adult retinal neurosphere-forming cells

(a) Experimental design of *in vivo* BrdU injections into adult mice and consecutive sacrifice for immunolabelling with an antibody against BrdU on neurosphere cultures.

(b) Neurospheres are derived from the population of slowly dividing cells present *in vivo*. In wildtype animals, neurospheres could only be derived from the adult CE and not the CNR. Scale bar 20  $\mu$ m.

N=3 independent experiments; counter-stained with Hoechst 33342.

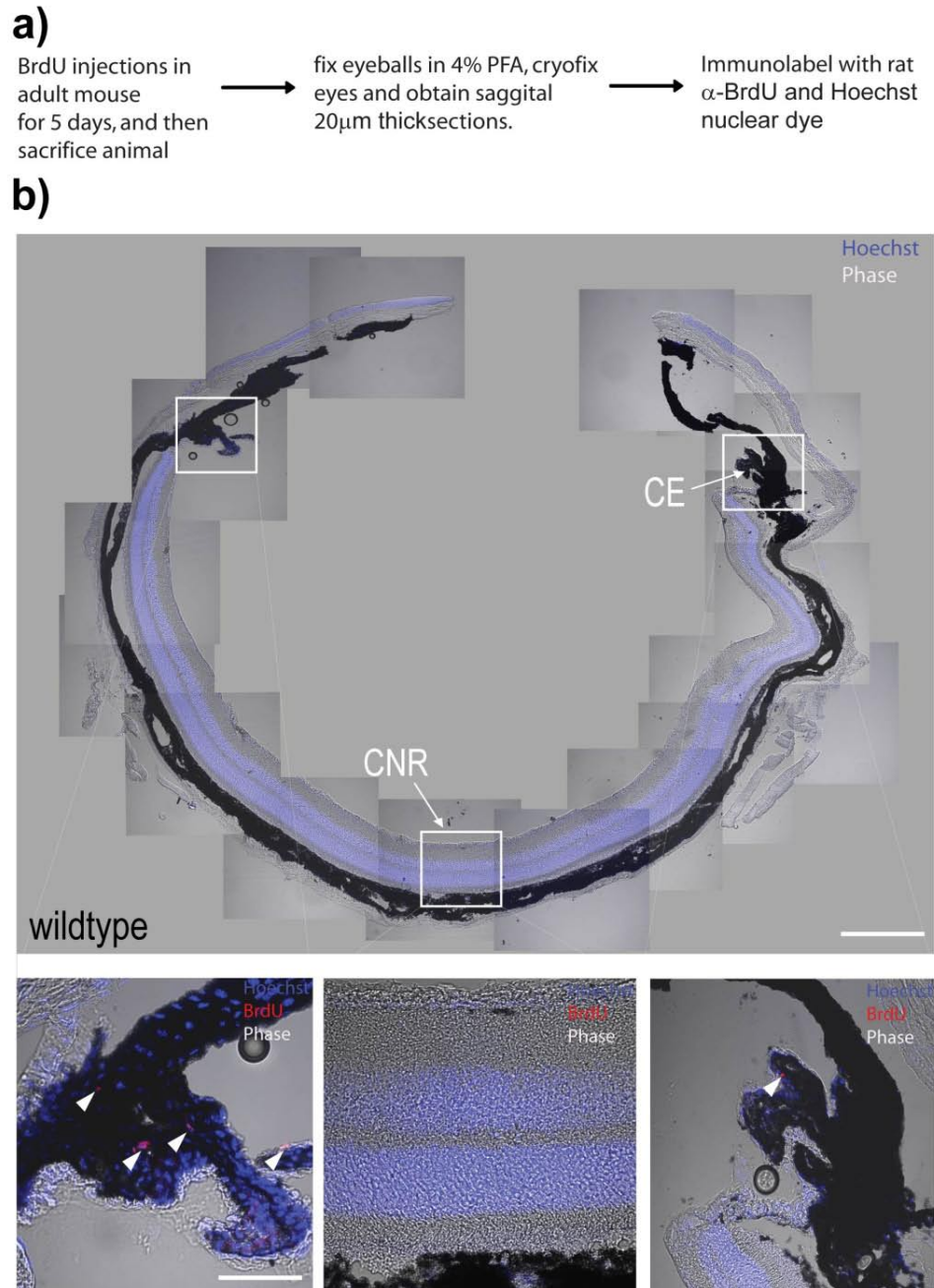


Figure 6.8 Proliferative cells in the adult murine retina  
**(a)** Experimental design of *in vivo* BrdU injections into adult mice and consecutive sacrifice for immunolabelling with an antibody against BrdU on sagittal ocular cryosections.  
**(b)** Confocal montage of a saggital section through a wildtype eye (lens removed). Sections show Normarski images in which nuclei are counter-stained with Hoechst 33342. Inserts, BrdU-positive (red) dividing cells are occasionally found in the ciliary epithelium (top and bottom). No BrdU-positive cells were found in the CNR (middle). Scale bars 200 mm (main image) and 50 mm (inserts).

#### 6.2.4 Developmental origin of the murine retinal cells

I also investigated the possibility of cells in the mature eye deriving from the *Hesx1*-expressing retinal progenitors. I was interested in the mosaic distribution of cells in the developing retina and the eye (see figure 6.3). Intriguingly, analysis of several (N=4) *Hesx1*<sup>Cre/+</sup>/*Rosa26*<sup>LacZ/+</sup> different animals showed an apparent random distribution of X-Gal stained cells. LacZ<sup>pos</sup> cells were observed in the ONL but not in the other two retinal layers (Figures 6.9 and 6.10), or a scattered expression covering all three layers and CE. No retinal sections were found with complete lack of LacZ expression, while RPE cells did not express LacZ.

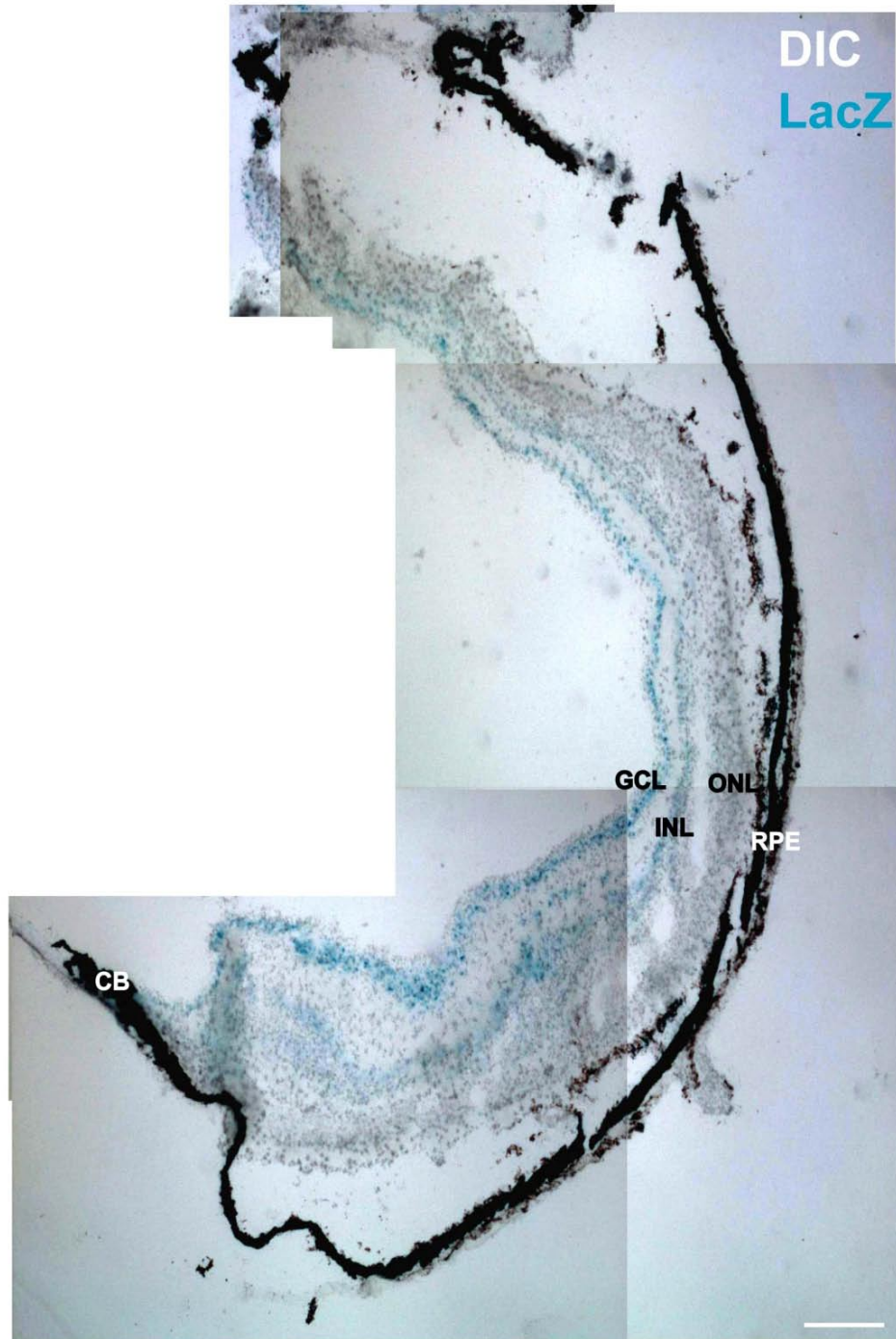
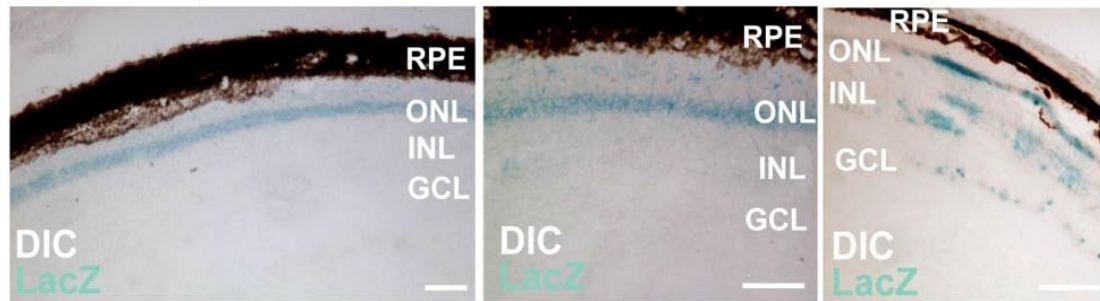


Figure 6.9 Origin of neural retinal and ciliary epithelium cells  
Representative microphotograph of a light microscope montage of a sagittal retinal section of a *Hesx1<sup>Cre/+</sup> / Rosa26<sup>LacZ/+</sup>* adult murine eye.

Scale bars= 100  $\mu$ m. Abbreviations; ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer, RPE = retinal pigment epithelium, CB = ciliary body



**a)**



**b)**

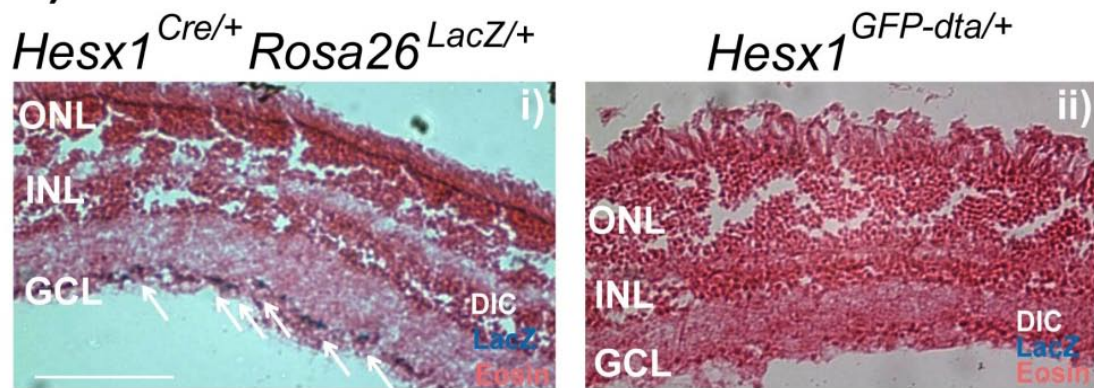


Figure 6.10 Comparative analysis of the expression of Hesx1-expressing cells, and descendants of Hesx1-expressing cells

(a) Light microscope microphotographs of sagittal CNR sections of  $Hesx1^{Cre/+} / Rosa26^{LacZ/+}$  stained with X-Gal.

(b) High magnification light microscope images of a sagittal retinal section of a  $Hesx1^{Cre/+} / Rosa26^{LacZ/+}$  (i) and  $Hesx1^{GFP-dta/+}$  (ii) adult murine eyes after staining with X-Gal and counterstained with Eosin.

Scale bars= 10  $\mu$ m. N=3 different animals for  $Hesx1^{Cre/+} / Rosa26^{LacZ/+}$  and N=1 for  $Hesx1^{GFP-dta/+}$  were examined. Arrows indicate where cells are  $LacZ^{pos}$

Abbreviations; ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer, RPE = retinal pigment epithelium, CB = ciliary body

## **6.3. Discussion**

### **6.3.1 Summary of findings**

In this chapter I performed a lineage tracing analysis of the adult CE-residing retinal stem/progenitor cells, by using the neurosphere forming assay. In addition by consecutive BrdU injections in adult mice the neurosphere-forming cells were attempted to be traced in culture. Finally the developmental origin of the mature retina and CE was investigated.

From this study the following conclusions can be drawn;

- i. Neurospheres observed *in vitro* likely arise from the proliferating cell population observed *in vitro*.
- ii. Some adult CB-derived neurosphere-forming cells are cells derived from the embryonic Hesx1-expressing forebrain neuroepithelial cells.
- iii. The adult murine retina includes progeny of Hesx1<sup>pos</sup> and Hesx1<sup>neg</sup> cells from the embryonic forebrain neuroepithelium.
- iv. Adult murine ocular tissues do not express Hesx1.

### **6.3.2 Origin of neurosphere-forming cells**

I sought to determine whether the CE spheres truly derive from the population of dividing cells observed *in vivo* (Dhomen NS 2006). Adult wildtype animals received injections of the nuclear label BrdU, marking cells undergoing mitosis *in vivo*. Histological analysis demonstrates the presence of proliferating cells *in vivo*, as shown by positive BrdU-labelling, within the CE of wildtype retinal sections. In addition, pre-labelled CE cells *in vivo* with BrdU, as described above, were also dissociated and placed in culture. After 3 days *in vitro* (without

BrdU), it was observed that all the resulting neurospheres contained one or two cells that were strongly labelled with BrdU, surrounded by a variable number of less strongly labelled cells and many more that bore no labelling at all. These results support, although do not prove, the idea that the neurospheres observed in culture derive from the different proliferating cell populations observed *in vivo*. They suggest that a pre-labelled, BrdU-positive parent cell divides to generate daughter progenitor cells that undergo successive rounds of division (hence diluting the BrdU) to generate the neurosphere.

### 6.3.3 Developmental origin of adult CE-derived neurospheres

The *Hesx1* homeobox gene is expressed in the forebrain neuroepithelium that contributes to the optic vesicle between E7.0 and E9 and thereafter is not expressed in any ocular tissue (Dattani MT 1999). I used a *Hesx1*<sup>Cre/+</sup> mouse model, generated by Dr Juan Pedro Martinez-Barbera (Martinez-Barbera JP 2000), to investigate the origin of the stem cells located in the CE of the adult retina. These mice contain *Cre recombinase* replacing the *Hesx1* locus, thus expressing Cre under the control of *Hesx1* regulatory sequences. By crossing the *Hesx1*-specific Cre-recombinase line with a mouse line carrying a stop-floxed  $\beta$ -galactosidase gene in the *Rosa26 locus* (R26R) (Soriano 1999), it was possible to perform lineage analysis of the cells that express *Hesx1* in the forebrain. When *Hesx1* activates Cre between E7.9 to E9 in the forebrain, CRE acts to remove the intervening LoxP flanked sequence which prevented transcriptional read through (neo expression cassette followed by a triple polyadenylation sequence, leading to constitutive expression of LacZ (from the *Rosa* locus) in the *Hesx1*-expressing cell population and their progeny in later stages of development.

When CB tissue was dissociated from *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* adult mice and cultured in NGM, neurospheres were formed deriving from proliferating cells found in the CB. Upon X-Gal staining all the cells within the neurospheres were LacZ positive, although some regions were difficult to visualise due to heavy pigmentation. Consequently, neurosphere-forming cells are descendants of forebrain neuroectodermal cells.

#### 6.3.4 Hesx1 is not expressed in the adult murine retina and CB

To confirm that Hesx1 is not expressed in the adult eye, a mouse line carrying a green fluorescent protein gene knock-in at the *Hesx1* locus was used (kindly provided by Dr Juan Pedro Martinez-Barbera). The reporter is an *eGFP-DTA* (enhanced green fluorescent protein - diphtheria toxin fragment A) cassette. The mouse expresses eGFP under control of the Hesx1 promoter, but DTA expression is prevented by the presence of eGFP, a Neo cassette, and a strong transcriptional stop sequence. Eyes obtained from *Hesx1<sup>GFP-dta/+</sup>* mice were normal and showed no expression of GFP in any ocular tissue. In addition, when immunolabelled with an antibody against GFP, no cells were found positive in retinal sagittal cryosections tested. From this set of data I conclude that Hesx1 is not expressed in the adult murine eye.

#### 6.3.5 The developmental origin of the murine retina

After confirming that Hesx1 is not expressed in the adult retina, I set out to investigate if retinal and CB cells derive from Hesx1-expressing cells. When adult retinal sections were stained with X-Gal, LacZ<sup>pos</sup> cells were observed in the retina and CB, but strikingly, in a non-uniform way. Rather, for each *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* eye the pattern of LacZ<sup>pos</sup> cell expression was different either being on the INL, ONL, or GCL. Similarly, within the CB, some cells were



LacZ<sup>pos</sup> but their location was not consistent between eyes. Of notice, no LacZ<sup>pos</sup> cells were found in the RPE on any eye investigated.

Vertebrate ocular development relies on co-ordinated initiation of morphogenic programs arising from inductive signals deriving from tissues of different embryonic origin. Our collective observations have shown that the murine retina and CE is formed from cells deriving not only from Hesx1-expressing cells, while the RPE showed no LacZ<sup>pos</sup> possibly due to pigment masking, although localisation and Cre-mediated  $\beta$ -galactosidase reporter results have been shown to overcome pigment masking (Le et al. 2008). Hesx1 is expressed early in embryonic development and eventually cells in the telencephalic and the diencephalic regions express mRNA transcripts for this homeobox gene. During the formation of the optic vesicle, the majority of cells were shown to derive from Hesx1-expressing cells within the neuroblastic layer but in a scattered fashion. This observation led me to investigate further into the adult retina, showing that not all retinal cells derive from a progeny of cells deriving from the Hesx1-expressing forebrain neuroepithelium.

During ocular formation there is an invasion of mesenchymal and neural crest precursor cells, especially in the anterior segment, generally contributing to the periocular mesenchyme. As Gage PJ *et al.* has elegantly showed, the stroma of the CB is of mesenchymal and neural crest origin, with some cells of non-ectodermal origin being present within the CE (Gage PJ 2005). However, there is an indication of neural crest descendants within the neuroblastic layer of the optic primordium at E10.5 (Gage PJ 2005). These observations suggest that cell fate specification is not only dependent on intrinsic cues but the micro-environment is crucial for cell fate specification, at least at the early embryonic stages. This hypothesis is supported by the fact that lack of Hesx1 does not confer only one optic dysplasia phenotype but there is a variable degree of abnormality from microphthalmia to anophthalmia (Dattani, Martinez-Barbera et

al. 1998; Dattani MT 1999). Thus, there is either a compensating mechanism in place and/or eye formation relies heavily on the presence of extrinsic cues placed in the correct position in the optic primordium that will instruct migrating cells to initiate their retinal specification cellular machinery.

Although it seems unlikely, it is possible that the non *Hesx1*-expressing retinal cells derive from sources other than the forebrain neural epithelium such as the mesenchyme or the neural crest, which upon arrival might acquire a neuronal fate, leading to the formation of the neural retina. Retinal transplantation experiments using ES cells over-expressing *Rx/rax* were showed to mimic the differentiation machinery already present in the developing retina and setup by the host cells (Tabata et al. 2004). *Rx/rax* is expressed in the anterior neural fold, including areas that will give rise to the ventral forebrain and optic vesicles in the early mouse embryo; and then, once the optic vesicles have formed, *Rx/rax* expression becomes restricted to the ventral diencephalon and the optic vesicles (Tucker et al. 2001). In addition, there has been a report indicating that late retinal progenitors, although normally differentiate in a time-dependent manner producing late-born neurons such as rod photoreceptors, bipolar cells and Müller glia, can alter their potential to give rise to early-born retinal neurons (James et al. 2003). These reports along with my findings illustrate that cells during the formation of the optic cup from the optic vesicle might have a high degree of plasticity in terms of fate determination and may not be of neuroectodermal origin. Rather, the epigenetic cues needed for correct cell fate specification might be retrieved from the microenvironment after the basic intrinsic machinery has been activated. Nevertheless, the most likely explanation is that *Hesx1*<sup>neg</sup> cells from the forebrain contribute to the retina and that the eye field microenvironment specifies the retinal fate to all cells in the region.

### 6.3.6 The developmental origin of multipotential retinal progenitors

Hitherto, the lineage tracing analysis using the *Hesx1*<sup>Cre/+</sup>/*Rosa26*<sup>LacZ/+</sup> and *Hesx1*<sup>GFP-Dta/+</sup> mouse models indicated that even if the neurosphere-forming cells derive from Hesx1-expressing cells, some regions of the CB and CE did not possess LacZ<sup>POS</sup> cells. The CE RSC population is rare (~0.6% and even lower in my LDA, see appendix), but it has not been reported to result from any clustering of any cell type within the CE of the adult mouse. That shows that some areas of the CE did not descend from the Hesx1-expressing anterior neuroepithelium rather, have a different developmental origin. The same effect was observed in the adult retina. This raises the question of whether there is a common retinal progenitor that gives rise to all retinal cell types and glia. As it was shown by injection of a BAG retroviral construct carrying an *E. coli*  $\beta$ -galactosidase gene in early-born mice, within the adult retina, variable size of clones were identified mainly indicating that P0-infected retinal progenitors could give rise to rod photoreceptors, bipolar cells and Müller glia (Turner and Cepko 1987). In addition the same laboratory showed that by retroviral injections earlier in development (E13.5) all retinal cell types were formed along with rare retinal astrocytes being LacZ<sup>POS</sup> (Turner et al. 1990), with the latter not deriving from the neuroblastic layer, rather astrocytic precursors migrate from the optic nerve to colonise the nerve fibre layer (Stone and Dreher 1987; Schnitzer 1988; Watanabe and Raff 1988).

Furthermore, it has been reported that cell death within clones might lead to overestimations in lineage relationships (Voyvodic et al. 1995). Specifically, it was shown there was an estimation of 50% of normal apoptosis occurring except in the case of rod photoreceptor differentiation, coinciding with data indicating the possibility of a distinct rod photoreceptor precursor and explaining the high variability in clone size found in cell lineage analysis in the frog retina (Wetts and Fraser 1988; Wetts, Serbedzija et al. 1989) and *Xenopus* (Holt,

Bertsch et al. 1988). Consequently, in conjunction with my data, there is a possibility that the existence of a single multi-potential retinal progenitor is not essential for retinal formation.

I propose that in the optic primordium there has been a pre-established retinal-specific microenvironment which holds the essential cues for cells migrating to acquire a retinal lineage. That is, the tissue acts as a permissive instructor. In this case, the emergence of retinal multipotential progenitors may arise later in development but their origin and competence are not identical. Rather, the activation of expression of TFs such as Pax6, Otx1 and Otx2, Six3 and Rax in these cells will be make them competent for optic cup formation, educating them to initiate further their intrinsic retinal specification machinery (Collinson et al. 2000; Martinez-Morales et al. 2001 ; Baumer et al. 2002; Tabata, Ouchi et al. 2004; Gestri et al. 2005 ; Canto-Soler and Adler 2006; Duparc et al. 2007; Reza et al. 2007). Finally, cell secreting factors will further induce nearby cells to follow a specific fate via instructive interactions. Perhaps there is a threshold amount of cells deriving from the anterior neural ectoderm (and thus descendants of Hesx1-expressing cells) populating the optic vesicle sufficient for these interactive instructions to occur and the optic cup to develop (Wessells 1962; Holtzer 1964).

## **Chapter 7 – Final Discussion**

## **7.1 CNR progenitors from the $Chx10^{orJ/orJ}$ give rise to neurospheres *in vitro***

The adult mammalian retina has no capability of regeneration. To date it has been reported that in the adult mice and humans the ciliary epithelium, a region of the CB, is a retinal stem cell niche. This region is analogous to the adult frog and fish CMZ along with the CB, the most extreme part of the retina, where retinal stem cells reside and give rise to new retinal neurons throughout life (Fernald 1990; Perron et al. 1998; Otteson and Hitchcock 2003). When cells from that region are cultured in a growth-factor rich medium they form neurospheres a characteristic of neural stem/progenitors (Reynolds BA 1992). The ocular retardation (orJ) mouse model is a microphthalmic mouse model due to the production of a non-functional Chx10 TF, leading to a defective proliferation of retinal progenitor cells during retinogenesis (Burmeister M 1996).

The adult  $Chx10^{orJ/orJ}$  murine retina is hypocellular and poorly demarcated with a complete absence of bipolar cells (Burmeister M 1996). Our lab has demonstrated that mitotic cells are located in the mutant CNR, a phenomenon that is not observed in the normal adult central retina (Dhomen NS 2006). I attempted to culture  $Chx10^{orJ/orJ}$  cells from the adult central neural retina in the presence of growth factors. Similar to CE and SVZ and DG cells they were able to form neurospheres *in vitro*.

In the chicken experimental model, retinal stem cells residing in the CMZ are able to differentiate to retinal neurons and glia and respond to injury during life by RPE transdifferentiation and increased cell division (Spence et al. 2007; Spence et al. 2007). In mammals, like human and mice, there has been no documented RSC population in the adult central neural retina (Perron M 2000). Interestingly, cells adjacent to the most peripheral part of the retina are enriched

for stem/progenitor markers such as Nestin, Pax6 and GFAP (Mayer, Hughes et al. 2003; Xu et al. 2007). Through several elegant studies and by the use of knock-out mouse models it has been shown that sonic hedgehog (Shh) is an important regulator in the area and inactivation of sonic receptor patched (ptc) prolongs peripheral retinogenesis in mice and sustains BrdU<sup>pos</sup> cells (Moshiri and Reh 2004).

Using the murine retina neurosphere assay as a model, several researchers have attempted to isolate retinal stem/progenitor cells from the human retina. Using fetal eyes derived from embryos at the beginning of the second trimester of gestation as a source, Yang *et al.* were able to isolate cells able to form neurospheres *in vitro* from the retinal tissue (Yang P 2002). These cells, expressed high levels of a variety of proliferative markers such as Ki-67 and BrdU which ceased after prolonged culture times and their expression profile was Nestin<sup>high</sup>Chx10<sup>high</sup>Vimentin<sup>high</sup>Sox2<sup>high</sup>Pax6<sup>high</sup>Six3<sup>pos</sup>MHC-Class-I<sup>pos</sup>Class-II<sup>neg</sup>Recoverin<sup>low</sup>β-III-tubulin<sup>low</sup> (Yang P 2002; Klassen HJ 2004).

My findings show that Chx10 is important in retinal stem cell biology. Absence of Chx10 may halt retinal stem cells in a more primitive stage, and as has been shown, hindering the overall retinal expansion (Dhomen NS 2006). Nevertheless, the retina is formed with the presence of all retinal neurons apart from bipolar cells. This interesting paradox indicates the complexity of the system and that an alteration in the RPC behaviour occurs, but does not explain sufficiently the small eye phenotype. To examine further the characteristics of Chx10-deficient cells, I dissociated and cultured them *in vitro*. Strikingly, CNR-derived cells from the *Chx10<sup>orJ/orJ</sup>* began cycling producing larger and more neurospheres than CE-derived neurospheres after seven days in culture. The normal CNR never gave rise to neurospheres in these conditions. These *in vitro* findings although not directly comparable to the *in vivo* developmental data, are contradictory; how Chx10 is needed for RPC proliferation upon development?

Why, *in vitro*, its absence gives rise to a large population of neurospheres? Although it has been reported that the adult  $Chx10^{orJ/orJ}$  CNR does not give rise to neurospheres (Tropepe V 2000) and that CE-derived mutant neurospheres are smaller in diameter than wildtype ones (Coles BL 2006), in my system that was not the case; it would be logical to suggest that the absence of Chx10 gives rise to smaller neurospheres either deriving from the CE or CNR in comparison to wildtype CE, but if Chx10 acts as a regulator, its absence will give rise to a highly proliferative population. As it was explained in chapter 3, the disruption of normal asymmetric-symmetric division balance could alter the RPC potential giving rise to a hypocellular retina.

From a more stochastic viewpoint, absence of Chx10 might induce a number of cell cycle inhibitors earlier than normal during retinogenesis, which shut down mitosis in RPC after E13.5. It has been shown recently that RGC differentiation delay occurs in the  $Chx10^{orJ/orJ}$  retina due to abnormal SHH signalling (Sigulinsky et al. 2008). The authors proposed that in the absence of Chx10, Hh signalling is active but negatively regulates RPC proliferation. In essence, Chx10 is needed for early RGC differentiation that would secrete SHH which will propel the remaining RPC to proliferate more. This positive feedback loop is disrupted when Chx10 is absent since it alters gene regulation. As it has been shown, the wildtype and  $Chx10^{orJ/orJ}$  RPC populations are similar at P0 (Green ES 2003), but the latter have a lengthier G1 phase during cell division (Konyukhov and Sazhina 1971; Dhomen NS 2006). Effectively, Chx10 is an important RPC regulator which might be implicated in the response of RPC in the feedback signals received from the developing retina. This can be shown by the *in vitro* neurosphere-forming data.  $Chx10^{orJ/orJ}$  neurospheres have derived from RPC that did not activate their Hh signalling pathway during development and in the presence of growth factors or absence of inhibitory signals show robust proliferation, *in vitro*.



Certainly, the data are not conclusive and more research is needed. Especially, it would be interesting to assess the gene and protein expression levels of EGFR and FGFR1 in these CNR *Chx10<sup>orJ/orJ</sup>* neurospheres assessing if they are more closely related to early RPC (increased EGFR) or late RPC (increased FGFR1) populations (James J 2004). An essential understanding of the different *in vitro* behaviour of early (E11.5) and later (after E13.5) RPC is important to pinpoint where in the developmental process Chx10 shows its effects on proliferation rate. In addition, investigation of cell cycle regulatory and enhancer protein and mRNA levels on the aforementioned developmental stages would be elucidative in how Chx10 affects mitosis. Also, BrdU pulsing at different embryonic stages could reveal if these cells derived from early or late RPC.

## **7.2 CNR progenitors from the *Chx10<sup>orJ/orJ</sup>* have Müller/progenitor characteristics**

Although cells of the retina are fully differentiated, recent reports have shown that Müller glia, comprising the major population of glial cells in the retina, might also act as end-stage progenitors. The Chx10-deficient adult retina is demarcated, although considerably thinner than normal, with early- and late-born populations being present, except bipolar cells that directly need Chx10 for their formation (Burmeister M 1996). How is it possible to have an early RPC proliferation dysfunction but still get late-born cells being produced such as Müller glia (the last cell population formed in mammals)?

My work shows that retinal stem/progenitor cells isolated from the murine adult CE and central retina of Chx10-deficient animals consistently showed a profile of Nestin<sup>high</sup>Pax6<sup>high</sup>Sox2<sup>high</sup>b-III-tubulin<sup>low</sup> expression common in neural stem-like cells along with a *Gfap*<sup>high</sup>GS<sup>pos</sup>CRALBP<sup>pos</sup> profile, typical of activated Müller glia. Further, cell cycle regulators, such as Cyclin D3 and p27<sup>Kip1</sup> gene and

protein expression fit a profile close to that reported for active Müller glia but with a strong progenitor characteristic profile (Dyer MA 2000). In addition, *Cyclin D1* and *PTEN* showed no alteration and a significant increase, respectively in mutant-derived neurospheres. The absence of Chx10 in the adult murine retina showed that CNR cells are capable of forming neurospheres *in vitro* and that a possible source is a GFAP<sup>pos</sup>Nestin<sup>pos</sup>RPE65<sup>neg</sup> progenitor. This implies that a candidate for being an endogenous neural progenitor in the CNR could be a Müller glial cell.

The possibility of Müller glia to be endogenous neural progenitors in the mammalian retina is intriguing and is supported from recent data derived from injury models in zebrafish (Bernardos, Barthel et al. 2007) and rodents (Ooto S 2004; Kohno, Sakai et al. 2006; Wan, Zheng et al. 2008). Upon laser-induced and neurotoxic injury, cells with Müller characteristics in the INL were able to uptake BrdU and co-express neural and retinal progenitor markers, with extensive differentiation potential towards rod photoreceptors in zebrafish and a significantly lower, but present, regenerative potential in rodents. Recently, adult human retinal explants cultures revealed that GFAP<sup>pos</sup>Nestin<sup>pos</sup> neurospheres deriving from the pars plana were able to give rise to a small population of BrdU<sup>pos</sup>Rhodopsin<sup>pos</sup> cells. In addition, anterior retinal explants showed an activation of a population of cells strongly expressing Nestin and migrating out of the explants. The combination of both FCS and growth factor supplements conferred to that progenitor-like behaviour (Mayer EJ 2005). Finally, by using spontaneously immortalized human cell line MIO-M1, it was shown that Müller cells could exhibit progenitor characteristics by expanding indefinitely and expressing post-mitotic retinal markers upon differentiation (Lawrence et al. 2007).

As described in Chapter 3 and discussed in section 7.1, the Chx10-deficient mice are microphthalmic with a hypocellular retina. My data showed that

reactive Müller glia markers, normally expressed later in histogenesis and only upon injury (after postnatal day 1) are found in the late but not early embryonic neural retina. Since Chx10 is expressed in a subpopulation of Müller glia (Rowan S 2004), it is feasible to assume that absence of Chx10 might alter that specific Müller subpopulation to a hybrid glial/progenitor state in the CNR. In addition, the presence of Müller glia earlier than normal, is indicative of the pathway of a RSC to a RPC and then a late end-stage progenitor (Müller glial cell), which is accelerated, partially explaining why late-born neurons are able to form in the *Chx10<sup>orJ/orJ</sup>* retina even if early RPC are defective in their proliferation potential. It is also possible that the early formation of Müller-like cells is due to accumulation of p27<sup>Kip1</sup> during retinogenesis that might drive cells to acquire that cell fate earlier than normal.

Recent unpublished data from Joel Rae, using mouse chimeras of wildtype and *Chx10<sup>orJ/orJ</sup>* mouse embryos suggests that Chx10 can act in a non-cell autonomous manner. Using a grade of 1 to 4 where 1 was scored as the last chimeric mouse, meaning most of the retina derive from a wildtype GFP-expressing transgene, while 4 is the opposite side of the spectrum with most of the retina deriving from the *Chx10<sup>orJ/orJ</sup>* mouse, it was shown that grade 1-2 mice had an E18.5 retina similar in size to wildtype. Immunohistochemistry confirmed that amacrine, retinal ganglion and photoreceptor cells were present with an absence of Müller glia cells. Interestingly, in grade 3 and 4 chimeras, there were considerably smaller ocular and retinal volumes at E18.5 in comparison to wildtype, with immuno-histochemical analysis showing all cell populations being affected, especially photoreceptors, where Recoverin<sup>pos</sup> cells were only found in the wildtype GFP-expressing retinal areas but not in the *Chx10*-deficient ones. Again, no GFAP<sup>pos</sup> cells were observed in these retinal sections. Perhaps the non-cell autonomous *Chx10* expression did not allow for the Müller/progenitors to develop as observed in the fully *Chx10<sup>orJ/orJ</sup>* CNR.

The above data indicate how crucial the developmental window is for Chx10 expression. Chx10 seems to be essential for photoreceptor expression *in vivo*, probably in a combination with structural and territorial extrinsic signals. Essentially, absence of Chx10 will not lead to a demarcated ONL and thus will give rise to abnormal photoreceptors. Later in development (postnatally), Chx10 needs to be switched off in order for cells not to acquire a bipolar cell fate (or an INL cell fate). In culture, for obtaining rod photoreceptors, it might be essential to transiently silence Chx10, instead of abolishing its expression completely. Perhaps this step will finely tune retinal precursors to acquire a photoreceptor precursor cell fate instead of a bipolar one.

### **7.3 The concept of retinal stem cell transplantation**

Human embryonic retinal progenitors show the ability to differentiate towards a neuronal and glial cell type, including rod and cone photoreceptors. Several lines of research showed that human retinal progenitors, as of others mammals, are influenced heavily by the surrounding microenvironment. The age of donors and the time cells are cultured for, as well as passage number influences their behaviour (Gamm, Nelson et al. 2005). Complex cellular programs interact, mediating RPC progression and competence. As has been elegantly shown, neurospheres formed from human retinal cell isolated from the first trimester of gestation have a stronger bias towards differentiation along the neuronal pathway while neurospheres from cells isolated towards the beginning of the second trimester show a bias towards a glial phenotype (Gamm, Nelson et al. 2005).

As mentioned previously, the adult human retina might possess a cell population that can give rise to neurospheres (Mayer EJ 2005; Lawrence, Singhal et al. 2007), and perhaps the anterior retina possesses a zone similar to the CMZ

found in lower vertebrates, judging by the presence of Nestin<sup>pos</sup>GFAP<sup>pos</sup> cells residing there (Mayer, Hughes et al. 2003). The adult retina is post-mitotic after day 11 in the *Mus musculus* (around the 3rd month after birth in humans). A specialised non-retinal structure, the CB has recently shown to possess cells that form neurospheres *in vitro* (Ahmad I 2000; Tropepe V 2000). Although these spheres possess similar characteristics with those isolated from the adult SVZ, they are significantly smaller in size, and more difficult to passage and expand. In humans, the most readily available source of adult ocular tissue is from cadavers, and CB cells have been cultured in conditions similar to human pre-natal and adult murine retinal neurosphere-forming assays. A study comparing mouse-, rat- and human-derived CB adult neurospheres showed similarities in diameter and stem/progenitor protein expression (Xu, Sunderland et al. 2007). Human CB-derived adult neurospheres showed similar differentiation profile when plated on laminated substrates, to that of adult murine-derived adult CB neurospheres, being able to produce cell types expressing retinal and glial proteins (Coles BL 2004). Furthermore, these spheres were able to be passaged as monolayers expanding their number, implying that they can be manipulated in culture to give rise to an adequate cell number for transplantation (Coles BL 2004). Apparently, and as expected, post-mortem time of dissection, donor age and interestingly donor sex, has an effect in neurosphere number (Carter, Mayer et al. 2007). Even if these data do indeed show a difference between donors and potentially patients prone for autologous transplantation, it is plausible to consider that the neurosphere assay involves the dissection of a very specific region which includes the pars plana and pars plicata, that have shown to give rise to different and unequal number of neurospheres (Coles BL 2004). Also, it is evident that cell-cell interactions are essential for neurosphere formation, since one-cell-one-neurosphere clonal experiments show a contradiction to those of cells cultured in concentrations of 20 cells/ $\mu$ l (Coles BL 2004).

In chapters 4 and 5 I attempted to characterise the Chx10 newly-found CNR-derived neurospheres that I showed to proliferate more *in vitro* in comparison to their wildtype CE counterparts (Kokkinopoulos et al. 2008). These results led me to construct a transplantation scheme shown in Figure 7.1. For retinal cell-based therapies, it is realistically essential to use either an *ex vivo* approach or allogeneic transplantation, which again requires an *in vitro* step. My approach was divided into three main steps; the first one was the expansion of RPC as neurospheres in the presence of growth factors. Following this 7-day neurosphere formation period, progenitors were exposed to key factors that would further expand them and commit them to the rod photoreceptor precursor cell fate by switching on the *Nrl* gene showing that they are in a potentially ideal stage for effective retinal transplantation (MacLaren, Pearson et al. 2006). Then, sub-retinal injections of these cells would effectively migrate and integrate, differentiating to mature rod photoreceptors.

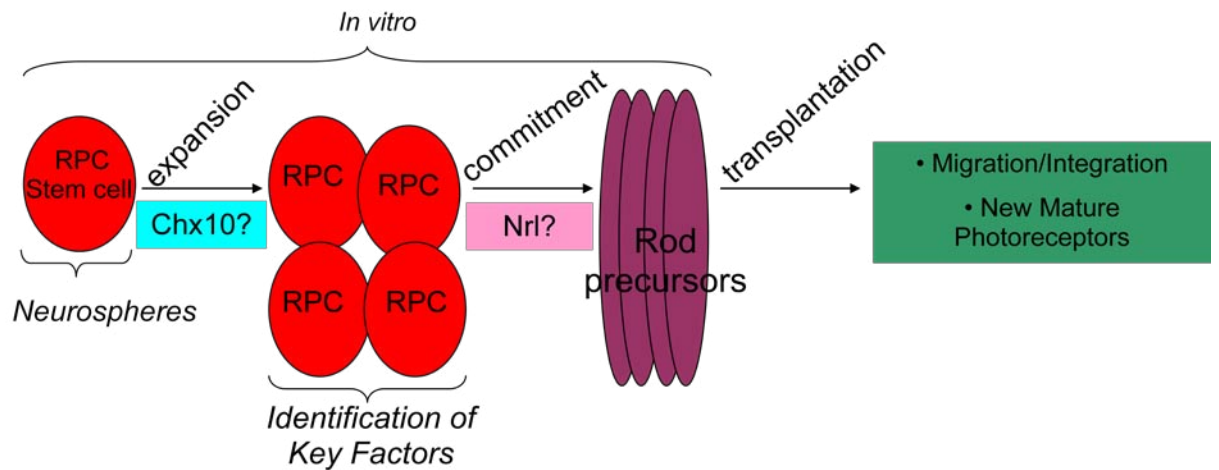


Figure 7.1 Overall ex vivo transplantation scheme

Results shown on chapter 5 suggest that neurosphere-derived cells may potentially be useful for cell-based therapies in the mammalian retina. The use of the *Nrl.gfp<sup>+/+</sup>* transgenic mouse model is an invaluable tool for detecting whether cells can switch to a rod photoreceptor cell, and lack of Chx10 might augment this progression from RPC or Müller/progenitor cells towards that of a

mature photoreceptor cell according to biochemical and developmental data (Dorval KM 2006; Livne-Bar I 2006). Therefore, the next step for the newly discovered neurosphere source was to assess their potential to give rise to retinal neurons *in vitro*. Their multipotentiality was similar to neurosphere-derived CE cells from both mutant and wildtype, suggesting that except the expected lack of bipolar cell differentiation, their potential is unaltered, as shown from immunocytochemical analysis. Interestingly, *Chx10<sup>orJ/orJ</sup>* neurospheres showed a trend towards a photoreceptor fate during differentiation judging by the increased gene expression levels for Crx and recoverin, but not  $\beta$ -III tubulin or Gap43, in comparison to wildtype.

The *in vitro* cocktail currently used for neurosphere differentiation involved FCS in a growth factor –free or -enriched medium. It was demonstrated that although the majority of neurosphere-derived cells exit the cell cycle in the conditions used, a considerable percentage was still undergoing mitosis. Interestingly, *Chx10<sup>orJ/orJ</sup>* CNR cells had a higher mitotic index with fewer cells expressing p27<sup>Kip1</sup> than CE, in differentiating conditions. This suggests that mutant CNR cells can maintain their proliferation state for longer, confirming results in Chapter 3, but it also shows the lack of effective differentiating conditions. Although this is a drawback in assessing neural stem cell multipotentiality, it may be used for obtaining precursor cells. It was recently shown from our lab that rod photoreceptor precursors are more efficient in successfully migrating and integrating in retinal transplantation and thus restoring visual function than embryonic stem cells or fully mature photoreceptors (MacLaren, Pearson et al. 2006; Canola and Arsenijevic 2007). I hypothesised that *Chx10*-deficient RPC would be a more potent source for cell-based retinal transplantation studies due to their robust proliferation potential *in vitro* and their shift towards a photoreceptor fate. The employment of the *Nrl.gfp<sup>+/+</sup>* transgene was invaluable in detecting rod photoreceptor precursors *in vitro* and *in vivo*.

As shown in Chapter 5, the expression of GFP transgene shows the initiation of Nrl protein since they utilise a similar promoter sequence. Neurospheres deriving from *Nrl.gfp*<sup>+/+</sup> and *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp* CE as well as *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp* CNR never expressed GFP during the neurosphere formation step. Upon monolayer and differentiation steps, immunocytochemical and PCR data indicated the presence of *Gfp* genomic DNA in a small population of cells. The use of different differentiation and monolayer cocktails did not alter the neurogenic potential in any of the three neurosphere sources. In conclusion, the current *in vitro* conditions are not sufficient in promoting a retinal cell fate in the majority of RPC. An important factor is the three-dimensional space in which neural progenitors need to exist in contrast to haematopoietic stem cells that can readily differentiate into functional cells *in vitro* in the absence of a specific organization (Spangrude GJ 1988; Wiles and Keller 1991; Nakano, Kodama et al. 1994). The use of Puramatrix 3D gel here, showed a transient expression of GFP suggesting that this factor is crucial for *in vitro* neural progenitor cell manipulation. This observation can be confirmed by the attempt of transplanting *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp* CE as well as *Chx10*<sup>orJ/orJ</sup>/*Nrl.gfp* CNR neurosphere-derived cells into the sub-retinal space of adult mice. As shown, GFP was detected in a small population of cells showing that lack of Chx10 does not hinder the production of rod photoreceptor precursors. Even if this set of data is preliminary, it shows that the current *in vitro* models lack vital factors and a three dimensional structure, which the adult ocular region apparently possesses. Further, it is important to improve the efficient delivery of cells in the sub-retinal space, since upon injection, inflammation occurs, recruiting macrophages, which endocytose and destroy donor cells, thus inhibiting efficient integration (Bull et al. 2008; Singhal et al. 2008). For that reason a pharmacological approach can be used to enhance engraftment (West et al. 2008). As shown, disruption of the outer limiting membrane using dl-alpha-aminoadipic acid (AAA) will perhaps allow a larger number of cells to readily integrate in the retinal layers instead of being phagocytosed by macrophages.



## **7.4 The origin of adult retinal stem cells**

The ocular structure derives from the developing forebrain. Cells participating in eye formation have a neural ectodermal, surface ectodermal and mesenchymal origin, (Gage PJ 2005). Upon discovery of an adult retinal stem cell niche in the CB, a region next to the Iris and outside the retinal tissue, the origin of these neurosphere-forming cells is currently under investigation. In Chapter 6, I attempted to identify the origin of these cells by employing two Hesx1 knock-in mouse models. I specifically used Hesx1 for my lineage analysis, since it has been shown that this homeobox gene is expressed specifically in the telencephalic region during development with its expression diminishing by E9.5, just before eye formation. The *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* mouse model provided a way to track daughter cells deriving from Hesx1-expressing cells in the adult retina and CB. With the combined use of a *Hesx1<sup>GFP-dta/+</sup>* to exclude any Hesx1 expression in the adult murine eye, I performed X-Gal staining on retinal tissues and neurospheres deriving from the CB of adult *Hesx1<sup>Cre/+</sup>/Rosa26<sup>LacZ/+</sup>* mice.

X-gal stained cells were identified in the CE indicating these cells arise from the eye field/forebrain. Strikingly, when the adult murine retina was X-gal stained, a mosaic of blue and non-blue cells was consistently observed in a random fashion in all eyes examined. Neurospheres deriving from the CB were also all stained for X-Gal, indicating that the proliferating cells are descendants deriving from Hesx1 progenitors in the eye field and are formed from X-Gal stained cells observed in the CE.

Although it has been shown 20 years ago that there is a common progenitor that gives rises to retinal neurons and glia, clone size variability and apoptosis were not taken into consideration (Turner and Cepko 1987; Turner, Snyder et al.

1990; Fields-Berry et al. 1992; Cepko CL 1996). Further, viral injections used for lineage tracing analysis were performed on E13 and E14 in rats which might be a late stage for identifying if there are one or more retinal stem/progenitors that give rise to the whole retina. These data suggest that eye field cells include Hesx1-expressing and non-expressing cells, both contributing to the retina and give rise to RPC. In this context, eye field cells might need only to have been in contact with Hesx1-expressing cells just before retinogenesis begins at E10.5 in order to become RPC.

## **7.5 Final thoughts and future work**

The work described here has revealed a variety of novel findings relevant to the *Chx10<sup>orJ/orJ</sup>* phenotype and retinal stem cell biology, in general. The characterisation of stem-like cells from the adult mutant retina raises the questions of how, why and when these cells are formed. The how would be directly linked to the presence of Chx10 during RPC proliferation and differentiation. Why these cells are formed is interesting. As described earlier, neurosphere cells have a Müller/progenitor phenotype while the absence of Chx10 causes a shift towards an active Müller/progenitor population, which is confirmed by another report emphasising this hybrid phenotype during postnatal retinogenesis (Rowan S 2004). Active Müller glia are present in E18.5 mutant retinæ. Why is this? Are these cells end-stage progenitors that due to the absence of Chx10 have derived from early RPC or Müller precursors abnormally produced earlier than normal? Are these the same cells that eventually will reside in the adult mutant retina and give rise to neurospheres? What is the origin of the BrdU<sup>pos</sup> cells residing in the mutant retina? As mentioned earlier, one way is to measure the gene expression levels for EGFR and FGFR1, which could be complemented by performing a lineage tracing analysis; preferably, a *GFAP<sup>Cre/+</sup>* mouse model could be very informative in the *Chx10<sup>orJ/orJ</sup>* mouse

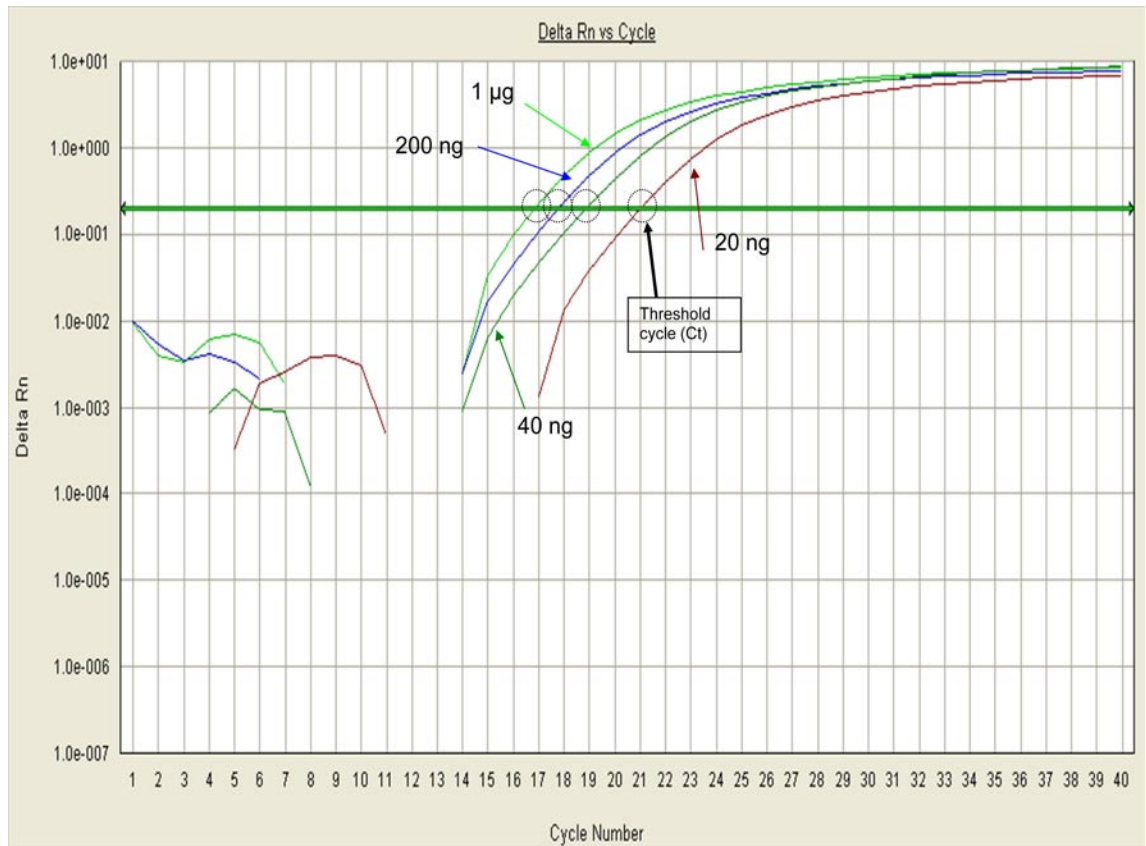
model for identifying the origin and end-stage of these cells. In a different perspective, it is possible that these cells have been halted in development due to their evolutionary program which protects the organism from cancer. This hypothesis would be very interesting to be examined in this system, since neurospheres can be considered as an uncontrolled growth of tumour-forming cells of the nervous system. Tumour suppressor genes and protein levels may be affected *in vivo*, including Rb and even Ras. Therefore, investigating these might be useful for cancer stem cell biology as well as how to expand a neural stem cell *in vitro*.

The differentiation potential of Chx10-deficient neurosphere-derived cells was also examined and showed a marked shift towards a photoreceptor potential which would be invaluable for future cell-based retinal therapies. Although promising, the conditions used are far from ideal for obtaining a large number of rod photoreceptor precursors. Ideally, more factors need to be examined as well as scaffolding system for these cells to differentiate properly.

The study conducted here has produced a number of interesting and novel findings and offered some valuable clues as to how Chx10 affects retinal progenitor cell proliferation and differentiation. In addition, it has contributed to the cutting-edge regeneration research field indicating the potential of the mammalian retina to possibly possess a glial population for regeneration as shown in other vertebrates. Finally, it has raised a number of new and interesting questions, which will form the basis of further work in this fast-evolving field. It is hoped that this work on Chx10 will contribute to the current knowledge of eye development and retinal stem cell biology.

## **Appendix**

## I. PCR normalisation



Quantitative *Re-Ti* PCR GAPDH normalisation using different amounts of WT NR cDNA

*Quantitative Re-Ti PCR Gap43 normalisation using different amounts of WT NR  
cDNA*

*Quantitative Re-Ti PCR Recoverin normalisation using different amounts of WT  
NR cDNA*

*Quantitative Re-Ti PCR Sox2 normalisation using different amounts of WT NR  
cDNA*



## II. *Nrl.gfp*<sup>+/+</sup> and *Nrl.gfp*<sup>+/-</sup>/*Chx10*<sup>orJ/orJ</sup> genotyping PCR data

Plate	Sample	Detector	Task	Avg Ct	Avg dCt	dCt std err	ddCt	RQ
exp093_G APDH_GF P_01	2	GAPDH	ENDO	18.363				
exp093_G APDH_GF P_01	3	GAPDH	ENDO	18.908				
exp093_G APDH_GF P_01	4	GAPDH	ENDO	21.631				
exp093_G APDH_GF P_01	5	GAPDH	ENDO	19.47				
exp093_G APDH_GF P_01	6	GAPDH	ENDO	19.093				
exp093_G APDH_GF P_01	7	GAPDH	ENDO	18.14				
exp093_G APDH_GF P_01	8	GAPDH	ENDO	19.612				
exp093_G APDH_GF P_01	9	GAPDH	ENDO	19.44				
exp093_G APDH_GF P_01	10	GAPDH	ENDO	18.584				
exp093_G APDH_GF P_01	11	GAPDH	ENDO	18.976				
exp093_G APDH_GF P_01	12	GAPDH	ENDO	20.829				
exp093_G APDH_GF P_01	13	GAPDH	ENDO	19.451				
exp093_G APDH_GF P_01	14	GAPDH	ENDO	20.862				
exp093_G APDH_GF P_01	15	GAPDH	ENDO	19.276				
exp093_G APDH_GF P_01	16	GAPDH	ENDO	17.962				
exp093_G APDH_GF P_01	17	GAPDH	ENDO	18.84				
exp093_G APDH_GF P_01	18	GAPDH	ENDO	18.063				
exp093_G APDH_GF P_01	19	GAPDH	ENDO	18.574				
exp093_G	20	GAPDH	ENDO	18.551				

Appendix

APDH_GF P_01								
exp093_G APDH_GF P_01	21	GAPDH	ENDO	17.856				
exp093_G APDH_GF P_01	22	GAPDH	ENDO	21.692				
exp093_G APDH_GF P_01	2	GFP	Target	26.216	7.853		-1.178	2.262
exp093_G APDH_GF P_01	3	GFP	Target	26.383	7.475		-1.556	2.94
exp093_G APDH_GF P_01	4	GFP	Target	28.589	6.957		-2.073	4.208
exp093_G APDH_GF P_01	5	GFP	Target	27.387	7.917		-1.114	2.164
exp093_G APDH_GF P_01	6	GFP	Target	24.787	5.694		-3.336	10.101
exp093_G APDH_GF P_01	7	GFP	Target	26.751	8.611		-0.419	1.337
exp093_G APDH_GF P_01	8	GFP	Target	27.491	7.879		-1.151	2.221
exp093_G APDH_GF P_01	9	GFP	Target	27.543	8.103		-0.927	1.901
exp093_G APDH_GF P_01	10	GFP	Target	25.15	6.566		-2.465	5.521
exp093_G APDH_GF P_01	11	GFP	Target	27.49	8.513		-0.517	1.431
exp093_G APDH_GF P_01	12	GFP	Target	27.052	6.223		-2.807	7
exp093_G APDH_GF P_01	13	GFP	Target	27.324	7.872		-1.158	2.232
exp093_G APDH_GF P_01	14	GFP	Target	27.696	6.834		-2.196	4.583
exp093_G APDH_GF P_01	15	GFP	Target	26.454	7.178		-1.853	3.612
exp093_G APDH_GF P_01	16	GFP	Target	25.795	7.833		-1.197	2.293
exp093_G APDH_GF P_01	17	GFP	Target	27.284	8.444		-0.587	1.502
exp093_G APDH_GF P_01	18	GFP	Target	25.336	7.273		-1.757	3.38
exp093_G APDH_GF P_01	19	GFP	Target	26.152	7.578		-1.452	2.737
exp093_G APDH_GF P_01	20	GFP	Target	26.009	7.459		-1.572	2.973

Appendix

exp093_G APDH_GF P_01	21	GFP	Target	26.887	9.03		0	1
exp093_G APDH_GF P_01	22	GFP	Target	24.356	2.663		-6.367	82.547

*Quantitative Re-Ti PCR data for Nrl.GFP genotyping using gDNA (I)*

Well	SampleName	Start	End	Ct
1	1A	5	18	20.6372
2	1A	5	17	20.2404
3	2A	5	13	15.8944
4	2A	5	12	15.3122
5	1B	5	20	23.0131
6	1B	5	19	22.264
7	2B	5	12	15.3945
8	2B	5	11	13.9979
9	3B	5	11	14.2509
10	3B	5	12	15.2496
11	4B	5	13	15.8596
12	4B	5	14	16.9169
13	5B	5	20	22.9537
14	5B	5	20	22.78
15	6B	5	13	16.0959
16	6B	5	13	15.9413
17	1C	5	14	17
18	1C	5	13	16.1442
19	2C	5	13	16.3044
20	2C	5	13	16.2289
21	3C	5	13	16.0206
22	3C	5	13	15.8126
23	1D	5	39	
24	1D	5	12	14.9186
25	2D	5	13	15.8032
26	2D	5	13	15.7563
27	3D	5	14	16.643
28	3D	5	14	17.1966
29	NEG	5	35	38.8374
Plate Name: Exp131GenotypingGFP_2ND				
Detector	Reporter	Start	End	Threshold

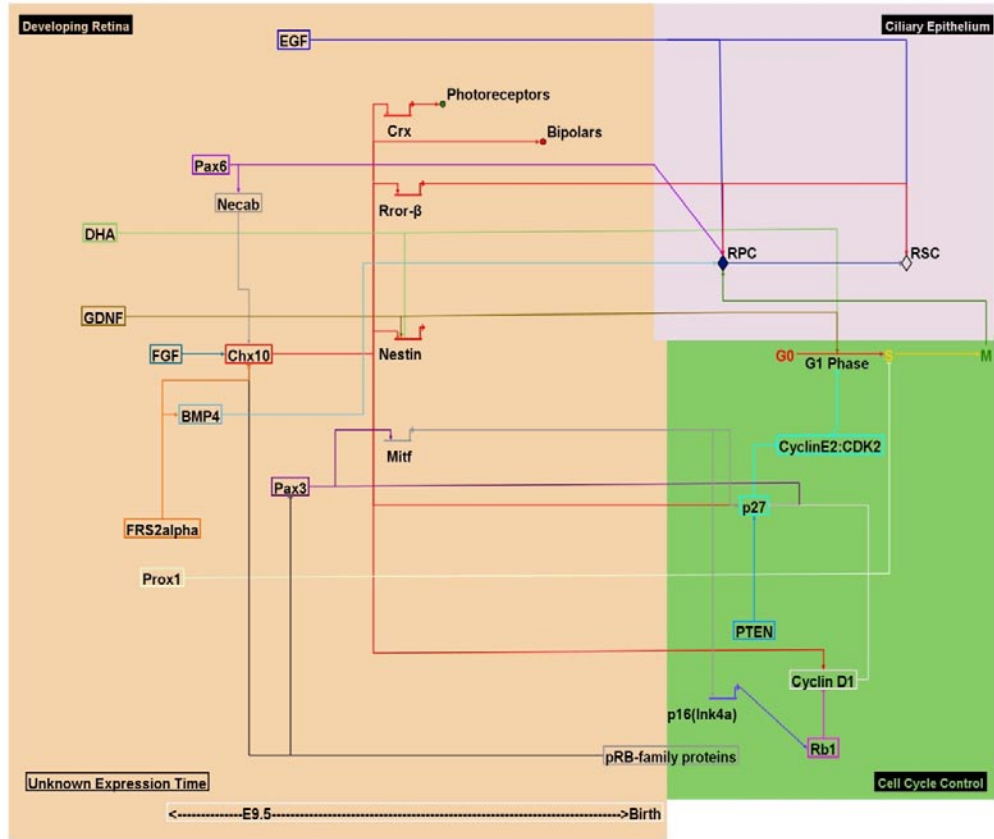
Appendix

GFP (SYBR)	SYBR	(Auto)	(Auto)	Auto:0.050111
Well	SampleName	Start	End	Ct
37	1A	5	23	26.2971
38	1A	5	23	26.3495
39	2A	5	19	21.8483
40	2A	5	19	21.7772
41	1B	5	27	29.6045
42	1B	5	26	29.4439
43	2B	5	19	21.8318
44	2B	5	19	22
45	3B	5	20	22.5619
46	3B	5	20	22.6654
47	4B	5	20	23.3816
48	4B	5	20	23.2972
49	5B	5	39	
50	5B	5	39	
51	6B	5	20	23.2325
52	6B	5	20	23.2672
53	1C	5	21	23.538
54	1C	5	21	23.6061
55	2C	5	21	23.9272
56	2C	5	21	23.9027
57	3C	5	20	23.3585
58	3C	5	21	23.6543
59	1D	5	25	27.7606
60	1D	5	20	22.8588
61	2D	5	31	34.1242
62	2D	5	31	34.1551
63	3D	5	21	23.7823
64	3D	5	20	23.3156
65	NEG	5	39	

*Quantitative Re-Ti PCR data for Nrl.GFP genotyping using gDNA (II)*

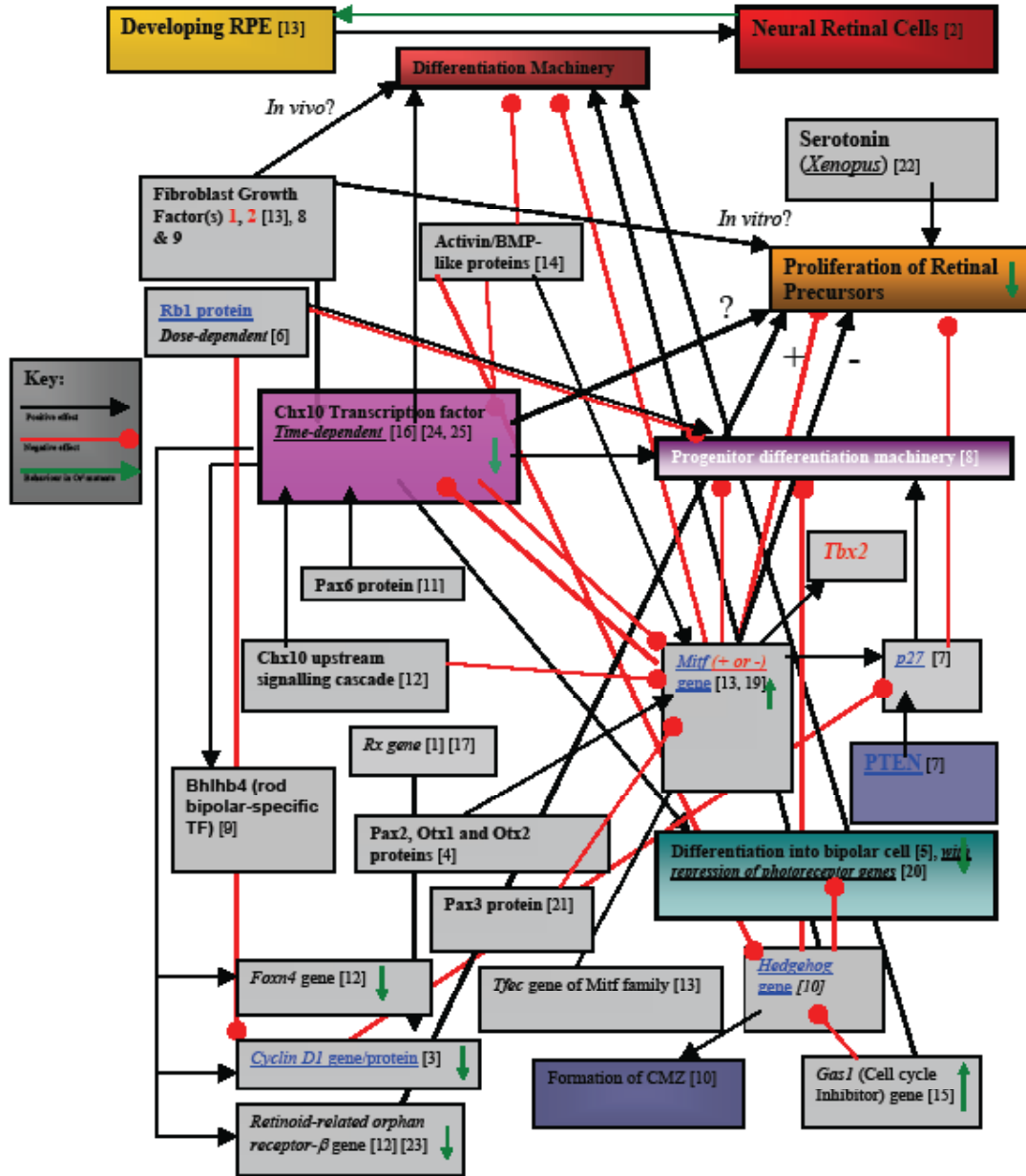


## IV. Chx10 signalling



*Potential actions of Chx10 TF*

## V. Chx10 biochemical pathway

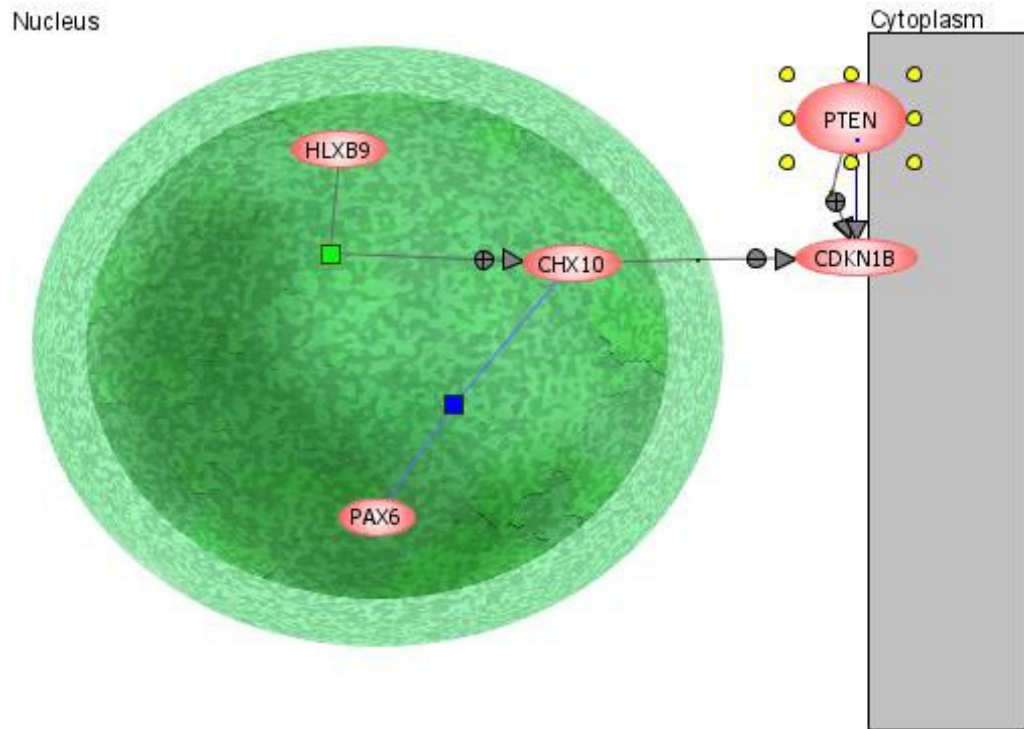


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*Potential collaboration action between Chx10 and PTEN on the cell cycle via CDK1B (p27<sup>Kip1</sup>)*

## **VI. Publication**

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