

**Influence of Oxygen tension on the Differentiation of
Embryonic Stem Cells Towards Photoreceptors and other
Retinal Phenotypes**

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“Learn from yesterday, live for today, hope for tomorrow. The
important thing is to not stop questioning.”
— **Albert Einstein**, *Relativity: The Special and the General Theory*

To my parents

To Fer

I, Marcela Garita Hernandez, 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.

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Abbreviations

Abbreviation	Meaning
ad	Autosomal dominant
AMD	Age-related macular degeneration
ar	Autosomal recessive
bp	Base pair
BSA	Bovine sérum albumin
cDNA	Complementary DNA
CNS	Central nervous system
DNA	Deoxyribonucleic acid
GCL	Gangion cell layer
INL	Inner nuclear layer
IPL	Inner plexiform layer
IS	Inner segment
mRNA	Messenger RNA
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segment
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PR	Photoreceptor
r.t.	Room temperature
RD	Retinal dystrophy
RNA	Ribonuclei acid
RPE	Retinal pigment epithelium
RT-PCR	Quantitative PCR
SS	Subretinal space
wt	Wild type

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Abstract

Retinitis pigmentosa (RP), a genetically heterogeneous group of diseases together with age-related macular degeneration (AMD), are the leading causes of permanent blindness and are characterized by the progressive dysfunction and death of the light sensing photoreceptors of the retina. Due to the limited regeneration capacity of the mammalian retina the scientific community has invested significantly in trying to obtain retinal progenitor cells from embryonic stem cells (ESC). These represent an unlimited source of retinal cells, but it has not yet been possible to achieve specific populations, such as photoreceptors, efficiently enough to allow them to be used safely in the future as cell therapy of RP or AMD. In this study we generated a high yield of photoreceptors from directed differentiation of mouse ESC (mESC) by recapitulating crucial phases of retinal development. We present a new protocol of differentiation, involving hypoxia and taking into account extrinsic and intrinsic cues. These include niche-specific conditions as well as the manipulation of the signaling pathways involved in retinal development. Our results show that hypoxia promotes and improves the differentiation of mESC towards photoreceptors. Different populations of retinal cells are increased in number under the hypoxic conditions applied, such as Crx positive cells, S-Opsin positive cells and double positive cells for Rhodopsin and Recoverin, as shown by immunofluorescence analysis. For the first time this manuscript reports the high efficiency of differentiation *in vivo* and the expression of mature rod photoreceptor markers in a large number of differentiated cells, transplanted in the sub-retinal space of wild type mice.

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1. INTRODUCTION

“The beginning is the most important part of the work.”

— **Plato**, *The Republic*

Retinal degenerative diseases such as RP and AMD are the leading causes of incurable blindness. They affect more than 2 million individuals of the worldwide population (Berger et al., 2010b). Photoreceptor loss commonly occurs due to dysfunction of the supportive layer of RPE. In many cases of retinal degeneration, the inner retina remains intact allowing for a window of opportunity for photoreceptors or RPE replacement treatments. Although it has been reported the possibility of using gene therapy approaches to treat patients with inherited RDs, before their vision is completely compromised, this will not restore vision into those whose photoreceptors or RPE have already degenerated (Bainbridge, 2009; Cai et al., 2009; Caplen, 2000; Dejneka et al., 2003; Dinculescu et al., 2005; Hauswirth et al., 2000; Hosch et al.; Kohno et al., 2005; McClements and Maclaren; Prentice et al.; Stout and Francis; Townes-Anderson; Zaneveld et al.). Stem cell therapy constitutes an alternative for such patients where existing pharmacological and surgical therapies are inadequate. Furthermore, embryonic stem cell biology holds the unique potential to provide comprehensive model systems to investigate the earliest stages of cellular ontogenesis. Nevertheless, the established methods to obtain early retinal cells typically present a low efficiency and result in a heterogeneous population. Therefore, we developed a protocol of differentiation yielding to a highly enriched population of retinal cells, which also mimics the stepwise fashion by which the retina is formed during normal development.

1.1 Early eye development

In mammals, eye development initiates at the moment of gastrulation, when the eye primordium is organized as a single eye field in the center of the proencephalon in

the anterior neural plate. During the formation of the midline this eye field, separates giving rise to two optic areas (Figure 1.1A.). In the neurulation stage (E 8.5 in the mouse), these two optic pits are formed as a consequence of the bilateral evagination of the neural tube corresponding to the first morphological sign of eye formation. Only 12 hours after, these two optic pits elongate to form the optic vesicles (Figure 1.1B.). The evagination will continue until the optic vesicles come in close contact with the surface ectoderm, driven by the surrounding mesenchyme from both sides. Thanks to the interaction with the OV, the surface ectoderm thickens and becomes the lens placode (Figure 1.1C. and D.), which progressively invaginates into the optic vesicle giving rise to the lens vesicle and forming the optic cup (Figure 1.1E. and F.). In the mouse this process begins at E10 and as a result two epithelial layers are formed, the inner most layer of the optic cup gives rise to the retina whereas its outer most layer will form the RPE (Figure 1.2; (Adler and Canto-Soler, 2007).

During this phase the immature RPE encapsulates the inner layer, narrowing the ventral part of the OV and forming the choroid fissure. The complete closure of the choroid fissure allows the formation of the optic nerve, through which ganglion cell axons transmit information to the brain. The ciliary body and the iris derive from the anterior margin of the optic cup, in the junction where NR and RPE meet. The remaining structures in particular the muscles are formed from cells in the surrounding mesenchyme, the same type of cells found in the neural crest. The surface ectoderm, henceforth in contact with the lens vesicle will form the cornea (Figure 1.2). In summary the major ocular structures derive from three major sources: neural ectoderm forms the retina, surface ectoderm gives rise to the lens and part of the cornea, and neural crest cells form the central part of the cornea

(Graw, 2010).

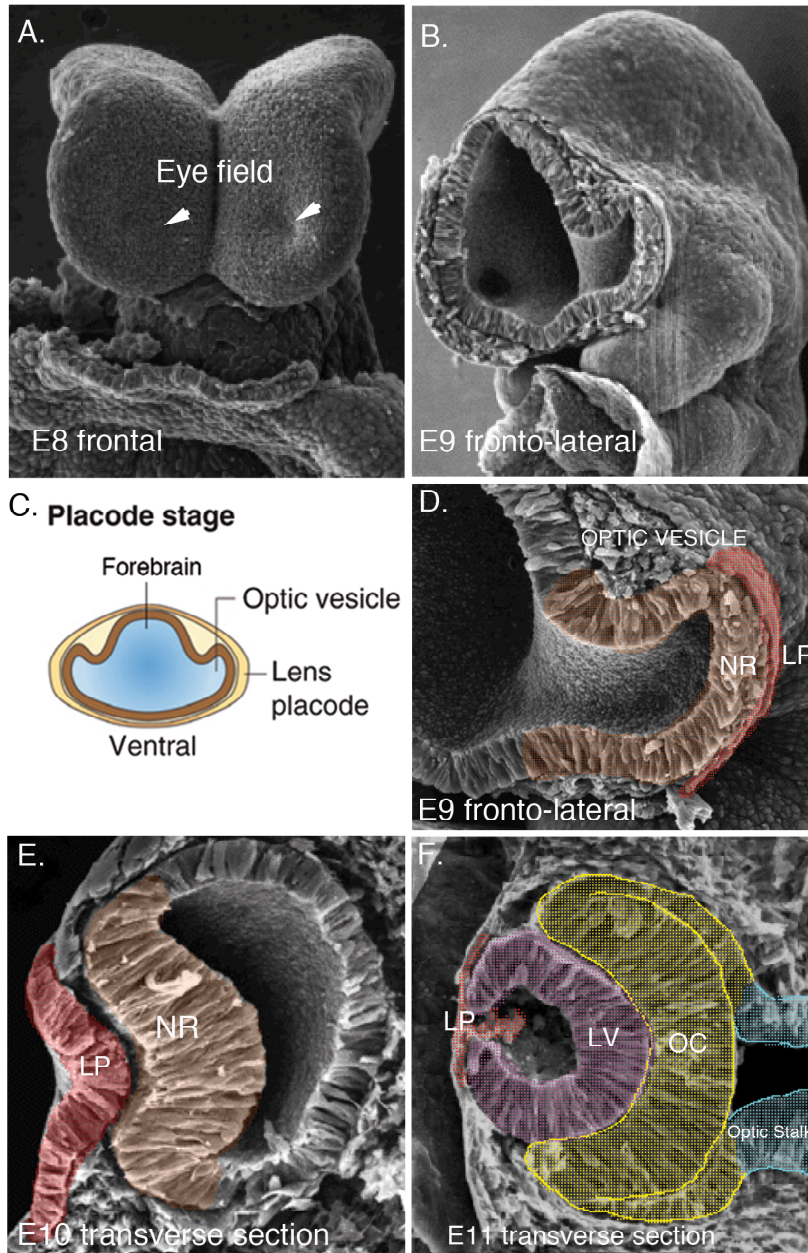


Figure 1.1 Scanning electron microscopy images of mouse embryos describing structural changes in early eye development. A. The optic grooves (arrows) are the first morphological signs of the eye primordium in the eye field region. **B.** Development expands the eye primordium to form the optic vesicles, where the presumptive neural retina (NR) comes in contact with the lens placode (LP, **C.** and **D.**) **E and F.** Contact with the LP promotes the invagination on the optic vesicle (OV) to form the lens vesicle and the Optic cup (OC). Electron micrographs reproduced and modified from http://syllabus.med.unc.edu/courseware/embryo_images/unit-eye/eyetoc.htm. Diagram in C. taken from

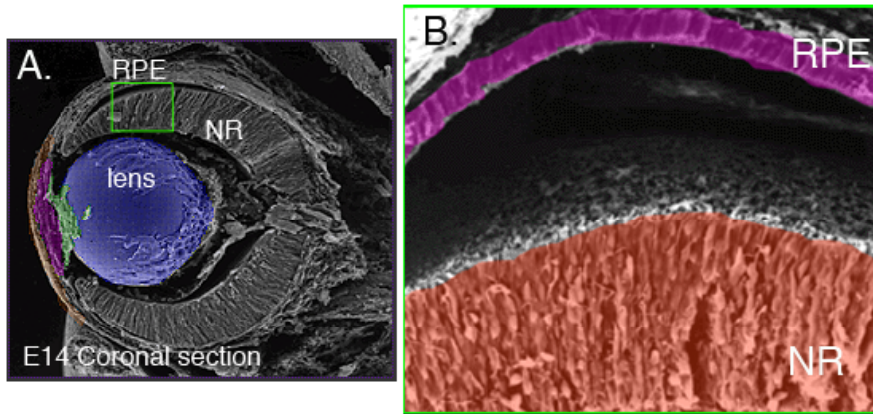


Figure 1.2 Scanning electron microscopy images of mouse embryos describing structural changes in early eye development. A. At E14 the anterior chamber of the eye (purple) forms as a space developed between the lens and its closely associated iridopupillary membrane (green) and the cornea (orange). B. Amplification of the section marked in A. with a green square that allows observing the very distinct structure of RPE and NR. Electron micrographs reproduced and modified from http://syllabus.med.unc.edu/courseware/embryo_images/unit-eye/eyetoc.htm.

1.1.1 Inductive interactions

The development of the eye field and the optic vesicles depends of the combined action of exogenous factors and transcription factors. These inductive signals actually start well before the first morphological indications of OV development (Kessler and Melton, 1994). Nearly half a century ago, it was shown that neural tissue from the salamander, isolated prior to OV formation, could initiate eye development in vitro (Lopashov and Stroeva, 1964). Hence the specification of the NR and RPE within the OV appears to be determined by inductive signals originating from the surface ectoderm and in the surrounding mesenchyme (Fuhrmann et al., 2000).

Nowadays it is well accepted that the cellular differentiation is a result of the

reciprocal action of extrinsic and intrinsic factors, which act in a spatiotemporal fashion that is tightly controlled by the genome (Cepko et al., 1996; Edlund and Jessell, 1999; Harris, 1997)

1.1.2 Exogenous factors

The main secreted factors involved in the regulation of eye development include only a few gene families, including Hedgehog molecules (Sonic, Indian and Desert), the molecules of Wnt family, morphogens molecules such as BMP and different growth factors (TGFB, FGF, EGF, etc). Many of these factors are present at different stages of eye development orchestrating different developmental events. Table 1 summarizes the role of some of these extracellular factors. Some factors will be described later in another chapter of the present thesis. FGF and the non canonical pathway of Wnt have long been considered to regulate the morphogenetic movements that allow the eye field specification in *Xenopus* (Lee et al., 2006; Moody, 2004; Moore et al., 2004). In vertebrates, Wnt introduce an element of complexity, while the activation of its non canonical pathway is required for the eye primordium formation, its canonical pathway has opposite effects and should be antagonized by Dickkopf (Dkk1) or secreted Frizzled proteins for eye field specification (Cavodeassi et al., 2005)

Eye field specification

The different gradients of factors ensure the eye field specification. The dorso-ventral specification of the eye field involves signals coming from FGF and Wnt which ventralize the field whereas BMP molecules exert a dorsalising effect (Lee et al., 2006; Moody, 2004; Moore et al., 2004). Molecules such as Noggin, Chordin and Follistatin play a role in the formation of the antero-posterior axis. The non-canonical Wnt pathway (Wnt11, Wnt 5) activates the eye field specification by

promoting the proliferation of progenitor cells within the eye field (Cavodeassi et al., 2005). Instead the canonical/ β -catenin Wnt pathway (Wnt1, Wnt10b and Wnt8b) must be downregulated or inhibited (Dickkopf or secreted Frizzled related proteins) in order to observe differentiation towards eye field from the diencephalon (Esteve and Bovolenta, 2006).

Optic vesicle/optic cup

The eye field separation relies on Nodal signaling as it was discovered in zebrafish mutants for cyclops (*cyc*), a secreted Nodal-related molecule, when all display cyclopia (Muller et al., 2000; Varga et al., 1999). Nodal effect is possibly acting through the induction of SHH expression (Chow and Lang, 2001; Marti and Bovolenta, 2002) since it was found in zebrafish and chick that the morphogen Shh is downstream to Nodal signaling, as it was absent in the early ectoderm of *cyc* mutants. It was also found that Shh promoter was activated in response to *cyc* signals and Shh enhancer elements were identified as *cyc* targets (Muller et al., 2000). Shh is also required for patterning along eye development. During early stages Shh controls the ventral fates of the neural tube, as a consequence its overexpression expands the forebrain and the optic stalk at the cost of neural retina. Later in development, Shh controls the dorso-ventral patterning of the optic vesicle and the optic cup instead (Peters, 2002; Zhang and Yang, 2001b).

As already mentioned the patterning of OV into NR and RPE depends on the inductive signals from the surface ectoderm and mesenchyme, making obvious the very tight relationship between tissue inductive signals and optic cup patterning. FGF family members are expressed in the surface ectoderm and induced the neural retina formation (Bharti et al., 2006; Chow and Lang, 2001; Martinez-Morales et al., 2004). In Addition, upon contact with the surface ectoderm the prospective neural retina itself expresses FGF8 and FGF9 both of which play a

role defining the boundary between NR and RPE (Adler and Canto-Soler, 2007; Crossley et al., 2001). Extraocular mesenchyme promotes RPE differentiation, on the other hand, possibly through an activin-like signal (Fuhrmann et al., 2000). BMP7 expression in the prospective RPE promotes the identity of this tissue by antagonizing effect of FGF (Adler and Canto-Soler, 2007) BMPs and RA also play an important role in the transformation of optic vesicle to the optic cup (Cvekl and Wang, 2009). Experiments with mouse embryos lacking RA synthesis in the optic vesicle showed a failure in optic vesicle invagination, (Duester, 2009; Mic et al., 2004; Molotkov et al., 2006)

Table 1. Extracellular molecules involved in eye development through early optic cup stages. Modified from Adler and Canto-Soler, 2007

Events during eye development	Extracellular molecules	References
Specification of the eye field	FGFs; Wnts; BMPs	(Cavodeassi et al., 2005; Esteve and Bovolenta, 2006; Lee et al., 2006; Moody, 2004; Moore et al., 2004; Wilson and Houart, 2004)
Splitting of the eye field and proximo-distal patterning of the OV	Cyclops; SHH	(Chow and Lang, 2001; Ekker et al., 1995; Li et al., 1997; Macdonald et al., 1995; Marti and Bovolenta, 2002; Muller et al., 2000)
Patterning of the OV	SHH; FGFs; Activin; BMP7; RA	(Martinez-Morales et al., 2004)
Invagination of the optic vesicle into an optic cup	RA	(Hyer et al., 2003; Matt et al., 2005; Mic et al., 2004; Molotkov et al., 2006)
Antero-posterior and Dorso-Ventral patterning of the optic cup	FGFs; SHH; BMPs; RA; Ventroptin; Follistatin, Chordin, Noggin; DAN	(Adler and Belecky-Adams, 2002; Belecky-Adams and Adler, 2001; Huillard et al., 2005; Koshiba-Takeuchi et al., 2000; Morcillo et al., 2006; Peters and Cepko, 2002; Sakuta et al., 2001; Sakuta et al., 2006; Yang, 2004)

BMPs: bone morphogenetic proteins; DAN: DAN domain family members; FGFs: fibroblast growth factors; RA: retinoic acid; SHH: sonic hedgehog; Wnts: members

of the Wnt family.

1.1.3 Transcription factors

Several transcription factors have been associated to each major stage of retinogenesis. The effect exerted by different transcription factor sets on the morphogenetic events necessary for the normal eye formation, are probably caused by the influence of extracellular molecules, as the ones describe in the previous section. Table 2 summarizes the transcription factors frequently involved in early eye development.

1.1.3.1 Eye field specification

Transcription factors involved in the early events of eye formation have long been known as eye field transcription factors (EFTF). On this regard, Otx2, Rax, Pax6, Six3, Lhx12, Six6, ET and tll appear essential for the specification of the eye field (Chow and Lang, 2001; Crossley et al., 2001; Zuber et al., 2003). Different publications dealing with knockout mice and misexpression experiments have postulated the regulatory network formed by these genes, where they regulate each other's expression (Chow and Lang, 2001; Crossley et al., 2001; Swaroop et al., 2010; Zuber et al., 2003). Interestingly, genes regulating forebrain development, such as Otx2 and Hes1, which are not present in the eye field, indirectly regulate early eye development (Bailey et al., 2004).

1.1.3.2 Optic vesicle evagination

Optic vesicle formation or optic vesicle evagination findings come from different knockout experiments. Rax mouse mutants fail to form optic vesicles and Pax6

mouse mutants presented abnormal formation through mechanisms still unknown, (Bailey et al., 2004; Chow and Lang, 2001) but it has been hypothesised from experiments with zebrafish that the effect is provoked by an impaired cell migration from the eye field (Loosli et al., 2003; Rembold et al., 2006)

1.1.3.3 Optic vesicle patterning

All the cells forming the adult retina derive from a common multipotent progenitor and the optic vesicle is not an exception in this matter. All the neuroepithelial cells of the early optic vesicle co-express Rax, Pax6, Hes1, Otx2, Lhx2, Six3, and Six9 while they are still capable of differentiating towards optic stalk, neural retina and RPE (Martinez-Morales et al., 2004). The patterning of the optic vesicle into NR or RPE progenitors depends of the differential expression of the above mentioned transcription factors as well as Chx10 and Mitf. The regulatory interaction between Chx10 and Mitf expression is mandatory for the establishment of boundaries between the developing territories. Cells expressing only Mitf and Pax6 will give rise to RPE while cells expressing Chx10 and Pax6 will give rise to the neural retina (Bharti et al., 2008; Horsford et al., 2005; Hyer et al., 2003; Rowan et al., 2004).

1.1.3.4 Optic vesicle invagination into optic cup

One of the most amazing morphogenetic events during eye formation is the invagination of the optic vesicle to give rise to the bilaminated optic cup. As it has been discussed in the previous section, this event is promoted by signalling molecules from the surface ectoderm, mainly by bFGF signalling pathway. In terms of transcription factors involved, Pax6, Hes1 and Lhx2 expression mediate the outgrowth of the optic vesicle and its conversion into an optic cup (Adler and Canto-Soler, 2007; Fuhrmann, 2010). The optic cup inner layer will form the mature retina while the outer layer will give rise to the RPE.

1.1.3.5 Neural retina patterning

Table 2. Transcription factors involved in eye development through early optic cup stages. Modified from Adler and Canto-Soler, 2007

Events during eye development	Transcription factors	References
Specification of the eye field	Rx; Pax6; Six3; Lhx2; Six6/Optx2; ET; tll; Hes1; Otx2	(Bailey et al., 2004; Chow and Lang, 2001; Esteve and Bovolenta, 2006; Viczian et al., 2006; Wilson and Houart, 2004; Zuber et al., 2003)
Optic vesicle evagination	Rx; Pax6; tll	(Bailey et al., 2004; Chow and Lang, 2001; Hollemann et al., 1998; Loosli et al., 2003; Loosli et al., 2001; Rembold et al., 2006)
Optic vesicle dorso-ventral patterning	Pax6; Rx; Lhx2; Chx10; Otx2; Mitf; Pax2; Vax	(Adler and Canto-Soler, 2007; Bharti et al., 2006; Chow and Lang, 2001; Horsford et al., 2005; Martinez-Morales et al., 2004; Schwarz et al., 2000)
Optic vesicle naso-temporal patterning	BF1/Foxg1; BF2/Foxd2; Pax6	(Baumer et al., 2002; Hatini et al., 1994; Yuasa et al., 1996)
Optic vesicle invagination into an optic cup	Pax6; Lhx2; Hes1	(Adler and Canto-Soler, 2007; Chow and Lang, 2001; Lee et al., 2006; Porter et al., 1997; Tomita et al., 1996)
Neural retina dorso-ventral patterning	Pax6, Pax2, Vax, Tbx5; Xbr1	(Adler and Canto-Soler, 2007; Chow and Lang, 2001; Leconte et al., 2004; Mui et al., 2005; Peters, 2002; Peters and Cepko, 2002)
Neural retina naso-temporal patterning	Pax6; BF1/Foxg1; BF2/Foxd2; SOH01; GH6	(Baumer et al., 2002; Chow and Lang, 2001; Peters, 2002; Takahashi et al., 2003; Yuasa et al., 1996)

1.2 Retinogenesis

The retina is a highly laminated structure comprised by seven different cell types distributed in three nuclear strata and two synaptic layers. (Figure1.3).

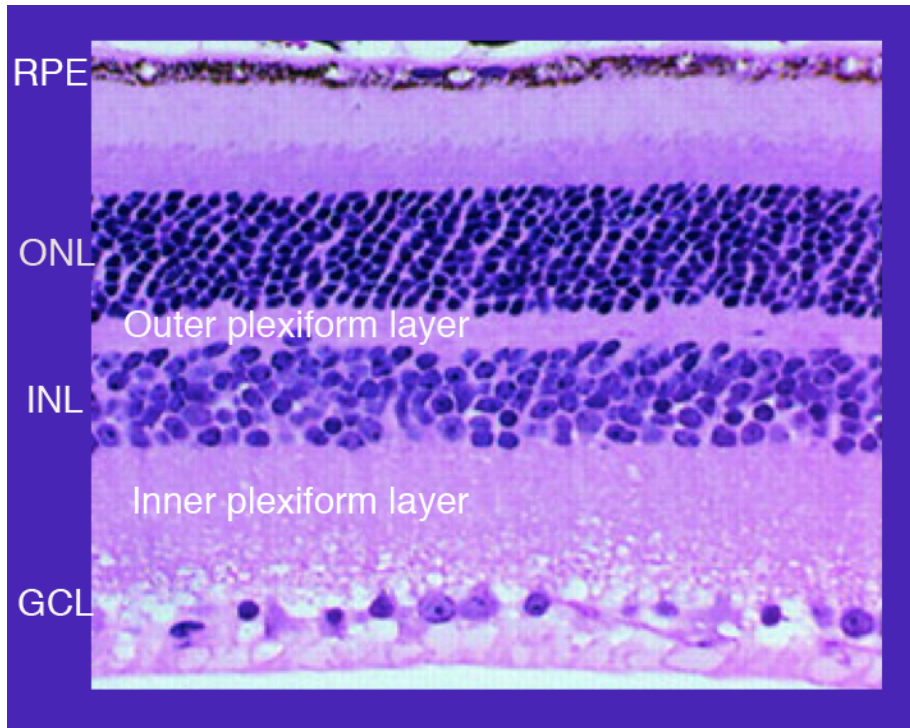


Figure 1.3 Histological section of an adult wild type mouse retina. The retina is organised in three nuclear ONL, INL and GCL and two synaptic strata, the outer plexiform layer and the inner plexiform layer.

As it was mentioned its development is a complex multistep process starting at the inner most layer of the optic cup. And this is the case for most of the vertebrates, nevertheless there are some differences between mice and human that can be summarize as follows.

In mice, cell proliferation commences around embryonic day (E10) and continues into the post-natal day (P14) week of life, when the principal retinal structure is completed (Sidman 1961; (Young, 1985a, b), nevertheless further condensation of photoreceptor chromatin and changes in cone opsin expression patterns carry on into adulthood (Solovei et al., 2009; Szel et al., 1994). The retinogenesis takes an extremely long period of time considering the whole gestation of small rodents such as mice lasts approximately 20 days.

On the other hand, human retinal development is completed before birth and there is none or little evidence of neural regeneration in the post-mitotic mammal retina (Lamba et al., 2009b).

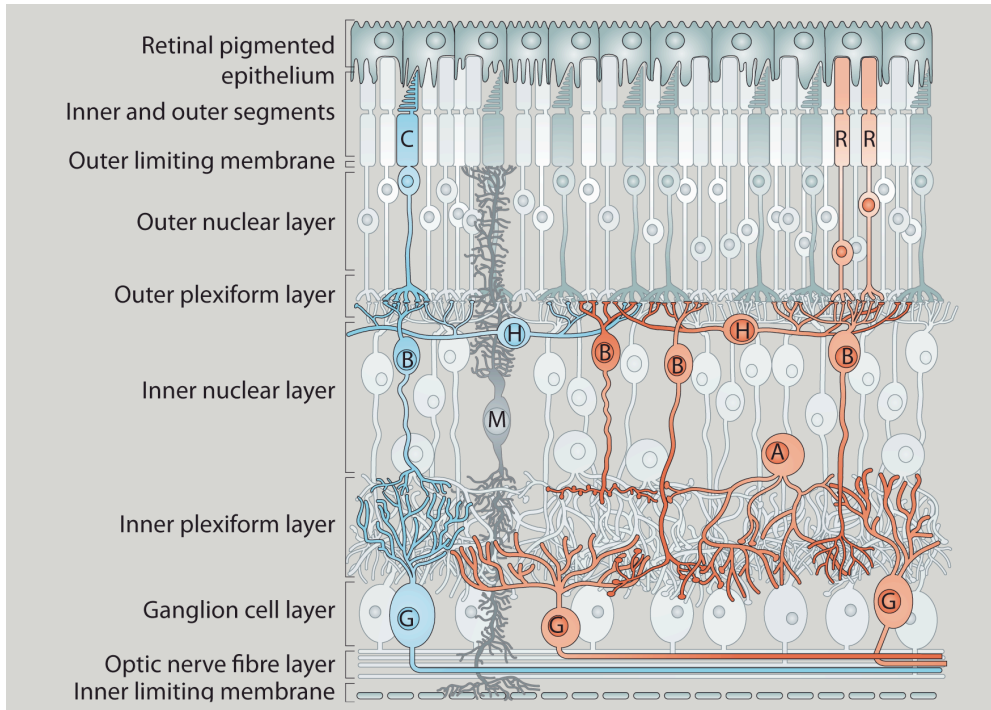


Figure 1.4 Structural and Functional circuitry of the retina. Organization of retinal circuits. Rod (R) and cone (C) photoreceptors cell bodies are located in the outer nuclear layer (ONL) and extend inner and outer segments (IS and OS), towards the RPE. Photoreceptor axons terminate in the outer plexiform layer (OPL) where they synapse onto horizontal (H) and bipolar (B) cells in the inner nuclear layer (INL), which also contains Müller glial (M) and amacrine (A) cells. Bipolar cells connect to amacrine and ganglion (G) cells in the inner plexiform layer (IPL). Ganglion cell axons form the optic nerve and carry signals to the brain. Rod (orange) and Cone (blue) pathways are represented. Modified from (Swaroop et al., 2010)

On this regard, long-term DNA labelling studies have shown that the different retinal cells are born in a stepwise but overlapping sequence which is conserved across many vertebrates (Prada et al., 1991; Rapaport et al., 2004).

Hence, Retinal ganglion cells (G), cone photoreceptors (C), as well as horizontal (H) and amacrine (A) cells are born embryonically, while bipolar cells (B), rod photoreceptors (R) and Müller cells (M) develop postnatally. When lineage tracing experiments were performed in retinal progenitor cells (RPC) of rat (Turner and

Cepko, 1987), mouse (Turner et al., 1990) and frog developing retinae, (Holt et al., 1988; Wetts and Fraser, 1988) absence of lineage-restricted progenitors was observed and it was concluded that all cell types derived from multipotent RPCs influenced by external cues (Cepko et al., 1996). These cells exit the mitotic cycle in a characteristic, stereotyped sequence that is highly conserved across vertebrates (Holt et al., 1988; Spence and Robson, 1989; Young, 1985a).

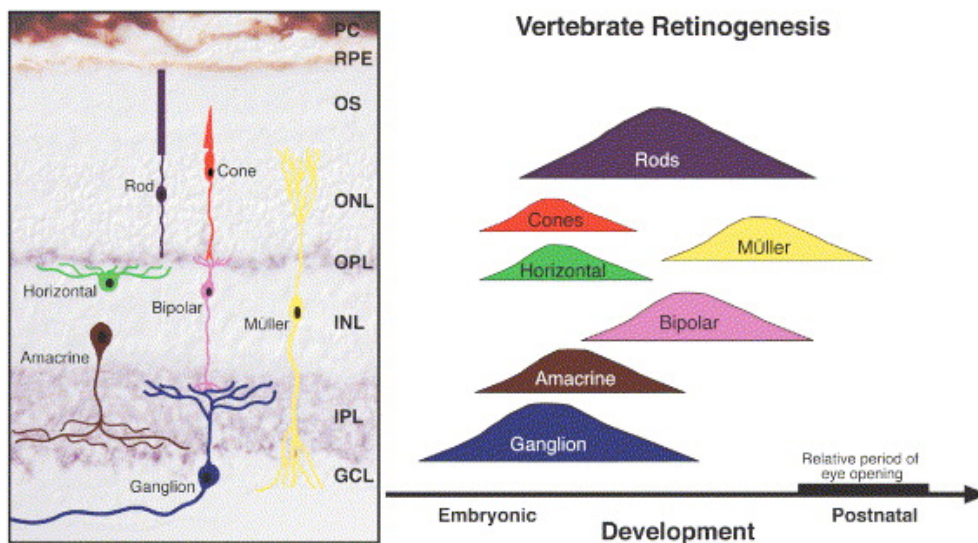


Figure 1.5. Order of birth of retinal cells in the mouse retina during normal development. Taken from (Klassen et al., 2004)

However, from co-cultures experiments it was established that RPCs must undergo a sequential process of intrinsic molecular changes throughout retinogenesis to give rise to all cell types (Harris, 1997; Livesey and Cepko, 2001). Yet, retinogenesis occurs thanks to an orchestrated balance between intrinsic signals and instructive extracellular cues, exemplified by some diffusible molecules modulating cell fate. On this regard, using P1 conditioned medium when culturing RPCs, Shh role in regulating ganglion cell genesis was discovered (Zhang and Yang, 2001a) and different publications determined the involvement of taurine, retinoic acid (RA) and

thyroid hormone (TH) on photoreceptor fate decision (Altshuler et al., 1993; Hyatt and Dowling, 1997; Kelley et al., 1995; Young and Cepko, 2004).

Interestingly, some factors may have different effects in different species; such is the case of ciliary neurotrophic factor (CNTF), which in chick promotes the differentiation of rod photoreceptors over bipolar cells (Fuhrmann et al., 1995), while in rat the opposite effect is observed (Ezzeddine et al., 1997). Fibroblast growth factor (FGF) and epidermal growth factor (EGF) signalling postpone a late rod photoreceptor towards the final Müller glia fate (Ahmad et al., 1998; Lillien and Cepko, 1992; McFarlane et al., 1998).

Nowadays, it is recognized the critical role played by Notch signaling pathway in gliogenesis and neurogenesis of RPCs. On this aspect, Notch activation through its ligand Delta in the retina, favors the differentiation towards Müller glia on expense of neurons, while a reduced activation of Notch signaling promotes the differentiation towards ganglion cells, reducing the number of glial cells. The mechanism by which Delta-Notch signaling seems to exert this effect is by the transactivation of specific downstream targets such as Hes1 and Hes5, which in turn induce the downregulation of proneural basic helix-loop-helix (bHLH) transcription factors (Dorsky et al., 1997; Rapaport and Dorsky, 1998). bHLH genes are part of well studied family of transcription factors whose many members are necessary for neurogenesis. Manipulations of individual genes shift the balance between neuronal cell fates, but also alter the timing of cell cycle exit of RPCs (Agathocleous and Harris, 2009). One Example of bHLH proneural is Ath5, a gene important for the final differentiation of RPCs to retinal ganglion cells. The path of differentiation toward horizontal cells goes under the influence of another transcription factor, Prox1, which induces cell cycle exit and horizontal cell differentiation and it can be

found as well in some amacrine cells (Dyer et al., 2003).

Table 3. Signalling pathways involved during retinogenesis

Signalling	Events during retinogenesis	References
FGF; EGF	Promotes neurogenesis and proliferation and is involved in gliogenesis	(Ahmad et al., 1998; Esteve and Bovolenta, 2006; Lillien and Cepko, 1992; McFarlane et al., 1998)
Notch	Inhibits neuronal differentiation, promotes RPC maintenance or gliogenesis	(Perron and Harris, 2000)
Hedgehog	Activates proliferation	(Agathocleous et al., 2007; Wallace, 2008)
BMP	Promotes proliferation and neurogenesis	(Murali et al., 2005)
CNTF	Promotes gliogenesis	(Ezzeddine et al., 1997; Goureau et al., 2004)
RA; Taurine; TH	Photoreceptor fate decision	(Altshuler et al., 1993; Hyatt and Dowling, 1997; Kelley et al., 1999; Young and Cepko, 2004)

Mature retinal phenotype acquisition requires the action of homeobox genes. In mice, cone-rod homeobox (Crx) and its homologue Otx2 are necessary for photoreceptor differentiation (Furukawa et al., 1997; Nishida et al., 2003), while bipolar cells rely on the expression of Chx10 (Burmeister et al., 1996).

As it has been mentioned earlier Pax6 is essential for eye field specification. Pax6 is a homeobox gene, which also play a role in late retinogenesis. Experiments with conditional knock outs for Pax6 shown, that due to the loss of several bHLH genes, only amacrine cells were produced (Marquardt et al., 2001). Other transcription factors important during the differentiation of progenitors to mature retinal phenotypes are shown in Table 4.

Table 4. Transcription factors involved during retinogenesis

Transcription factors	Events during retinogenesis	References
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Hes1; Hes5	Promotes gliogenesis	(Dorsky et al., 1997; Dorsky et al., 1995; Furukawa et al., 2000; Rapaport and Dorsky, 1998)
Ath5	RGC differentiation, activates BarH1 and Brn3	(Liu et al., 2001)
Prox1	Cell cycle exit and horizontal cell differentiation	(Dyer et al., 2003)
Crx; Otx2		
Chx10	Bipolar cell differentiation	
Pax6		(Marquardt et al., 2001)

In sum, retinal fate decisions share some common features like cell cycle exit of RPC and the induction of differentiation by manipulation of signalling pathways and the composition of transcription factors. Nevertheless, the most significant feature is the lack of a define linear sequence of events form a prgenitor to a postmitotic cell. Instead several processes work in paralel and interact at multiple levels, ensuring coordination of proliferation and differentiation. Furthermore, it remains unclear if the different extrinsic factors involved during retinogenesis are instructive or permissive in regard to cell fate acquisition.

1.3 Photoreceptors development

The PRs are found in the outer most part of the retina, were they relay in close contact and interaction with the RPE. PRs are highly specialised light-sensitive neurons with elaborated outer segments carrying discs packed with pigments. There are two different types of PRs, cones and rods, which are distinguished by their shape, type of pigment, retinal distribution and pattern of synaptic conections (Figure 1.6)

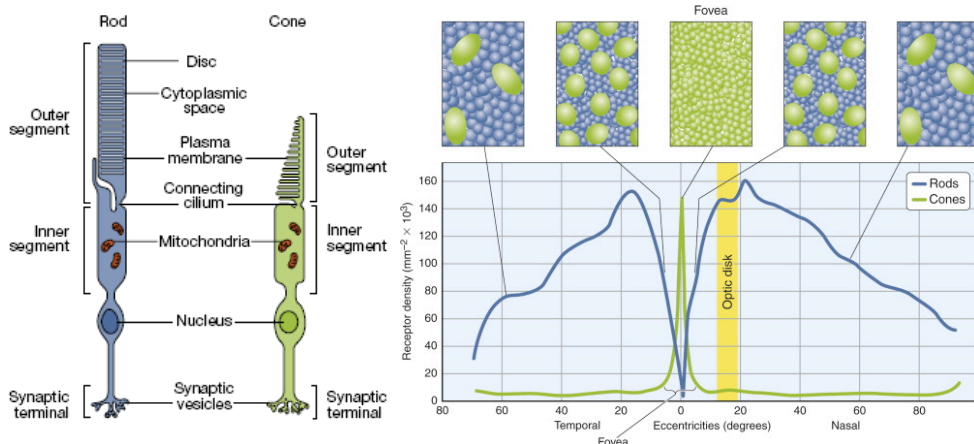


Figure 1.6. Types of photoreceptors. Webvision. Distribution of photoreceptors in the human eye. Overall, rods outnumber cones by a ratio of 20:1 or greater in the retina. However, in the fovea, the cone density is the highest and is correlated with visual acuity.

In contradiction to the well understood photoreceptor function and biochemistry, which will be describe later on this chapter, the information regarding the sequence of events that lead to either cone or rod PR is more limited.

There are two proneural bHLH transcription factors that are thought to play a role in photoreceptor development: the mammalian achaete-scute homologue 1 (Ascl1) and NeuroD. Ascl1 May play a role at early stages of development of specific neural lineages in most regions of the CNS. In the rodent retina instead, Ascl1 is confined to a subset of proliferating late progenitors giving rise to rods and other late-born cell types and later in development, becomes restricted to the inner nuclear layer (Tomita et al., 1996). In 2001, Hatakeyama and colleagues demonstrated that overexpression of Ascl1 in E17.5 retinae resulted in the predominant generation of rod PRs (Hatakeyama et al., 2001). On the other hand, NeuroD expression during normal development is mainly found in retinal cells committed to either PRs or amacrine cells.

Several transcription factors form part of the complex network around the cone-rod homeobox gene, *Crx*. *Crx* gene is essential for the maintenance of mammalian photoreceptors. A minimal network of interaction can be seen in the Figure 1.7, where blue lines show the direct binding of *Crx* on other genes, such as *Nrl*, *Nr2e3* and *Atxn7*. Yellow lines represent the inhibition of expression and green arrowheads show direct activation of expression, such is the case for *Rho*. However, fully mature amacrine cells lose *NeuroD* expression and in the mature retina *NeuroD* is confined to only a subset of PRs (Morrow et al., 1999). As well as with the *Ascl1* knock-outs, inactivation of *NeuroD* is lethal and mice die at birth. Nevertheless, from explant cultures of *NeuroD* knock-outs, it was observed an increase in Müller glia and bipolar cells and a delay in amacrine cell differentiation. In the case of PRs they are generated at the expense of an increase in apoptosis among the cells in the ONL (Morrow et al., 1999; Tomita et al., 1996). Consequently, *NeuroD* may play a role in the commitment to PRs or amacrine cell fate and in the particular case of PRs, on its survival. Additionally, in *Crx* knock-outs *NeuroD* expression was decreased (Hennig et al., 2008).

1.3.1. Photoreceptor lineage determination, *Crx* positive cells

In vertebrates, as it has been mentioned, the formation of the forebrain and the eyes rely on three ortholog genes; *Otx1*, *Otx2* and *Crx*. The latter two are essential in the lineage specification and maturation of PRs. Experiments from knockouts have revealed that *Otx2* inactivation is lethal in mice due to impaired forebrain development and the heterozygous *Otx2* knock-out presented a severe ocular malformation.

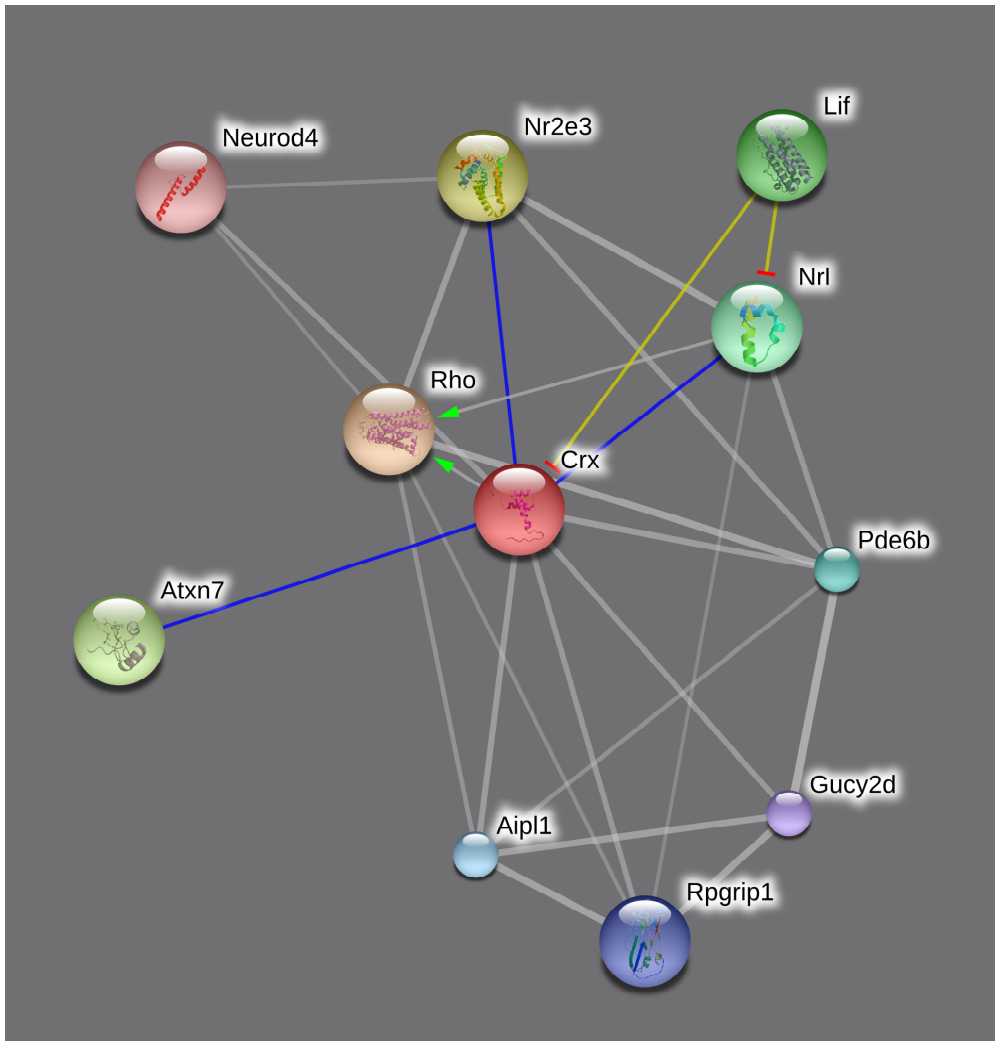
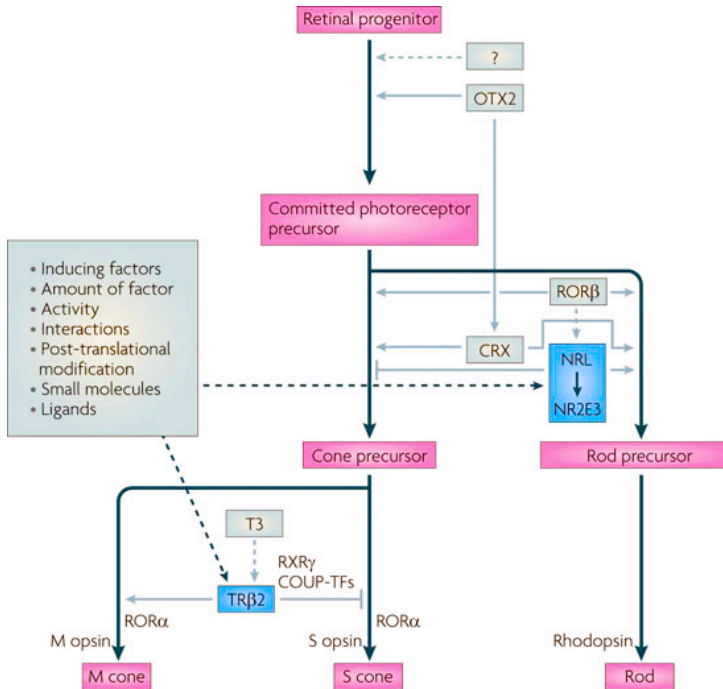


Figure 1.7. Cone-rod homeobox gene (*Mus musculus*) network of interaction; Binds and transactivates the sequence 5'-TAATC[CA]-3' which is found upstream of several photoreceptor-specific genes, including the opsin genes. Essential for the maintenance of mammalian photoreceptors. Built with String 9.05 and UniProt.

1.3.2. Cones and Rods, a fate decision



1.4 Photoreceptor function

1.4.1 Phototransduction

1.5.2 Differences in rod and cone photoreceptor function

Gene	Name	Function
<i>Gucy2d</i>	Guanylate cyclase 2d	Cyclic nucleotide metabolism
<i>Rpgrip1</i>	Retinitis pigmentosa GTPase regulator	Essential for RPGR function and is also required for normal disk morphogenesis

interacting protein 1		
<i>Rho</i>	Rhodopsin	Photoreceptor required for image-forming vision at low light intensity. Required for photoreceptor cell viability after birth. Light-induced isomerization of 11-cis to all-trans retinal triggers a conformational change leading to G-protein activation and release of all-trans retinal
<i>Aipl1</i>	Aryl hydrocarbon receptor-interacting protein-like 1	May be important in protein trafficking and/or protein folding and stabilization (By similarity)
<i>Nrl</i>	Neural retina leucine zipper gene	Transcription factor which regulates the expression of several rod-specific genes, including RHO and PDE6B (By similarity)
<i>Atxn7</i>	Ataxin 7	Involved in neurodegeneration. Acts as component of the STAGA transcription coactivator-HAT complex. Mediates the interaction of STAGA complex with the CRX and is involved in CRX- dependent gene activation (By similarity)
<i>Crx</i>	Cone-rod homeobox containing gene	Binds and transactivates the sequence 5'-TAATC[CA]-3' which is found upstream of several photoreceptor-specific genes, including the opsin genes. Essential for the maintenance of mammalian photoreceptors
<i>Neurod4</i>	Neurogenic differentiation 4	neurogenic differentiation 4 Gene; Appears to mediate neuronal differentiation (By similarity)
<i>Pde6b</i>	Phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide	This protein participates in processes of transmission and amplification of the visual signal. Necessary for the formation of a functional phosphodiesterase holoenzyme
<i>Nr2e3</i>	Nuclear receptor subfamily 2, group E, member 3	Orphan nuclear receptor of retinal photoreceptor cells. Transcriptional factor that is an activator of rod development and repressor of cone development. Binds the promoter region of a number of rod- and cone-specific genes, including rhodopsin, M- and S-opsin and rod-specific phosphodiesterase beta subunit. Enhances rhodopsin expression. Represses M- and S-cone opsin expression
<i>Lif</i>	Leukemia inhibitory factor	LIF has the capacity to induce terminal differentiation in leukemic cells. Its activities include the induction of hematopoietic differentiation in normal and myeloid leukemia cells, the induction of neuronal cell differentiation, and the stimulation of acute-phase protein synthesis in hepatocytes

Table 5. Genes involved in photoreceptor function

Gene	Protein function in photoreceptors
<i>AIP1</i>	Chaperone, nuclear export
<i>CABP4</i>	Synaptic terminal, voltage-gated Ca ²⁺ channel modulation
<i>CEP290</i>	Connecting cilium, 20% of LCA
<i>CRB1</i>	OLM, 9-13% of LCA
<i>CRX</i>	Transcription factor, 1-3% of LCA
<i>GUCY2D</i>	Cyclic nucleotide metabolism
<i>IQCB1</i>	Cyclic nucleotide metabolism
<i>KCNJ13</i>	Potassium channel, membrane potential
<i>LCA5</i>	Connecting cilium
<i>LRAT</i>	Retinol metabolism, visual cycle (expressed in RPE)
<i>OTX2</i>	Transcription factor
<i>RD3</i>	Unknown
<i>RDH12</i>	Retinol metabolism, visual cycle, 4% of LCA
<i>RPE65</i>	Retinol metabolism, visual cycle, 16% of LCA
<i>RPGRIP1</i>	Cyclic nucleotide metabolism
<i>SPATA7</i>	Unknown
<i>TULP1</i>	Connecting cilium, opsin transport from IS to OS

RetNet (<http://www.sph.uth.tmc.edu/Retnet/sum-dis.htm#B-diseases>, accessed 09/2011)

1.5 Retinal dystrophies

Inherited retinal dystrophies are the main cause of incurable blindness in the developing world. These group of diseases affect as many as 1 in 2.000 births, and unfortunately in many cases, no treatment can be offered (Berger et al., 2010a). The most common forms of retinal dystrophies are retinitis pigmentosa (RP) and acute macular degeneration (AMD). In both cases, the main pathological hallmark is the PR degeneration and the consequent implantation of blindness. As expected,

many gene mutations associated to either RP or AMD, have been found in genes that play a crucial role in PR functions, nevertheless, mutations in other cells such as RPE, also can cause the diseases. Interestingly, classical apoptosis is not the main cell death mechanism in PR. Instead, a necrosis-like cell death mechanism seems to be the responsible for the PR degeneration, where the cells experience energy depletion, increased oxidative stress and also show a deregulated DNA repair. (Sancho-Pelluz et al 2008, Marigo 2007 and Reme 1998). According to Berger and colleagues retinal diseases can be categorized in three major groups:

1. stationary and progressive rod dominated diseases
2. stationary and progressive cone dominated diseases
3. non-syndromic generalized PR diseases (affecting both PR cell types, rod and cones; (Berger et al., 2010a).

Disorders primarily affecting the rod system present initially night blindness and may progress to involve the peripheral visual field, with relative preservation of central vision. In contrast, cone dystrophies will manifest initially with loss of central visual acuity and colour vision and should not significantly affect peripheral vision unless the rod system is also involved (cone-rod dystrophy, CORD). Macular dystrophies (MDs) affect both rods and cones within the macula, whilst leaving the peripheral populations intact.

1.5.1 Retinitis Pigmentosa

Retinitis pigmentosa is the most common form of inherited RD, in which patients lose both rod and cone PRs. In most cases of RP, hallmark features include night

blindness, attenuated retinal vessels, waxy pallor of the optic disc, and bone spicule-like pigmentation in the fundus. It has a worldwide prevalence of 1 in 3.500. The functions of the different RP-associated genes may be grouped into mainly five categories: (i) phototransduction, (ii) retinal metabolism, (iii) tissue development and maintenance, (iv) cellular structure, and (v) splicing. More than 50 genes have been associated to RP to date, most of them are rod-specific genes, yet some RP genes are ubiquitously expressed (RetNet, <http://www.sph.uth.tmc.edu/retnet>). Autosomal dominant RP is the most frequently inherited type of RP, accounting for approximately 20% to 25% of cases (Ferrari et al., 2011). Among the most prevalent are rhodopsin (*RHO*) and peripherin/*RDS*. *RHO* gene was the first gene linked to RP. Its mutations are responsible for 30% to 40% of the autosomal dominant cases (Ferrari et al., 2011). More than 100 mutations have been identified in *RHO*, causing variation within the clinical presentations. Another common cause of adRP is attributed to the peripherin/*RDS* gene mutation, which accounts for 5% to 9.5% of adRP (Ferrari et al., 2011). Mutations in transcription factors (*NRL*, *CRX*) and pre-mRNA processing factors (*HPRP3*, *PRPC8*, *PRPF31*) have been also implicated in adRP (RetNet, <http://www.sph.uth.tmc.edu/retnet>). Mutations in *TOPORS*, a ciliary protein, were identified in patients with adRP (Chakarova et al., 2007) linked to a previously reported locus, *RP31* (Papaioannou et al., 2005).

Autosomal recessive RP is the second most frequently inherited type of RP, accounting for approximately 15% to 20% of cases. The majority of arRP-causing genes are involved in the phototransduction cascade (*CNGA1*, *PDE6A*, *PDE6B*, *SAG*) or in vitamin A metabolism and its recycling in the eye (*RLBP1*, *ABCA4*, *LRAT*, *RPE65*) (Retinal Information Network: <http://www.sph.uth.tmc.edu/retnet>).

X-linked (XIRP) is the least frequently inherited type of RP, accounting for only 10%

to 15% of cases (Ferrari et al., 2011). To date, only 2 genes have been identified (RetNet, <http://www.sph.uth.tmc.edu/retnet>). The *RPGR* and *RP2* genes.

1.5.2 Acute Macular Dystrophy

One of the most common macular degenerations (MDs) is age-related macular degeneration (AMD) and results in progressive loss of visual acuity in the center of the visual field (the macula), colour vision abnormalities and central scotomas (shadows or missing areas of vision). AMD usually, affects the elder with a prevalence of 0.05% before the age of 50, increasing up to a 12% in patients above 80 years old (Friedman et. Al. 2004). A combination of genetic and environmental factors such as hypertension, smoking, diet, obesity and chronic inflammation are responsible for the development of AMD. A pathological feature of the disease is the deposit of lipids in the Bruch's membrane underlying the RPE, a sign known as drusen. The the condition can progress by further accumulations of drusen (dry AMD) or intrusion of fluid leading to the detachment of the RPE or the retina (wet AMD, de Jong PT 2006) *ARMS2*, *HTRA1* and *PLEKHA* among other genes have been identified as susceptible genes for AMD and are summarized in Table 4 (RetNet, <http://www.sph.uth.tmc.edu/retnet>).

Table 4. Known mutations to cause age-related macular degeneration (AMD)

Gene	Protein function in disease
<i>ABCA4</i>	Phototransduction. Expressed in rod outer segment and foveal cones
<i>ARMS2</i>	Mitochondrial protein found in the inner segment of PR
<i>C2</i>	Innate immunity and inflammation
<i>C3</i>	

<i>CFB</i>	Gene contiguous to C2. Innate immunity and inflammation
<i>CFH</i>	Factor H. Regulation of complement activation
<i>ERCC6</i>	Protein is involved in DNA nucleotide excision repair
<i>FBLN5</i>	
<i>HMCN1</i>	
<i>HTRA1</i>	Protein found in drusen, Regulates degradation of extracellular matrix
<i>RAX2</i>	Modulator of photoreceptor gene expression. Binds to <i>Rho</i> promoter
<i>TLR3</i>	
<i>TLR4</i>	

RetNet (<http://www.sph.uth.tmc.edu/Retnet/sum-dis.htm#B-diseases>, accessed 09/2011)

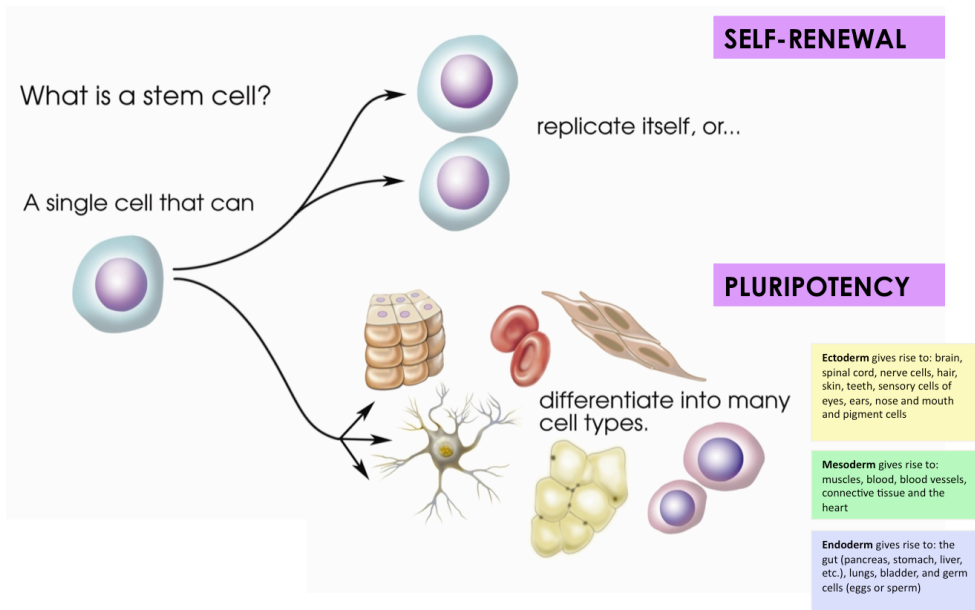
1.6 Cell-based therapy for retinal dystrophies

Effective treatment for retinal degeneration has been widely investigated. Gene therapy has shown to improve visual function in inherited retinal disease. However, this treatment was less effective in advanced cases and due to its nature is too specific, increasing the cost and reducing the spectrum of patients whom can benefit from it. One of the main drawbacks of gene therapy is that photoreceptor loss cannot be reclaimed, for this reason it has become so important to design protocols of differentiation more efficient and safe for cell replacement therapies. Cell-based therapy pursue to find an appropriate source of cells which could give rise to photoreceptors after transplantation into the diseased

1.7 Stem cells

A stem cell is defined as a cell that can renew itself for the lifetime of the organism

while also producing a cell progeny that matures and differentiates into more specialized organ specific cells. The balance between populations of stem cells and differentiating cells is critical during embryonic development and for the maintenance and regeneration of adult tissues.



1.7.1 Concept and definition

As mentioned above, there are two main features to define a stem cell. Any cell capable of self-renewal that under certain physiological or experimental conditions can be induced to become cells with specialized functions, which is known as pluripotency (Evans and Kaufman, 1981; Thomson et al., 1998). Self-renewal and pluripotency make stem cells unique. Pluripotency is perhaps the most exciting feature of stem cells from the therapeutic point of view. Pluripotent stem cells have the ability to differentiate into any cell type constituting an adult organism, since they have proven to differentiate into the three germ layers, ectoderm, mesoderm and

endoderm cells. Nevertheless, there are other stem cells found in adult tissues, which do not follow this rule strictly and this will be discussed in the following section.

1.7.2 Classification

1.7.3 Cellular differentiation

1.7.3.1 Chromatin and differentiation

1.7.3.2 Apoptosis and differentiation

1.8 Retinal differentiation of mouse embryonic stem cells

1.8.1 Extrinsic Factors

1.8.2 Intrinsic Factors

1.8.3 Coculture experience

1.9 Oxygen Tension

1.9.1 Early development

1.9.2 Neuronal development

1.9.3 pluripotency and differentiation

1.9.4 Retinal development

Eye development is a complex multistep process rather than a simple one-step phenomena, which is why it is rational to consider that instead of transcription factors or signalling molecules acting individually is the interaction of both of them along with microenvironmental factors that achieves the development of a normal eye.

1.10 Summary

Stem cell therapy is a potential treatment for dysfunction and death of photoreceptor

cells of retinal dystrophies. Retinitis pigmentosa (RP) and age-related macular degeneration (AMD), the leading causes of permanent blindness in humans are characterized by the progressive the retina. For such diseases, the replenishment of functional photoreceptor precursors may be a good strategy for retinal regeneration, as gene therapy or growth factor supplement cannot regenerate dying photoreceptor cells. Retinal development is a multi-step process involving cell cycle exit, migration, and changes of cell morphology (Malicki, 2004). These changes result from a reciprocal relationship between tissue-tissue interaction and cell intrinsic factors (Fuhrmann, 2010). Additionally, accumulating evidence suggests that other components of the niche, such as oxygen tension, play an important role in cell fate determination during the development of many tissues, including the nervous system and the retina (Arden et al., 2005; de Gooyer et al., 2006; Evans and Kaufman, 1981).

Mouse embryonic stem cells (mESC) allow us to recapitulate retinal development in vitro. These cells are derived from the early embryo and are characterized by their two unique features of pluripotency and self-renewal (Evans and Kaufman, 1981). In particular, during implantation and fetal development, stem cells live at oxygen tensions between 2% to 8% (Maltepe and Simon, 1998).

Early embryonic formation during mammalian development occurs in a precise environment, where the O_2 tension plays a critical role (Dunwoodie, 2009). In comparison to the atmospheric O_2 tension (20%) the uterus environment is hypoxic. Mammals, including rabbits (8.7% oxygen tension) and monkeys (1.5% oxygen tension; (Fischer and Bavister, 1993) as well as humans develop embryos under low oxygen tension. Up until the second trimester in humans this ranges from 0-3% (Burton and Caniggia, 2001; Burton and Jauniaux, 2001). The retina is not an

exception and recent studies have shown the important role that hypoxia may play in neuroprotection and development of the human retina (Grimm and Willmann, 2012). This relative hypoxia or tissular normoxia is relatively low compared with traditional in vitro culture conditions (20% O₂; (Simon and Keith, 2008). Once, the pluripotency agents such as Leukemia inhibitory factor (LIF) is removed, ESC spontaneously differentiate following a reproducible temporal pattern of development, that in many ways recapitulates early embryogenesis (Keller, 1995). Due to these special characteristics, ESC are considered an unlimited source for cell replacement therapies. The formation of embryoid bodies (EBs), which are three-dimensional aggregates of ESC, is the initial step in ESC differentiation. Therefore, EB culture has been widely utilized as a trigger for the in vitro differentiation of ESC. Numerous groups have recently demonstrated that ESC can be converted into cells that resemble retinal progenitors (Banin et al., 2006; Hirami et al., 2009; Lamba et al., 2006), photoreceptors (Osakada et al., 2008) or RPE (Klimanskaya et al., 2004; Vugler et al., 2008). Furthermore, Meyer and colleagues have very elegantly mimicked the early retinal development in a stepwise fashion typical of normal retinogenesis (Meyer et al., 2009) and high yield of cells differentiated towards photoreceptors were achieved by Mellough and colleagues (Mellough et al., 2012). Others went further attempting to obtain 3D structures of early optic cup using scaffolds (Eiraku et al., 2011; Nistor et al., 2010). However, evidence of fully characterized high yield populations of photoreceptors or mature RPE cells has not yet been accomplished. The main drawback of the differentiation methods available is the very low efficiency along with the lack of reproducibility. Most of the protocols published in recent years have been geared towards the induction of expression of retina-specific transcription factors, but very few

publications have included information regarding in vivo integration of differentiated cells into the mouse retina, suggesting cell survival, migration and functionality of the grafted cells (Lamba et al., 2009a; Lamba et al., 2010). In this study, we have optimized and fully characterized an original protocol of differentiation that allows us to obtain photoreceptors at a high efficiency in a reproducible way.

2. AIMS

“Research is what I'm doing when I don't know what
I'm doing.”

—**Wernher von Braun**

The work presented in this thesis investigates whether lower oxygen tensions mimic the retinal microenvironment increasing the yield of photoreceptors and improving the modeling of retinogenesis *in vitro*.

Furthermore, the work attempts to resolve if the retinal cells differentiated under hypoxic conditions, could survive and integrate more efficiently given the similarities between the culture conditions and the physiological O₂ tension found in the retinal niche.

Specific aims

To evaluate whether D3-mESC line is suitable for the generation of retinal cells in a stepwise manor that mimics normal development.

To determine if low O₂ tension has a positive effect on mESC's spontaneous differentiation, loss of pluripotency and favors the generation of a retinal phenotype.

To improve the differentiation of mESC towards photoreceptors and other retinal cells by taking into account micro-environmental cues such as O₂ tension.

To study the transplantation of retinal cells obtained from mESC and determine if they survive, migrate and integrate into the host retina.

3. MATERIALS AND METHODS

“No amount of experimentation can ever prove me right; a single experiment can prove me wrong.”

—**Albert Einstein**

Unless otherwise specified, all the reagents used in this thesis were acquired from Sigma® or Invitrogen®. Table 8. Contains detailed media description.

3.1 Maintenance of mESC pluripotent cultures.

All experiments conducted in this thesis were carried out using ES-D3 cells (ATCC CRL 1934, (Doetschman et al., 1985; Mellough et al., 2012) passages 18-35. ESC were maintained modifying already published protocols (Zhao et al., 2002, 2006) and were incubated at 37°C under 20% Oxygen tension. Cultures were passaged every 4-7 days and grown at low confluence at a 1:1000 split ratio (Figure 3.1). Fresh medium (Table 8) was exchanged every 48 hours. Appropriate Master and Working Cell Banks were generated to allow all the experiments to be accomplished using early passages.

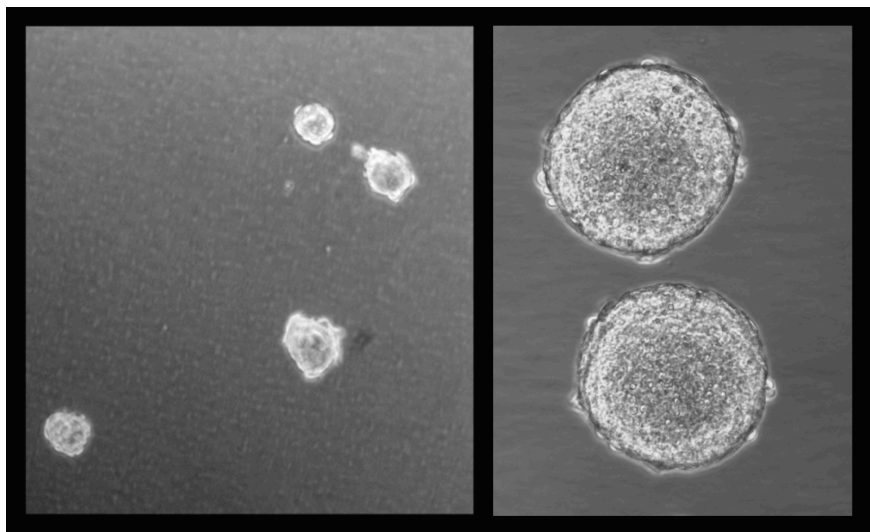


Figure 3.1. ES-D3 cell cultures. Bright field images showing the undifferentiated ES-D3 cell line 2 days (left) and 6 days (right) after passage.

Table 6. Formulations of media used.

Maintenance Medium (500mL)	EB Medium (500mL)	Progenitors Medium (500mL)	Retinal Medium (500 ml)
DMEM GLUTAMAX (GIBCO32430) 409ml	DMEM GLUTAMAX (GIBCO32430) 459ml	DMEM GLUTAMAX (GIBCO32430) 459ml	MEM-HEPES 66% 320ml HBSS 33% 50 ml
FBS (Hyclone) 15% 75 ml	KSR (GIBCO 10828) 5% 25ml	NEAA (GIBCO 11140) 0.1mM 5ml	GLUCOSE SOL. 2.88g Glucose in 115ml HBSS
NEAA (GIBCO 11140) 0.1mM 5ml	NEAA (GIBCO 11140) 0.1mM 5ml	ANTIBIOTICS (GIBCO 15140) 1% 5ml	L-GLUTAMINE (Sigma) 200 µM 500 µl
ANTIBIOTICS (GIBCO 15140) 1% 5ml	ANTIBIOTICS (GIBCO 15140) 1% 5ml	Na+ PYR (GIBCO 11360) 1mM 5ml	N2 SUPPLEMENT (GIBCO 17502) 1% 5mL
Na+ PYR (GIBCO 11360) 1mM 5ml	Na+ PYR (GIBCO 11360) 1mM 5ml	2-ME (GIBCO 31350) 0.1mM 1ml	FBS (Hyclone) 1% 5 ml
2-ME (GIBCO 31350) 0.1mM 1ml	2-ME (GIBCO 31350) 0.1mM 1ml	KSR (GIBCO 10828) 5% 25ml	ANTIBIOTICS (GIBCO 15140) 1% 5ml
LIF (Hyclone)			

3.2 ES cell differentiation

One week prior to starting the differentiation protocol cells were incubated at 37°C in 5% CO₂ under either Normoxic (20% Oxygen tension) or Hypoxic (2% Oxygen tension) conditions in a Thermo Fisher incubator (CO₂/O₂ WJ IR Model 3141, Thermo Electron Corporation, Fisher Scientific). Both conditions were maintained during the whole time of differentiation. Oxygen tension control was monitored daily.

3.2.1 Spontaneous Differentiation: Model of Embryoid Bodies

EBs were generated following an optimized protocol of the hanging drop method described by Wobus and colleagues (Wobus et al., 1991). mESC were dissociated using 0.05% Trypsin for 4 min at 37°C. Trypsin was washed away adding EBs medium (Table 8). The cell suspension generated was spun down by centrifugation (Beckman coulter) and pelleted cells were resuspended in ES medium at the desired concentration (1000 cells per 30 µl). Hanging drops of 30 µl were plated onto the lid of a 150 mm² ultralow attachment plate (Soria Greiner) using a multichannel pipette (Eppendorf).

NORMOXIA / HYPOXIA

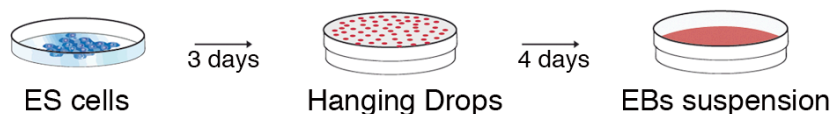


Figure 3.2. Embryoid bodies Generation. Diagram Showing the procedure followed to obtain a homogenous sample of embryoid bodies from mouse embryonic stem cells.

ESC were allowed to aggregate in hanging drops for 3-4 days before

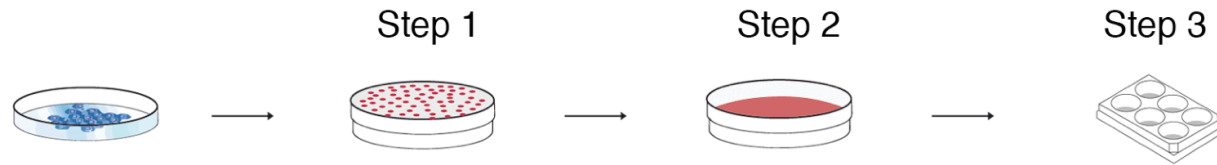
transfer to a suspension culture. After 3 days identical spherical EBs were formed and each drop was collected individually with a 100 μ l pipette and deposited into a 10-cm ultra-low-attachment dish (Soria Greiner) containing 10 ml of EB medium to a final concentration of 100 EBs per dish (Figure 3.2). The EBs were cultured for 5 and 7 days and the medium was changed every 2-3 days.

3.2.2 Directed Differentiation of mESC Towards Retinal Progenitors and Retinal Mature Phenotypes

Retinal differentiation of mESC was accomplished using an optimized protocol encompassing growth factors described in previously published protocols (Hirami et al., 2009; Ikeda, 2005; Osakada et al., 2008) combined with the manipulation of the microenvironment. All the growth factors and media supplements were bought from R&D Systems, unless otherwise specified. EBs generated from mESC were induced to differentiate in Progenitors medium (Table 8) supplemented with 100 ng/ml Dickkopf-related protein 1 (Dkk1) and 500 ng/ml Lefty-A for 5 days at 37 °C with 5% CO₂ under Normoxic (20%) or Hypoxic (2%) conditions. Media was changed 72 hours later and fresh aliquots of the growth factors were added along with 5% fetal bovine serum (FBS) and 100 ng/ml Activin-A. On day 5, media was changed and EBs were cultured for 5 more days in Progenitors media without the addition of any growth factor. Fresh Progenitors medium was changed every 48 hours. On day 10, EBs were plated in 6-well plates or coverslips coated with human recombinant 30 μ g/cm² Laminin (Sigma) and 150 μ g/cm² Poly-L-Ornithine (Sigma) and cultured in Retinal medium

(Table 6) supplemented with 10 μM N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT; Calbiochem) at 37 °C with 5% CO_2 for 48 hours. Ideal cell density was established in 150 EBs per 9.6 cm^2 . To allow better attachment of the EBs, 10% FBS was added to the medium for 48 hours. Retinal medium was exchanged every 2 days. On day 16 and until day 24, Retinal medium was supplemented with 10 μM DAPT, 50 ng/ml acidic fibroblast growth factor (aFGF), 10 ng/ml basic fibroblast growth factor (bFGF, Millipore), 3 nM Sonic hedgehog homolog (Shh), 0.5 μM retinoic acid (RA; Sigma) and 100 μM Taurine (Sigma). From day 24 to day 28 Retinal medium was supplemented with 10 μM DAPT, 3 nM Shh, 0.5 nM RA and 100 μM Taurine. Samples were collected on day 0, day 5, day 10, day 16, and day 28 for molecular biology analysis and immunocytochemistry. Figure 3.3 provides an schematic of the protocol of differentiation and a diagram with all the growth factors and small molecules, distributed by day of addition, used to direct the differentiation towards retinal cells during the development of this thesis.

A. NORMOXIA / HYPOXIA



B.

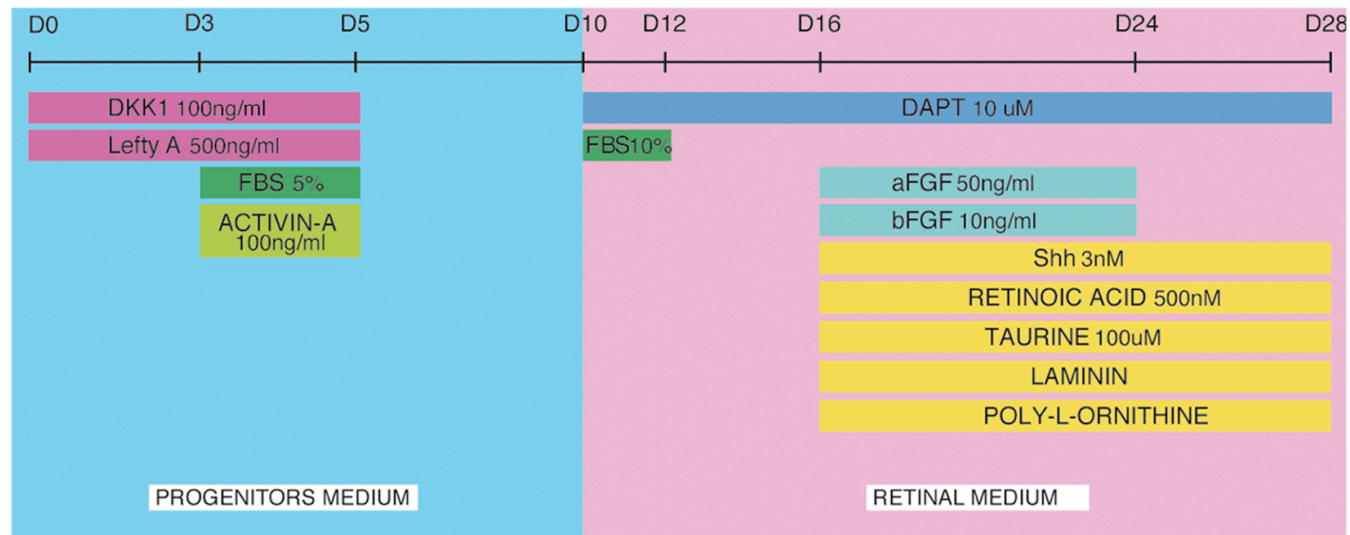


Figure 3.3. Protocol of differentiation. A. Schematic Diagram of the 3 step differentiation protocol used to generate retinal cells form mESC. B. Detail of the protocol of differentiation used to generate retinal cells.

The integrity of total RNA was qualitatively assessed on an Agilent 2100® Bioanalyzer (Figure 3.4). Reverse transcription was carried out with 1 µg of total RNA using the Superscript III RT kit (Invitrogen). Quantitative PCR (RT-PCR) reactions were performed using SensiFAST™ SYBR No-ROX Kit (Bioline, London, UK). All samples were normalized against a housekeeping gene (β-actin). The primer sets, as well as the annealing temperatures are listed in Table 7. All RT-PCR reactions were run at 40 cycles and data analysis was done using the CFX Manager v2.1 software (BioRad) by the $\Delta\Delta CT$ method. The $\Delta\Delta CT$ method is widely used to present relative gene expression and is also referred as the comparative C_T method, where the changes in steady-state mRNA levels of a gene across multiple samples are expressed relative to the levels of an internal control RNA (Livak and Schmittgen, 2001).

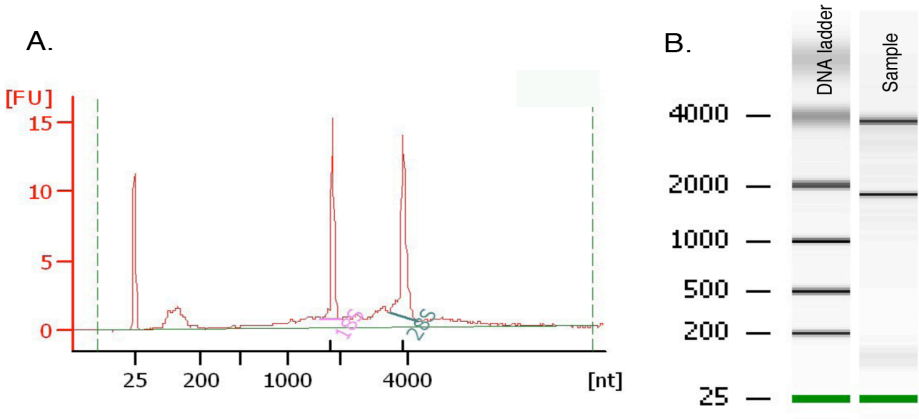


Figure 3.4. RNA sample analysed in Agilent 2100 bioanalyzer. Example of a typical sample of total RNA extracted for the analysis of gene expression. A. Electropherogram of a high quality total RNA sample. The 18S and 28S peaks are clearly visible. The microchannels of the Bioanalyzer are filled with a sieving polymer and fluorescence dye. Samples are detected by their fluorescence and translated into electropherograms or into gel-like images. B. Gel representation of total RNA.

Table 7. PCR primers used to study the loss of pluripotency and the retinal differentiation

Gene	Primers (5'-3')	Ta (°C)	Product size (bp)
------	-----------------	---------	-------------------

B-Actin	F:TCCTGTGGCATCCACGAACTACA R:ACCAGACAGCACTGTGTTGGCATA	60	93
Cdkn1a	F: TTGTCGCTGTCTTGCCTCTGGT R:AGACCAATCTGCGCTTGGAGTGAT	62	141
Vegfa	F: GGAGAGCAGAAGTCCCATGA R: ACTCCAGGGCTTCATCGTTA	60	184
Pax6	QT01052786 Qiagen	60	120
Chx10	QT00112056 Qiagen	60	134
Nrl	QT00109298 Qiagen	60	71
Crx	QT00115402 Qiagen	60	127
Rax	QT01775193 Qiagen	60	88
Rhodopsin	QT00099022 Qiagen	60	78
Nanog	QT01743679 Qiagen	60	190
Sox2	QT00249347 Qiagen	60	103
Tert	QT00104405 Qiagen	60	145
Pou5f1	QT00109186 Qiagen	60	101
Six3	QT01045219 Qiagen	60	117
Otx2	QT01079771 Qiagen	60	115
Mitf	QT00131313 Qiagen	60	87
ZO-1	QT00493899 Qiagen	60	150

3.4 Immunocytochemistry

Cell cultures were washed in PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (r.t.). Fixed cells were washed twice with PBS before staining. For nuclear staining, permeabilization within ice methanol was accomplished for 30 min at -20°C. After permeabilization, cells were blocked with 3% Donkey serum, 3% Goat serum in 0.5% Triton-PBS for 30 min at r.t. Immunostaining was performed overnight at 4°C in 0.5% Triton-PBS using the antibodies listed in Table 8 and 9. Cells were counter-stained with

300 nM DAPI for 10 min at r.t. For negative controls, primary antibodies were omitted and the same staining procedure was carried out. Positive cells were detected using either Alexa488-, Alexa594-, Alexa633- (Invitrogen) or Dylight549- conjugated secondary antibodies in a Leica DM 5500 microscope (Leica Microsystems, Wetzlar Germany) and a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar Germany). Specificity of each antibody was determined in mouse embryonic and adult retinal tissue (Annex 2).

Table 8. List of primary antibodies used to study the retinal differentiation of embryonic stem cells

Antibody	Type	Source	Dilution
Ki67	Rabbit polyclonal	Abcam	1:200 (1:100 FACS)
Mitf	Mouse monoclonal	Abnova	1:500
Opsin-s	Rabbit polyclonal	Abcam	1:200
Otx2	Rabbit polyclonal	Millipore	1:500
Rhodopsin	Mouse monoclonal	Abcam	1:100
Recoverin	Rabbit polyclonal	Millipore	1:1000
RAX	Rabbit polyclonal	Abcam	1:300
ZO1	Rabbit polyclonal	Invitrogen	1:50
Crx	Mouse monoclonal	Novus Biologicals	1:100
RPE65	Mouse monoclonal	Abcam	1:250
Chx10	Sheep polyclonal	Exalpha	1:1000
Pax6	Mouse monoclonal	Hybridoma Bank	1:100
Nrl	Rabbit polyclonal	Gift	1:1000
Hif1a	Rabbit polyclonal	Santa cruz	1:50
Tuj1	Mouse monoclonal	Covance	1:500

Table 9. List of primary antibodies used for analysis of pluripotency and stemness.

Antibody	Type	Source	Dilution
SSEA-1	Mouse monoclonal	Cell Signaling	1:100
OCT-4	Rabbit monoclonal	Cell Signaling	1:100
Nanog	Goat monoclonal	R&D Systems	1:50
Sox2	Mouse monoclonal	R&D Systems	1:50
PE-Nanog	Mouse monoclonal	BD Pharmingen™	
PerCP-Cy5.5-Oct3/4	Mouse monoclonal	BD Pharmingen™	
Alexa Fluor® 647-Sox2	Mouse monoclonal	BD Pharmingen™	
PE-Mouse IgG1, κ	Mouse Isotype control	BD Pharmingen™	
PerCP-Cy5.5-Mouse IgG1, κ	Mouse Isotype control	BD Pharmingen™	
Alexa Fluor® 647-Mouse IgG2a, κ	Mouse Isotype control	BD Pharmingen™	

3.5 Image analyses

The immunocytochemistry experiments were repeated at least 3 times. For Image analysis at least 8 microscopic fields from each sample were taken randomly using a 40X lens objective in a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar Germany). To reduce human bias, a semi-automated image analysis system was used to determine the percentage of immunoreactive cells from digital images using the MetaMorph NX® v 7.5.1.0 Software (Molecular Devices, Downington, PA). First, cells and processes of interest were outlined to exclude adjacent cells or areas of nonspecific immunoreactivity.

3.6 FACS

EBs were washed twice with DPBS (Gibco 14190) and enzymatically digested by incubation with the Embryoid Body Dissociation Kit from Miltenyi Biotech (Bergisch Gladbach, Germany) for 15 minutes at 37°C. The single cell suspension obtained was washed in DPBS and fixed with 4% paraformaldehyde in PBS for 20 min at r.t. Fixed cells

were washed twice with PBS before staining. Cells were then immunostained using the BD Stemflow™ Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit (BD Biosciences) following the manufacturer's instructions. Appropriate isotype controls provided with the kit were used to immunostain the negative populations. At least 10,000 events were analysed in each experiment using FACSCalibur system (BD Biosciences). Results correspond to at least 6 individual runs and the number of positive cells within the gated population was analysed using CellQuest™ Pro (BD Biosciences) software. RPE-1 and D3 cells were washed twice with PBS and enzymatically digested by incubation with Trypsin for 4 minutes at 37°C. The single cell suspension obtained was washed in PBS and fixed with 4% paraformaldehyde in PBS for 20 min at r.t. Fixed cells were washed twice with PBS before staining. Cells were then permeabilized with 1X Perm Wash Buffer (BD Biosciences) at r.t. for 10 min and incubated overnight with the Rabbit Polyclonal Hif1α primary antibody (1:50 dilution) and the corresponding IgG Isotype control in staining buffer containing 1% FBS in PBS. Immunostaining was completed with Goat anti rabbit Alexa488-conjugated secondary antibody (1:500). 10.000 events were analysed in each experiment (n=3) using FACS Calibur system (BD Biosciences). The number of positive cells within the gated population was analysed using Cell Quest™ Pro (BD Biosciences) software.

3.7 ApoTox-Glo™ Triplex Assay

This kit combines three assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. In the first part of the assay, it measures two protease activities simultaneously; one being a marker of cell viability and the other being a marker of cytotoxicity. Peptide substrate (glycylphenylalanylaminofluorocoumarin; GF-AFC) enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease

becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. Peptide substrate (bis-alanylalanylphenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Bis-AAF-R110 is not cell-permeable, so intact, viable cells generate no signal from this substrate. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously. In the second part of the assay, the Caspase-Glo[®] 3/7 Reagent, added in an "add-mix-measure" format, results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal produced by luciferase. RPE-1 cells were seeded in a flat 96-well micro-plate (approximately 500/well) (Nunc) as triplicates. Three different types of controls, namely: positive, untreated, and negative controls were used throughout the study. Positive control had cells with culture medium exposed to 10 μ M MG132 for 16 hours to induce apoptosis. Control cell cultures contained cells untreated with MG132. Negative control (background) contained only culture medium without cells. After 7 days of exposure to either normoxic or hypoxic conditions, 20 μ l of Viability/Cytotoxicity reagent containing both GF-AFC and bis-AAF-R110 substrates was added to each well, and briefly mixed by orbital shaking at 500 rpm for 30 seconds and then incubated at 37°C for 30 minutes. Fluorescence was measured at 400Ex/505Em (Viability) and 485Ex/520Em (Cytotoxicity) by using Thermo Scientific Varioskan[®] Flash Spectral Scanning Multimode Reader. After that 100 μ l of Caspase-Glo 3/7 reagent was added to each well, and briefly mixed by orbital shaking at 500 rpm for 30 seconds and then incubated at room temperature for 30. Luminescence was measured using a Thermo Scientific Varioskan[®] Flash Spectral Scanning Multimode plate reader. Luminescence RFUs are proportional to the amount of caspase activity present. Figure 3.5 provides a schematic of the mechanism of action of ApoTox-Glo[™].

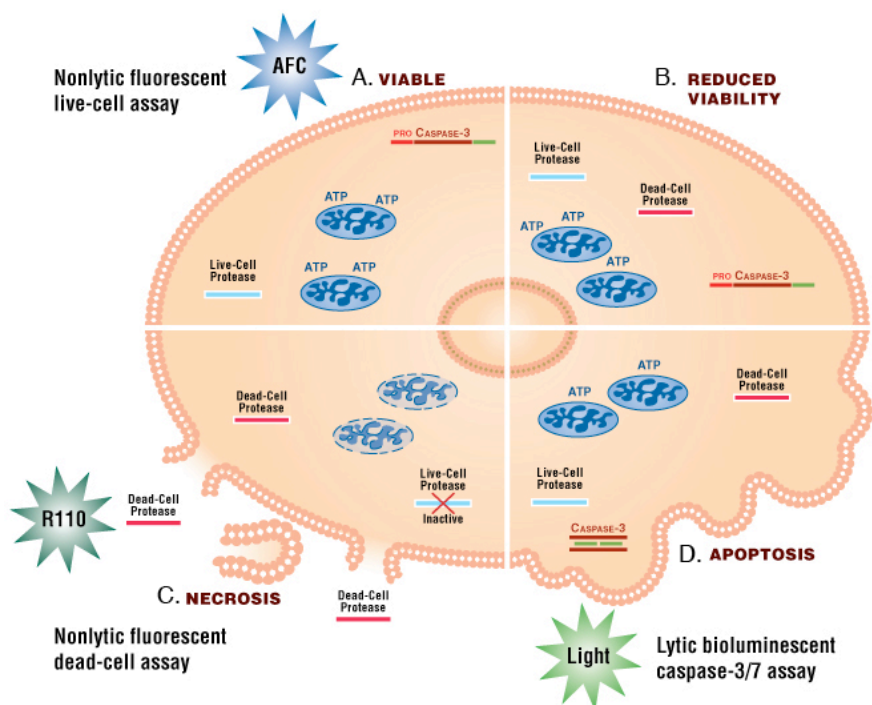


Figure 3.5. ApoTox-Glo™ Triplex Assay Schematics. The ApoTox-Glo™ Triplex Assay is a multiplexed, sequential addition assay that measures biomarkers associated with cell viability, cytotoxicity and apoptosis. Cell viability and cytotoxicity are measured first by adding two substrates that detect viable and membrane-compromised cells (GF-AFC and bis-AAF-R110). A. GF-AFC is cell permeant and measures a protease associated with live cells. C. The live-cell protease becomes inactivated once it is released into the medium and produces only a minimal signal from dead cells. The bis-AAF-R110 measures a dead-cell protease. D. Activated Caspase 3 is detected in apoptotic cells. Taken and modified from www.promega.com

3. 8 RPE-1 cell cultures

Telomerase-immortalized human retinal pigment epithelia 1 (RPE-1, ATCC CRL-4000), cells were cultured in DMEM-Ham's F12 supplemented with 10% fetal bovine serum (FBS) and L-Glutamine (Figure 3.6). RPE-1 cells were maintained at 37° C in a humidified 5% CO₂ environment under either Normoxic (20% oxygen Tension™) or Hypoxic (2% Oxygen tension) conditions in a thermo Fisher incubator (CO₂/O₂ WJ IR Model 3141, Thermo Electron Co, Fisher Scientific). Cell cultures were passaged every 7 days to 1:10 split ratio and fresh medium was exchanged every 48 hours.

ATCC Number: **CRL-4000**
Designation: **hTERT RPE-1**

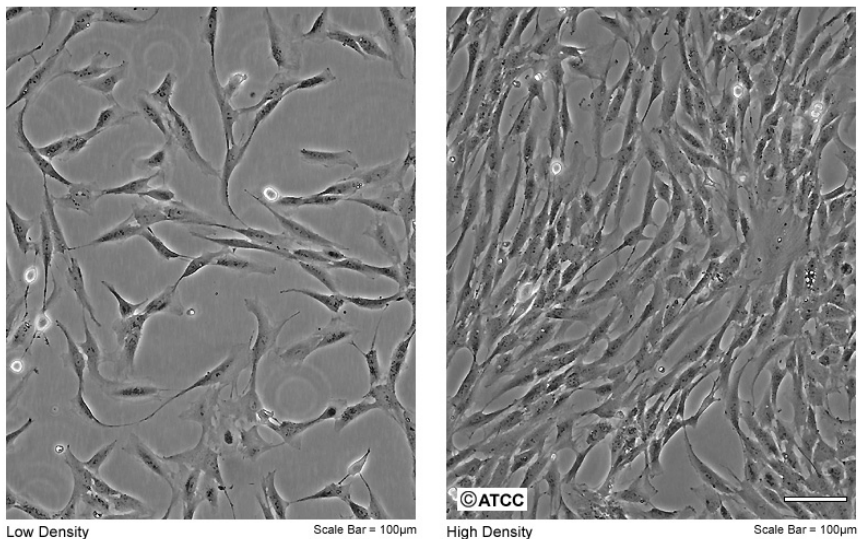


Figure 3.6. RPE-1 cultures. Representative images of the appearance of RPE-1 cell cultures. Left image shows a low confluence culture and right image a high confluence culture. Images taken from ATCC website. <http://www.lgcstandards-atcc.org/en.aspx>

3. 9 Statistical analyses

Data are the mean \pm standard error of mean (SEM) of at least three independent experiments, except for the immunocytochemistry, for which a representative image from three assays is depicted in the figures. Comparisons between values were analysed using one-way analysis of variance (ANOVA); $p \leq 0.05$ was considered statistically significant.

3.10 Preparation of cells for transplantation

Cells, after 20 days of *in vitro* differentiation were trypsinized to obtain a single cell suspension. Harvested cells were labeled using a 2 μ M PKH26 solution (Sigma-Aldrich) and washed in DPBS (Gibco 14190). Stained cells were counted with a hemocytometer and the suspension to be transplanted was diluted to an appropriate cell density of 50,000 cells/ μ l.

3.11 Transplantation procedure

10-week old C57BL/6NCrI mice were used in this study. Animals were distributed in 2 groups of 7 animals each, according to the culture conditions of the cells, Normoxia or Hypoxia. All animal procedures were accomplished following the guidelines of the local ethics committee of animal experimentation. Surgical procedures were performed under general anesthesia with 100 mg Ketamine and 5 mg diazepam per kilogram bodyweight. Additionally, the eye was topically anesthetized with 0.1% tetracaine and 0.4% oxybuprocaine. One drop of each 10% phenyleprine and 1% tropicamide were used to dilate the pupils. Following complete dilation, the anesthetized animal was placed in lateral recumbency under the SMZ-1 Nikon dissecting microscope and positioned with one hand holding mice. The mice fundus could be visualized with the application of a drop of 2.5% methylcellulose to the eye. The fundus observation served to evaluate the condition of the eye before injection and to compare with the postoperative condition of the retina. The needle with bevel up was advanced full thickness 1 mm posterior to the sclerocorneal limbus into the posterior chamber. At least 50% of the bevel was pushed through the choroid to produce a hole sufficiently large to insert the 33 gauge blunt needle (Hamilton Company, Reno, NV). The blunt needle tip was inserted through the choroidal puncture and advanced into the posterior chamber, avoiding trauma to ciliary body or lens. Subsequently, the needle shaft was aimed slightly nasally toward the posterior chamber and it was advanced toward the desired injection location in the posterior retina. A 10 μ l syringe (Hamilton, Switzerland) with a 33-gauge needle attached to an ultra-micropump (World Precision Instruments, Sarasota, FL) was used to inject 1,5 μ l of cell suspension (75,000 cells) slowly, at a rate of 0.05 μ l/second, into the subretinal space (SS) (Figure 3.7). Immediately after injection, the fundus was examined and any animals with massive subretinal hemorrhage or vitreous hemorrhage were removed from the study. Injected animals developed a retinal detachment and small amount of bleeding in the same area of

injection. Finally, a drop of antibiotic (0.3% ciprofloxacin) was administered on each eye and animals were kept on a 37°C pad until recovery from anesthesia.

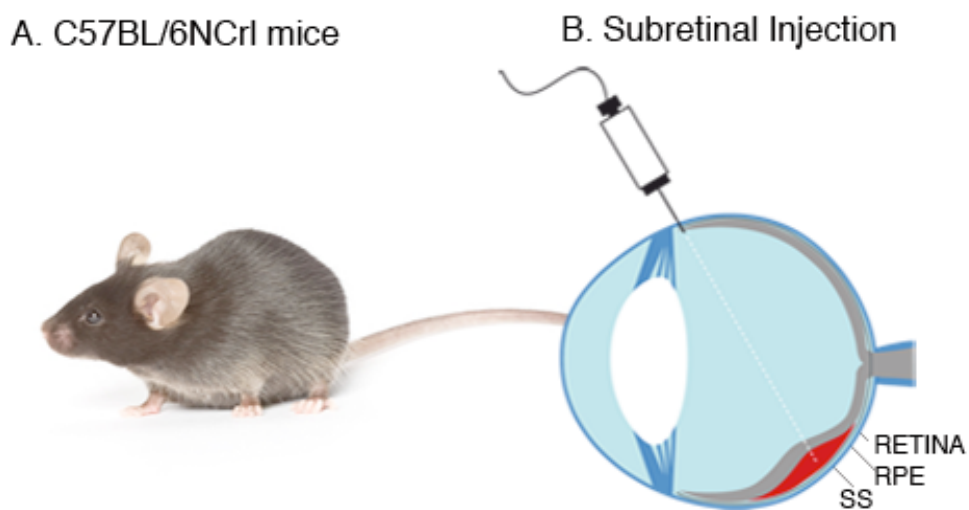


Figure 3.7. Transplantation procedure. A. Photograph of the mice used for the transplantation studies. B. Diagram showing the anatomical site where the subretinal injection was performed for the transplantation studies.

3.12 Tissue preparation

Animals were sacrificed by cervical dislocation after 24 hours and 1 and 4 weeks of transplantation. Eyes were enucleated and immediately fixed overnight at 4°C in freshly prepared 4% paraformaldehyde solution. Eyes were then washed in PBS and transferred into 30% sucrose in PBS solution for at least 12 hours before inclusion in OCT and cryosectioning. Retinal sections (18 μm) were mounted in SuperFrost Ultra Plus® slides (MENZEL-GLÄSER, Braunschweig, Germany) and stored at room temperature (RT) until further processing.

3.13 Immunohistochemical Analysis

Sections were blocked in PBS containing 10% goat serum and 0.1% Triton for 1 hour at RT and incubated with primary antibodies overnight at 4°C. Primary antibodies used are listed in Table 8 and Table 9. After incubation with primary antibodies, sections were

washed with PBS containing 0.1% triton and incubated with secondary antibodies for 1 hour at room temperature. After successive washing in PBS, nuclei were counterstained with DAPI (4',6-diamino-2-phenylindole, dilactate; Invitrogen-Molecular Probes, Eugene, OR). Immunofluorescence was observed using a Leica DM 5500 microscope (Leica Microsystems, Wetzlar Germany) and a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar Germany).

4. RESULTS

“Science never solves a problem without creating ten more.”

— **George Bernard Shaw**

4.1 Effect of Hypoxia on spontaneous differentiation

We wanted to assess whether hypoxic conditions could induce retinal fate of mESC. EBs were generated from ESC using the hanging drop method followed by culture in suspension under either normoxic or hypoxic conditions (Figure 3.2). Time course (day 0, day 5 and day 7) of spontaneous differentiation of EBs were analysed for expression of pluripotency markers along with other markers of retinal commitment. FACS analysis showed significant differences in the number of cells positive for pluripotency markers in hypoxic conditions when compared to the corresponding number under normoxia during the protocol (Figure 4.1 A).

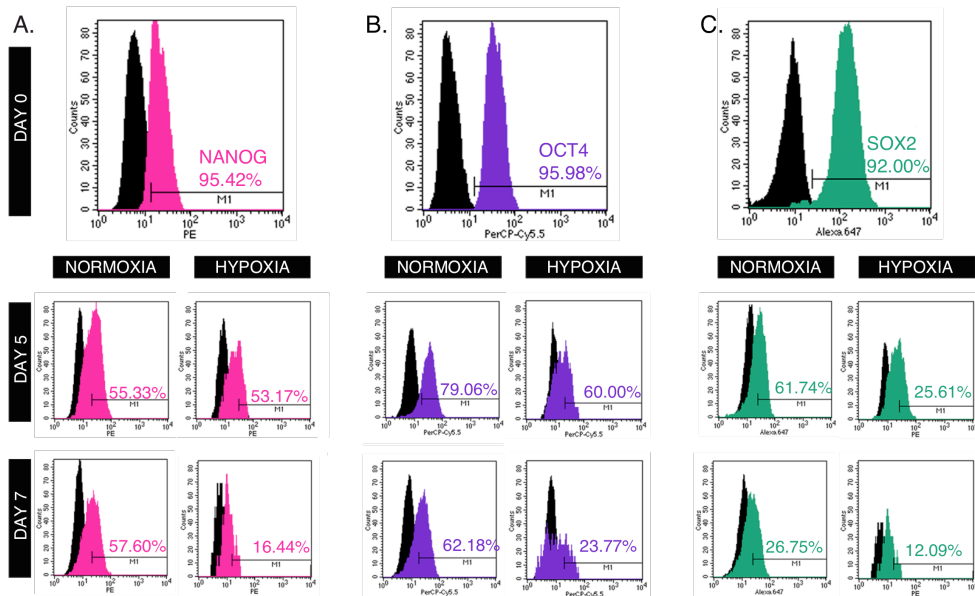


Figure 4.1. Effect of low oxygen tension during early spontaneous differentiation. FACS analysis showing the loss of pluripotency markers is more efficient after 7 days of spontaneous differentiation in low oxygen when compared to normoxia. A. Nanog. B. Oct4 and C. Sox2

When the FACS analysis data in hypoxia was normalized against normoxia, data showed a significant decrease of Nanog⁺ (46±18%), Oct4⁺ (50±14%) and Sox2⁺ (42±17%) cells after 7 days under hypoxic conditions (Figure 4.2.A). Besides, RT-PCR analysis demonstrated that retinal specific genes, such as Pax6 and Crx, were

significantly upregulated (4.9 ± 1.1 and 4.2 ± 0.6 fold, respectively) when EBs were cultured under hypoxia for 7 days compared to normoxia (Figure 4.3 B). The upregulation of Pax6 and Crx genes in cells cultured under hypoxia corresponded with the downregulation of pluripotency genes (Figure 4.3A) and the loss of pluripotency markers at a protein level as demonstrated by FACS analysis at this time point (Figure 4.1 and Figure 4.2).

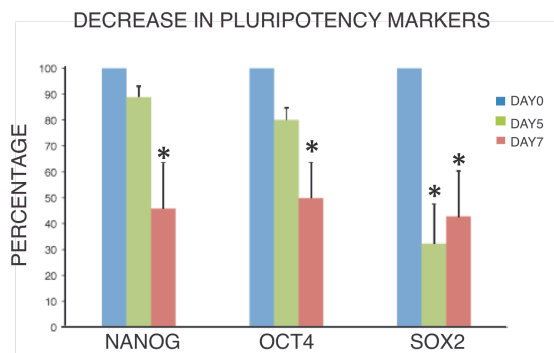


Figure 4.2. Low oxygen tension decrease pluripotency during early spontaneous differentiation. A. Decrease in number of positive cells for pluripotency markers under hypoxia when data was normalized against the normoxia values. * $p\leq 0.05$ was considered statistically significant.

Other retinal genes such as Six3, a well characterized eye field transcription factor (Liu et al., 2010), Chx10, a neural retina marker (Horsford et al., 2005) and Nrl, a photoreceptor precursor marker (Oh et al., 2007), showed a slight but not significant increase under hypoxia condition (Figure 4.3B). These results have shown that hypoxia significantly decreases pluripotency and increases the expression of different retinal genes in spontaneous differentiation.

A. DOWNREGULATION OF PLURIPOTENCY MARKERS B. UPREGULATION OF RETINAL MARKERS

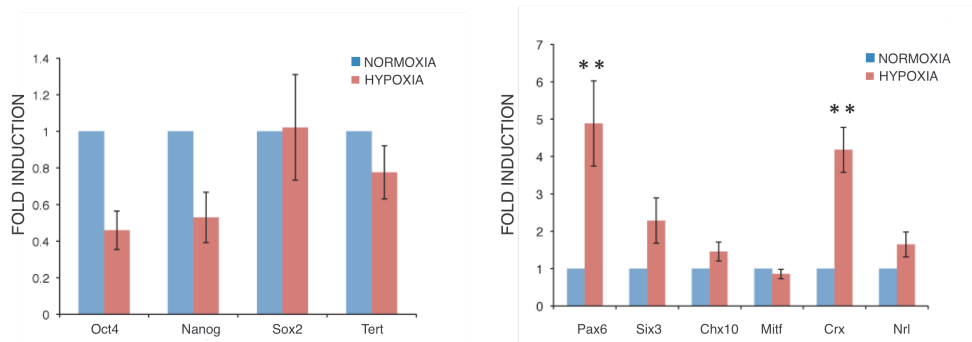


Figure 4.3 Effect of low oxygen tension during early spontaneous differentiation. **A.** RT-PCR analysis comparing the relative levels of expression of pluripotency marker genes after 7 days of spontaneous differentiation in normoxia (blue bar) and hypoxia (red bar). **B.** RT-PCR analysis comparing the relative levels of expression of retinal specific genes after 7 days of spontaneous differentiation in normoxia (blue bar) and hypoxia (red bar). * $p \leq 0.05$ or ** $p \leq 0.01$ was considered statistically significant.

4.2 Directed differentiation

The results observed with spontaneous differentiation led us to believe that hypoxic conditions could improve the differentiation of ESC towards a retinal fate, but obviously a more sophisticated protocol for retinal differentiation had to be applied. In order to demonstrate our hypothesis and corroborate whether we could obtain a higher yield of retinal cells, we have optimized a differentiation protocol that involves the culture of ESC under hypoxia (2% O_2), and the use of small molecules previously described by different authors (Ikeda, 2005; Osakada et al., 2008). Our 3-step approach is a novelty in differentiation protocol. We included EB step where EBs were generated as a hanging drops because previously published data show (Dang et al., 2002) that this is an efficient and scalable system which allows uniform distribution of culture parameters such as oxygen tension due to the homogeneous size of the generated EBs (Figure 4.5). Briefly, ES-cells characterized by the typical expression of Oct4, Nanog, Sox2 and SSEA-1 were induced to differentiate into neural retina cells or Chx10 positive cells and more mature phenotypes of retinal commitment, such as Rhodopsin in the case of rod cells, in a sequential manner

(Figure 4.4).

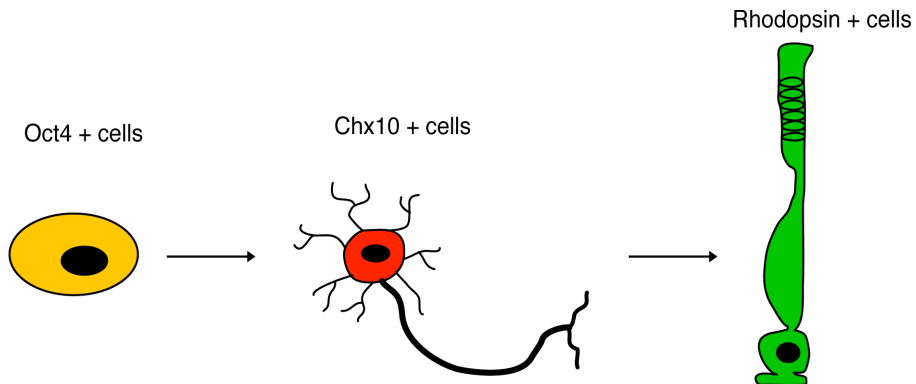


Figure 4.4. General Approach to obtain a stepwise differentiation protocol. Immunofluorescence analysis against pluripotency markers; OCT4, B. Ssea-1, C. Sox2 and D. Nanog before differentiation. E. FACS analysis showing levels of pluripotency markers Nanog, Oct4 and Sox2 in the undifferentiated cultures

To induce *in vitro* retinal differentiation, ESC were cultured as hanging drops for 3 days in the presence of Dkk1 and Lefty A. Hanging drops containing the EBs were collected and allowed to differentiate in suspension for 7 more days in progenitors medium, after which, EBs were plated and allowed to further differentiate for 18 more days in adherent culture in Retinal Medium (Figure 3.3 and 4.5). From Day3 to Day5 FBS and Activin A were added in culture. Different growth factors such as DAPT, Fibroblast growth factors (FGFs), Shh, RA and taurine were added to the protocol of differentiation as described in Materials and methods (Figure 3.3B) to promote differentiation toward photoreceptors (Ikeda, 2005; Osakada et al., 2008; Zhao et al., 2002).

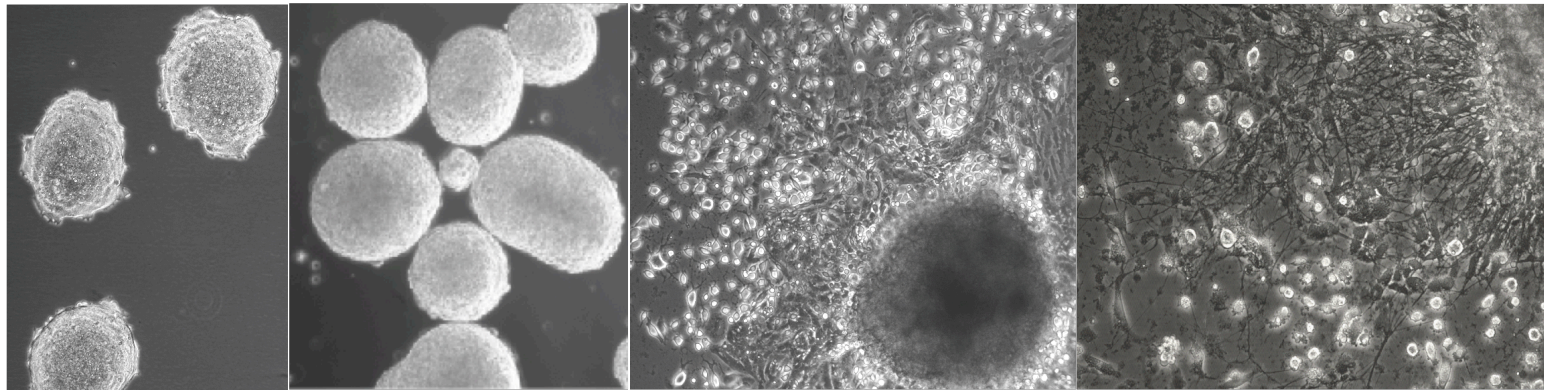
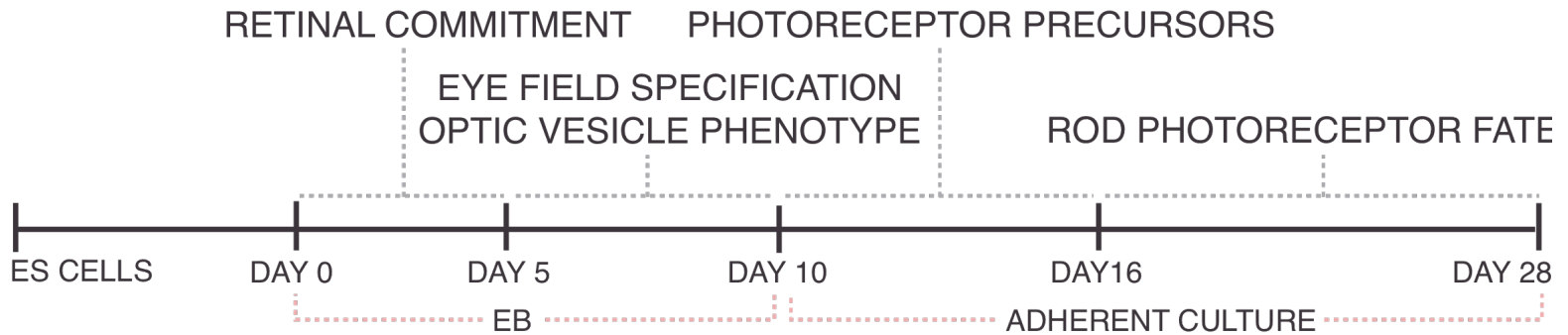


Figure 4.5. Photomicrographs of the different stages of retinogenesis in vitro. (Diagram of major stages of retinogenesis and the corresponding days of *in vitro* differentiation (Above). Photomicrographs of ES-D3 cell colonies, EBs, monolayer cultures deriving from cell clusters and monolayer cultures at day 22 of differentiation (Below).

All results were compared to ESC differentiated under normoxic conditions (20% O₂). During the 28 days of differentiation the expression of various transcription factors associated to each major stage of retinogenesis was analysed (Figure 4.6).

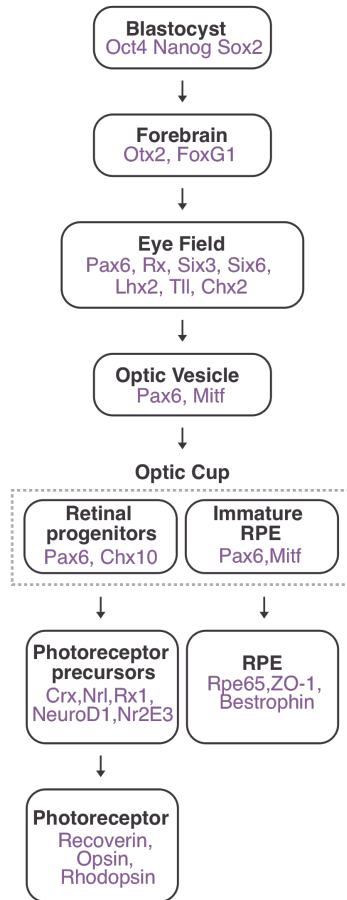


Figure. 4.6. Schematic of transcription factors associated to each major stage of retinogenesis.

4.3 Stemness, loss of pluripotency and eye field specification

Before the start of differentiation, undifferentiated ESC cultures were characterized. All cultures regardless of passage number showed normal morphology. ESC grew forming colonies and expressed pluripotency markers as expected; all colonies

were positive for Oct4, Ssea-1, Nanog and Sox2 as determined by immunofluorescence (Figure 4.7 A-D) and the level of expression of these markers was determined by FACS each time to assure the purity and stemness of the culture prior to differentiation. Nanog (98.4%) expression was higher than Oct4 (94.5%) and Sox2 (83.5%) but no levels lower than 80% were found in any passage of ESC used (18-35 passages, Figure. 4.7 E).

To direct mESC toward rostral fate, the EBs were treated with Dkk1, an antagonist of Wnt/ β -catenin signaling, the nodal antagonist, Lefty-A, and Activin A in presence of FBS. The loss of pluripotency, as a hallmark of ESC under differentiating pressure, was measured by RT-PCR. ESC rapidly lost expression of the pluripotency genes Oct4 and Nanog and even downregulated the neural induction marker Sox2 on day 5 (Figure 4.8). Sox2 levels of expression were higher than the levels of Oct4 and Nanog at all time points analysed and its expression was upregulated on day 10, corresponding to differentiation efforts towards ectoderm (Figure 4.8). Indeed, we have observed a majority of cells expressing the anterior neural marker Otx2, and a greater number of Otx2 positive cells in hypoxia (Figure 4.10B), which is consistent with a developmental *in vivo* study (Baumer et al., 2003) (Figure 4.10 A).

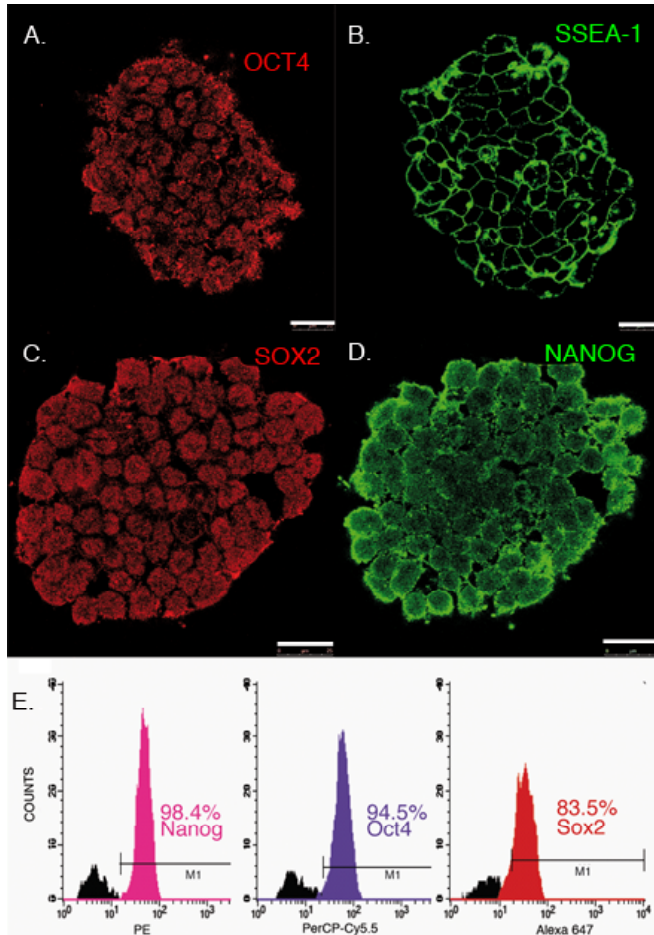


Figure. 4.7. Stemness characterization. A. Immunofluorescence analysis against pluripotency markers; OCT4, B. Ssea-1, C. Sox2 and D. Nanog before differentiation. E. FACS analysis showing levels of pluripotency markers Nanog, Oct4 and Sox2 in the undifferentiated cultures

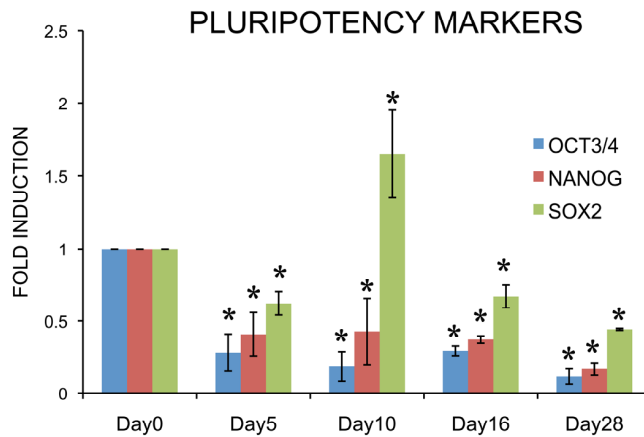


Figure 4.8. Loss of pluripotency. RT-PCR analysis showing efficient loss of pluripotency markers. * $p \leq 0.05$ was considered statistically significant.

Furthermore, ESC acquired the expression of transcription factors associated with

eye field specification. RT-PCR analysis revealed that Six3, gene required for early eye field specification, as well as Pax6 and Rax expression was upregulated as early as on day 5 under hypoxia (Figure 4.9A, 4.9B and 4.9C respectively).

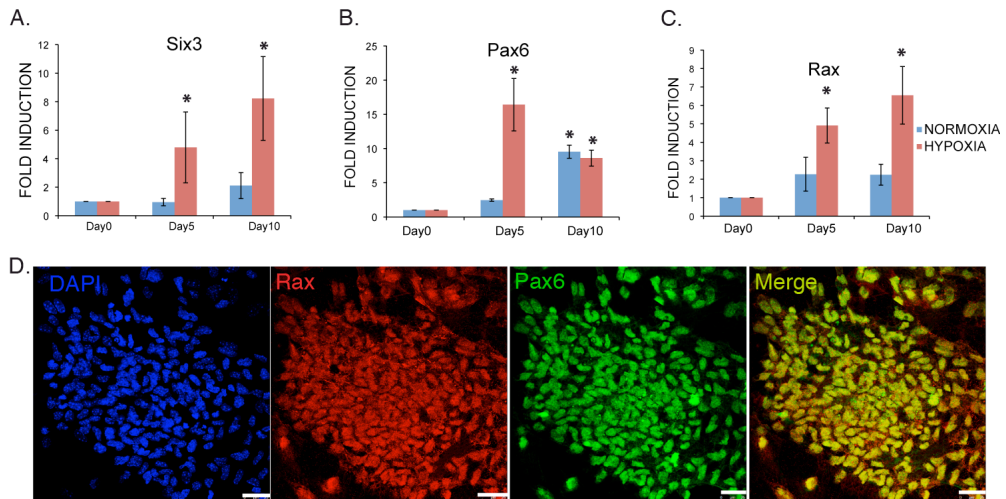


Figure 4.9. Hypoxia improves eye field specification. A.B.C. The time course expression of the eye field transcription factors Six3, Pax6 and Rax by RT-PCR analysis. D. Immunofluorescence analysis showing Rax positive cells colocalize with Pax6 after 10 days of differentiation under hypoxia. $*p \leq 0.05$ was considered statistically significant.

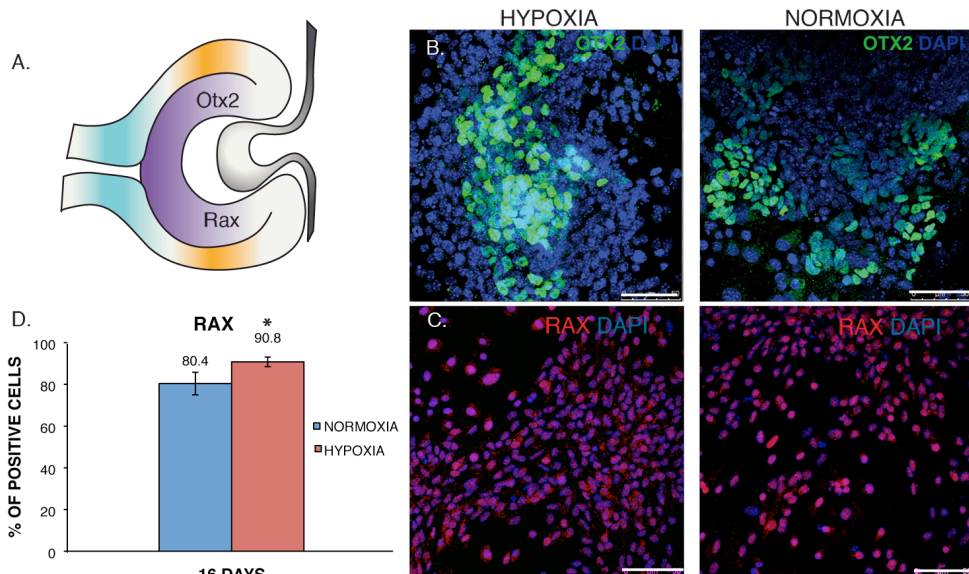


Figure 4.10. Hypoxia improves early retinogenesis. A. Schematic of neural retina (purple) on embryonic day 14, where the differentiating cells are expressing the transcription factors Otx2 and Rax. B. Comparative immunofluorescence analysis showing positive cells for anterior neural specification marker OTX2 under hypoxia and normoxia. C. Comparative immunofluorescence analysis showing positive cells for the eye field transcription factor Rax under hypoxia and normoxia. D. Quantification of Rax positive cells derived under normoxic (blue bar) and hypoxic condition (red bar). $*p \leq 0.05$ was

considered statistically significant.

Eye field cells are characterized by coexpression of Pax6 and Rax (Mathers et al., 1997; Mathers and Jamrich, 2000) (Figure 4.9D). As a matter of fact, on day 10 of the differentiation protocol high expression of Rax coincided with the high expression of Pax6. Not only the expression of these markers was upregulated earlier using our protocol but the levels of expression of each individual gene at their peak of expression were higher under hypoxia when compared to normoxia (Figures 4.9B and 4.9C). Additionally, by day 16, hypoxia significantly increased the number of Rax⁺ cells (91% of total cells vs. 80% in normoxia; Figure 4.10C and 4.10D). On day 10 the fold induction of Six3 in hypoxia was 6 times higher than in normoxia (8.2±2.9 against 2.1±0.91 fold change. Figure 4.9A). RT-PCR analysis on day 5 revealed a difference of 14 fold change of Pax6 gene expression under hypoxia (16.4±3.8 against 2.4±0.2; Figure 4.9B) and the expression of Rax was induced 4 times (6.6±1.5 against 2.2±0.6; Figure 4.9C) above the level observed in normoxia on day 10. Our results showed that hypoxic conditions significantly increased the expression of the eye field genes.

4.4 Retinal commitment, Optic vesicle and Optic cup phenotype

To induce *in vitro* differentiation, ESC were cultured in suspension as EBs for 10 days in progenitors medium after which, EBs were plated and allowed to differentiate for another 18 days in adherent culture in Retinal Medium (Figure 4.5). The next stage of *in vivo* retinal specification occurs when optic vesicles from the paired eye fields are formed. At this stage all cells express the transcription factors Mitf and Pax6 and give rise to either neural retina or retinal pigment epithelium (Figure 4.6; (Bharti et al., 2008).

Furthermore, the fact that Mitf positive cells destined to become neural retina derive from the subset of Rax positive cells (Fujimura et al., 2009; Horsford et al., 2005) was confirmed with coexpression of these two markers in culture (Figure 4.11).

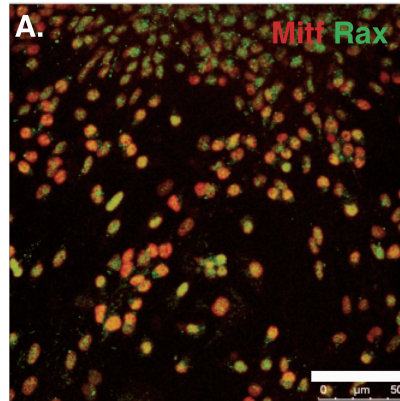


Figure 4.11 Hypoxia modulated optic vesicle phenotype towards neural retina commitment. A. Immunocytochemical analysis showing that all Rax positive cells were also positive for Mitf by day 16 under hypoxia.

In fact, RT-PCR analysis revealed that Mitf expression was upregulated since day 5 coinciding with the peak of expression of Pax6 (Figure 4.9B). During development, Mitf is downregulated in response to the onset of Chx10, a neural retina specific gene (Horsford et al., 2005). The dynamic expression of Mitf and Chx10 was examined over time and it was determined that neural retina phenotype was acquired as early as day 16 (Figure 4.12A and 4.12B).

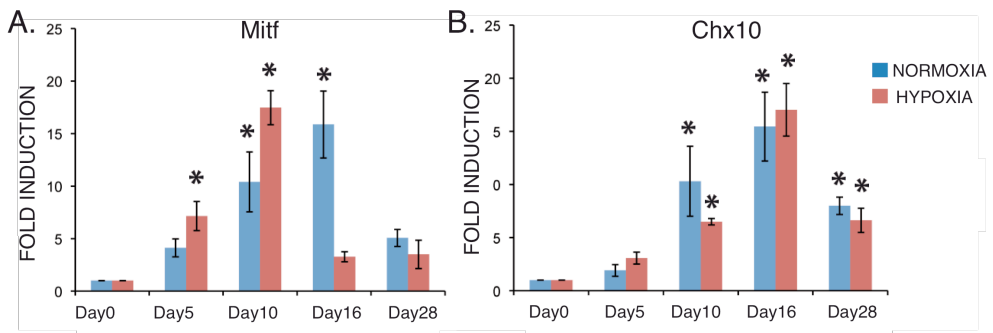


Figure 4.12 Hypoxia promotes the neural retina commitment. The time course of expression of the neural retina transcription factors, Mitf (A.) and Chx10 (B.), respectively, by RT-PCR in normoxia (blue

bar) and hypoxia (red bar). * $p \leq 0.05$ was considered statistically significant.

Also, our cells at day 16 coexpressed Mitf and Rax (Figure 4.11A), revealing RPE commitment (Osakada et al., 2009), while the clusters (Figure 4.13A, 4.13B and 4.13C) formed by day 16, of Chx10 positive cells maintaining expression of Pax6 revealed their commitment to retinal fate (Figure 4.13B and 4.13C). This corresponded with the peak of expression of Chx10 as checked by RT-PCR (Figure 4.12B).

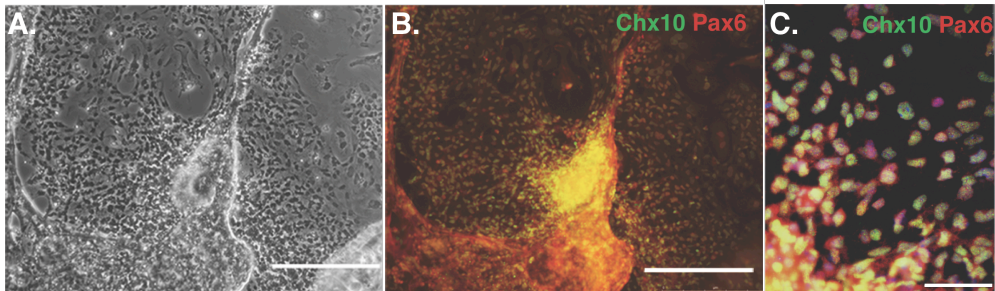


Figure 4.13 Hypoxia improves neural retina phenotype acquisition. **A.** Photomicrograph showing clusters formed by day 16 of differentiation. **B.** Immunocytochemical analysis showed Chx10+/Pax6+ cells deriving radially away from the clusters by day 16 of differentiation. **C.** Higher magnification showing Chx10 positive cells were also positive for Pax6.

4.5 Photoreceptor fate

Prolonged culture of Chx10⁺ and Pax6⁺ cells allowed for further maturation of these cells towards a photoreceptor phenotype. A crucial gene for rod specification is Nrl (Mears et al., 2001), highly induced since day 16 under hypoxia (Figure 4.14A) coinciding with high expression of Crx (Figure 4.14B) as observed by RT-PCR analysis.

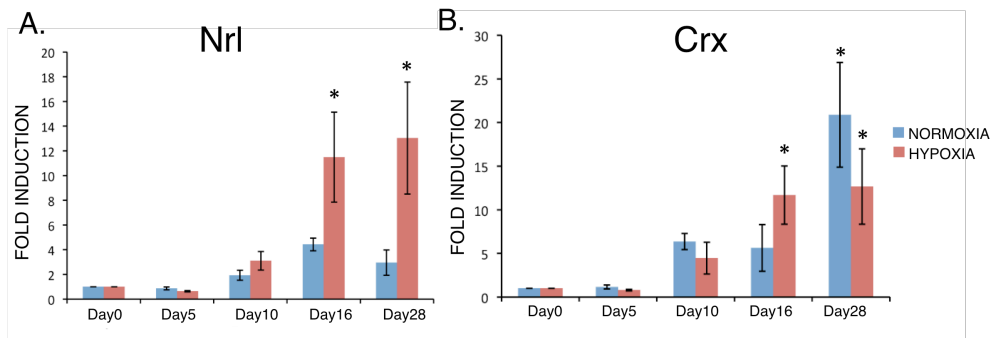


Figure 4.14. Hypoxia induces rod photoreceptor specific transcription factors. A. Comparative RT-PCR analysis of *Nrl* gene expression during differentiation in normoxic (blue bar) and hypoxic (red bar) conditions. B. Comparative RT-PCR analysis of *Crx* gene expression over the 28 days of differentiation under normoxia (Blue bars) and hypoxia (red bars). * $p \leq 0.05$ was considered statistically significant.

These data was confirmed by immunocytochemistry analysis for these two markers

at the end of the protocol where a high number of cells have shown to be positive for *Crx* (Figure 4.15A and 4.15B) and *Nrl* (Figure 4.17A). The importance of this finding is because during development *Nrl* interacts with *Crx* to induce the expression of rod specific genes such as Rhodopsin (Swaroop et al., 2010). As it has been mentioned, the primitive cone and rod photoreceptor-specific transcription factor *Crx* was upregulated since day 16 as well (Figure 4.14B), and the immunoreactive cells against *Crx* antibody accounted for 75% of the total cells ($75.1 \pm 1.9\%$ in hypoxia vs. $64.4 \pm 2.3\%$ in normoxia; Figure 4.15B) at the end of the protocol.

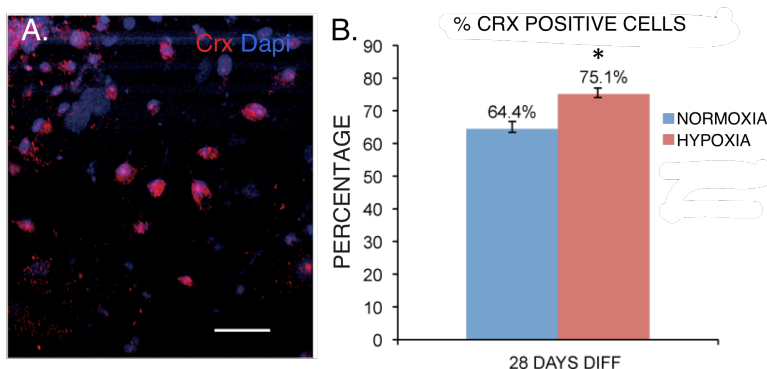


Figure 4.15 Hypoxia promotes photoreceptor phenotype. A. Immunocytochemical analysis showing *Crx*⁺ cells in hypoxic culture by day 28 of differentiation. B. Quantification of the percentage of *Crx*⁺ cells determined by 3 independent experiments under both conditions of differentiation. * $p \leq 0.05$ was

considered statistically significant.

Moreover, it seems that our differentiation protocol applying normoxia did not promote high yield of cone cells because from the Crx⁺ population, only about 8% ($8.1 \pm 5.5\%$) of cells were immunoreactive for the cone photoreceptor-specific protein Opsin-S (Figure 4.16A and 4.16B).

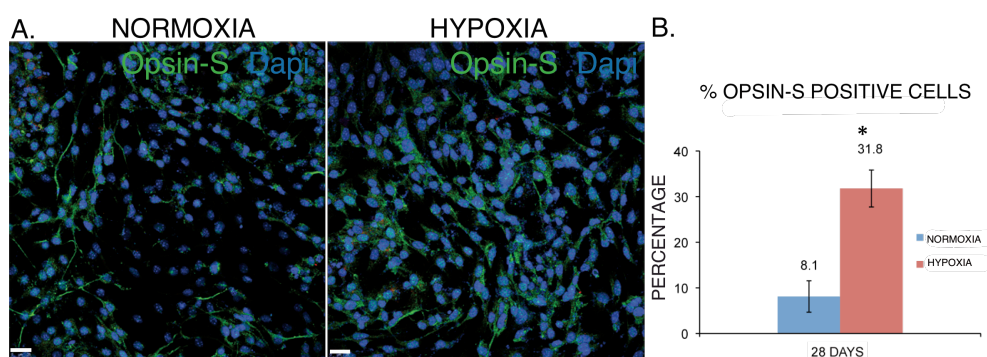


Figure 4.16. Hypoxia improves cone differentiation A. Comparative immunofluorescence analysis showing positive cells for cone specific marker Opsin-S after 28 days of differentiation under normoxia or hypoxia. B. Quantification of the percentage of Opsin⁺ cells in normoxic (blue bar) and hypoxic (red bar) conditions. * $p \leq 0.05$ was considered statistically significant.

On the other hand, the percentage of Opsin-S⁺ cells was significantly increased to 32% of total cells under hypoxic conditions ($31.8 \pm 11.6\%$; Figure 4.16A and 4.16B). Consistently, differentiation of ESC in hypoxic conditions significantly increased their retinal commitment toward rod photoreceptors, as determined by coexpression of Rhodopsin and Recoverin at the end of the protocol (Figure 4.17B and 4.18A), when approximately 53% of total cells showed double positive staining for these markers (53% in hypoxia against 30% in normoxia. (52.9 ± 1.5 against 29.4 ± 3.5 ; Figure 4.17C).

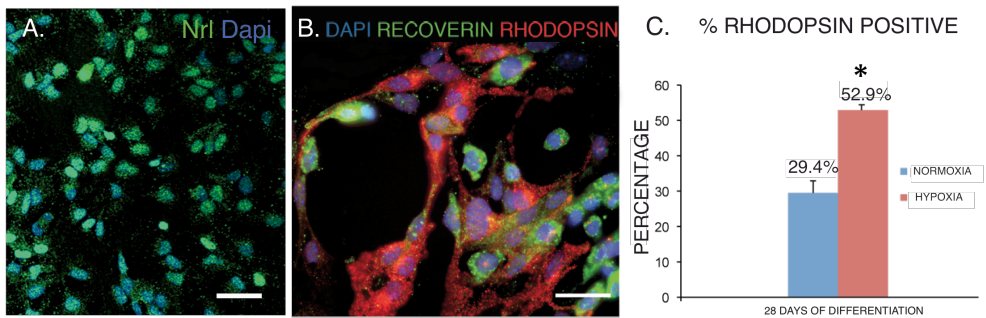


Figure 4.17. Hypoxia increases the yield of Rhodopsin positive cells. A. Immunocytochemical analysis showing Nrl+ cells in hypoxic culture by day 28 of differentiation. B. Immunocytochemical analysis showing the coexpression of rod photoreceptor markers Rhodopsin and Recoverin after 28 days in hypoxic culture conditions. C. Quantification of the percentage of Rhodopsin+ cells in normoxic (blue bar) and hypoxic (red bar) conditions. * $p \leq 0.05$ was considered statistically significant.

The induction of Rhodopsin by hypoxia was confirmed by RT-PCR. The analysis showed significant increase in fold change of Rhodopsin since day 16 in hypoxia (28.5 ± 3.6 vs. 11.1 ± 2.9) compared to normoxia (Figure 4.18B).

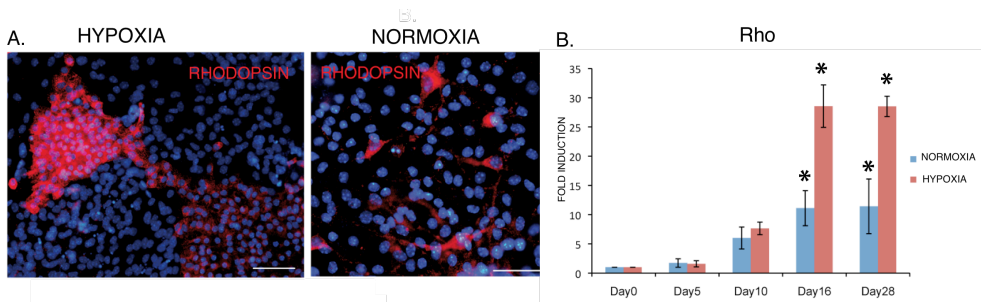


Figure 4.18. Hypoxia induces Rhodopsin expression. A. Comparative immunofluorescence analysis of expression of Rhodopsin during the differentiation in hypoxic and normoxic conditions. B. Comparative RT-PCR analysis of Rhodopsin gene expression during the differentiation in normoxic (blue bars) and hypoxic conditions (red bars). * $p \leq 0.05$ was considered statistically significant.

Recoverin expression did not localize with Tuj1 indicating the presence of immature neurons and mature photoreceptors in our culture (Figure 4.19A). This data reveals that the generated photoreceptors are not immature neurons but mature retinal cells.

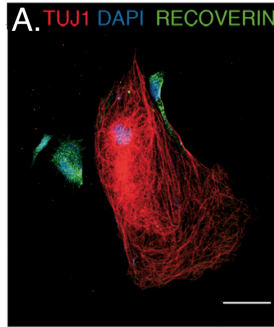


Figure 4.19. Inmature neurons are present in our protocol of differentiation. A. Immunofluorescence analysis of other cell types present after 28 days of differentiation Tuj1+/Recoverin- cells.

Furthermore, using hypoxic conditions, morphology typical of rods was observed in isolated cases, in sections of the culture where cell density allowed for the outgrowth of structures that suggest the formation of outer segments *in vitro* (Figure 4.20A and 4.20B).

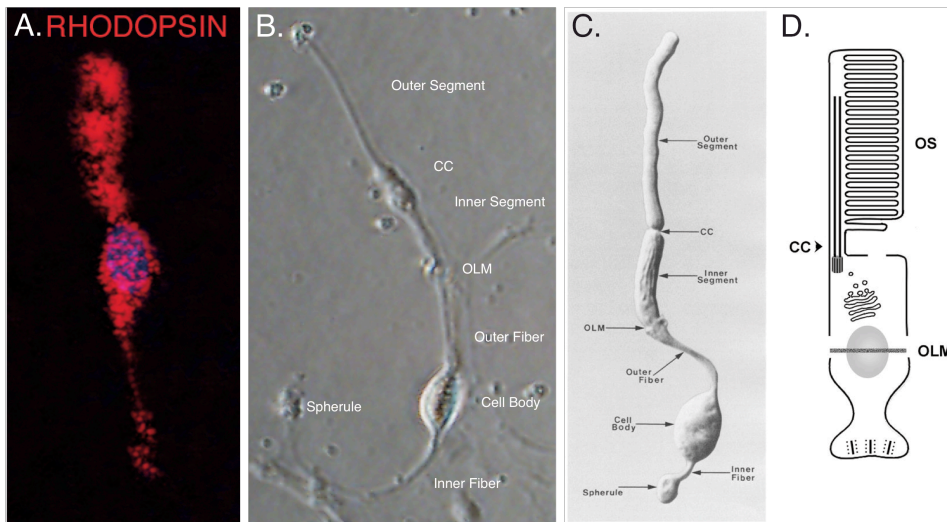


Figure 4.20. Hypoxia promoted rod-like morphology. A. A representative Rhodopsin+ cell in hypoxic conditions. B. Bright field image showing the rod-like morphology in a representative cell in hypoxic cultures. C. Electron microscopy photograph of an isolated rod photoreceptors from the rabbit retina. D. Schematic of the anatomical features of a Rod photoreceptor, C and D are taken from (Kennedy and Malicki, 2009).

To assess the proliferation capacity of differentiated cells, we stained the cells after 28 days of differentiation against the operational marker, Ki67 (Scholzen and Gerdes, 2000). We have found that after 28 days of differentiation under hypoxic

conditions, there was a significant decrease in the number of proliferating cells ($14.8 \pm 1.8\%$) against the amount of proliferating cells differentiated under normoxia over the same time (22.8 ± 1.7 , Figure 4.21A and 4.21B).

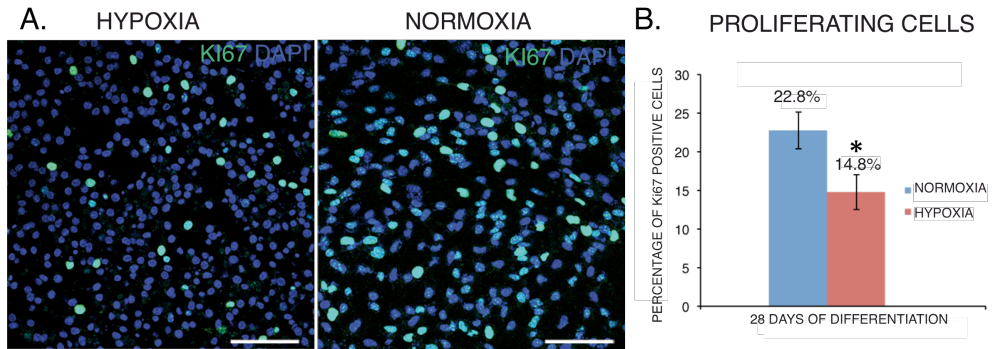


Figure 4.21. Proliferation is reduced in hypoxia. A. Comparative immunofluorescence analysis of expression of Ki67 during the differentiation in hypoxic and normoxic conditions. B. Quantification of the percentage of Ki67+ cells in normoxic (blue bar) and hypoxic (red bar) conditions. * $p \leq 0.05$ was considered statistically significant.

All cell phenotypes generated at the end of our protocol are depicted in Figure 4.22.

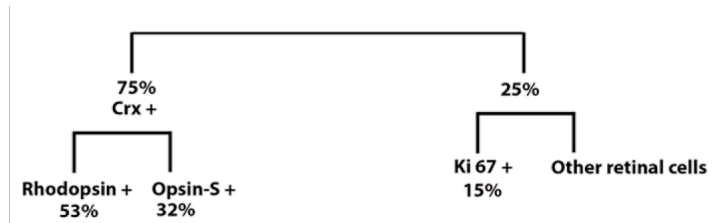


Figure 4.22. Schematic representation of obtained retinal cells.

To assess the possible mechanisms underlying the increased differentiation capacity of mESC in hypoxia, we observed that, a higher proliferation rate was not related with an increased yield of Rho^+ cells. Hence, we did not observe any coexpression of Ki67 and Rho after 28 days of differentiation, neither under hypoxic or normoxic conditions (Figure 4.23A). Furthermore, this phenomena was also observed in other cellular models, RPE-1 cells showed a significant decrease in their proliferation capacity after 3 weeks of hypoxic culture, as determined by the percentage of Ki67 + cells in culture and compared to the number found in normoxic

conditions (Figure 4.23B).

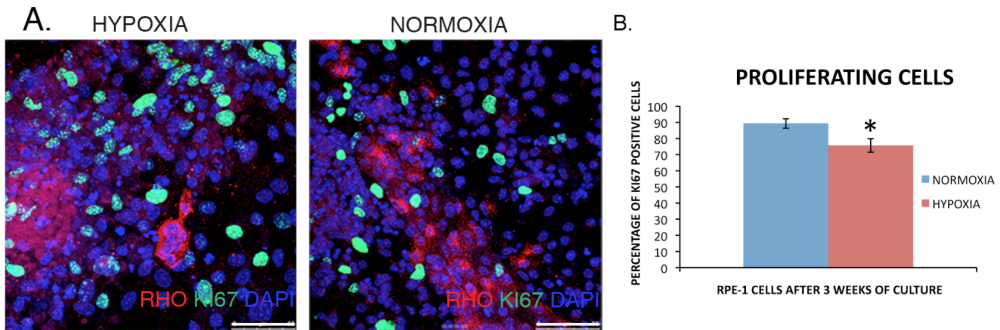


Figure 4.23. Poliferative cells are not Rhodopsin positive A. Comparative immunofluorescence analysis of expression of Rhodopsin and Ki67 positive cells during the differentiation in hypoxic and normoxic conditions of ESC. B. Quantification of the percentage of RPE-1 cells positive for Ki67 after 3 weeks of culture in normoxic (blue bar) and hypoxic (red bar) conditions. * $p \leq 0.05$ was considered statistically significant.

To assess the safety of low oxygen tension in our cultures we performed cytotoxicity and apoptosis (presented by Caspase 3/7 activity) tests and found that, after 1 week of hypoxic culture, RPE-1 cells showed a significant decrease in cytotoxicity and Caspase 3/7 activity compared to normoxia (Figure 4.24A and 4.24B).

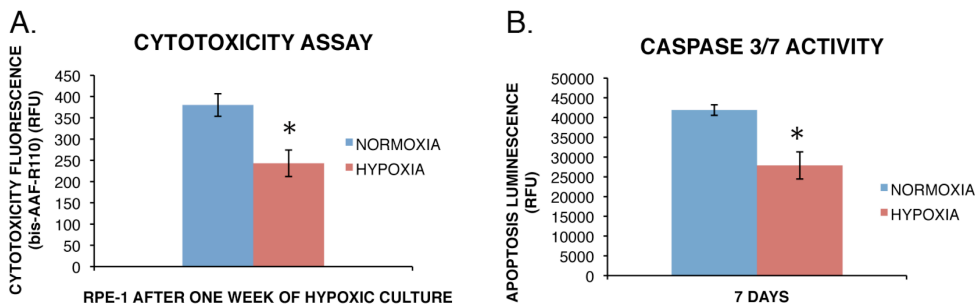


Figure 4.24. Hypoxia reduces cytotoxicity and apoptosis. A. Cytotoxicity assay showing a decrease in toxicity in RPE-1 cells after one week in hypoxia (red bar) when compared to normoxia (blue bar). B. Apoptosis assay showing a decrease in caspase 3/7 activity when RPE-1 cells were cultured for one week in hypoxia (red bar) compared to normoxia (blue bar). * $p \leq 0.05$ was considered statistically significant.

To correlate hypoxia and retinal development we followed the expression of Vegfa and Cdkn1a. The expression of these two genes is very well-known to be induced under hypoxia and recently they have been directly related with retinal hypoxia

(Crosson et al., 2009; Ishikawa et al., 2010). We observed that hypoxia significantly increased the fold induction of Vegfa (6.1 ± 1.0 in hypoxia vs. 4.6 ± 0.7 in normoxia; Figure 4.25A) and Cdkn1a (11.7 ± 1.0 in hypoxia vs. 5.5 ± 1.1 in normoxia; Figure 4.25B) at day 28 of differentiation, revealing possible mechanisms through which hypoxia promotes retinal development.

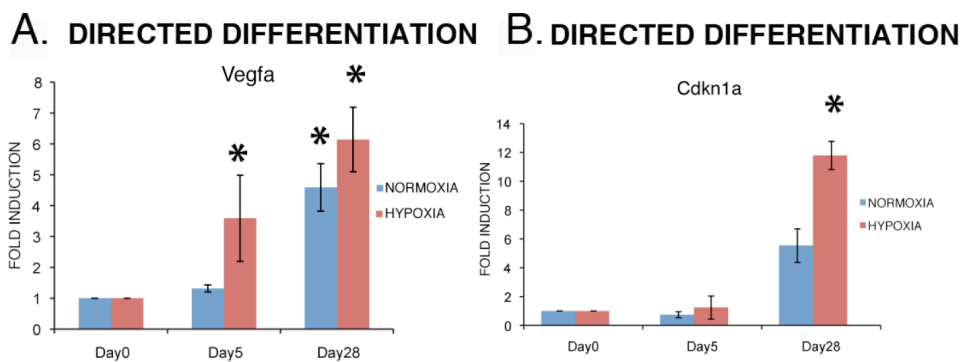


Figure 4.25 Hypoxia induces the expression of targets of Hif signaling pathway. A. RT-PCR analysis showing upregulation of Vegfa gene after 5 and 28 days of differentiation in normoxia (blue bar) and hypoxia (red bar). B. RT-PCR analysis showing upregulation of Cdkn1a gene after 5 and 28 days of differentiation in normoxia (blue bar) and hypoxia (red bar). * $p \leq 0.05$ was considered statistically significant.

The exposure of cells to hypoxia leads to the activation and stabilization of hypoxia inducible factor Hif1 α as determined by FACS analysis. The percentage of Hif1 α cells in RPE-1 cells was higher after 3 weeks of hypoxia when compared to normoxia culture (69.6 ± 4.18 vs 55.6 ± 10.4 ; Figure 4.26A) and significantly higher in D3-mESC cells after 1 week of hypoxia in the presence of LIF (79.9 ± 11.5 vs. 49.1 ± 10.7 ; Figure 4.26B).

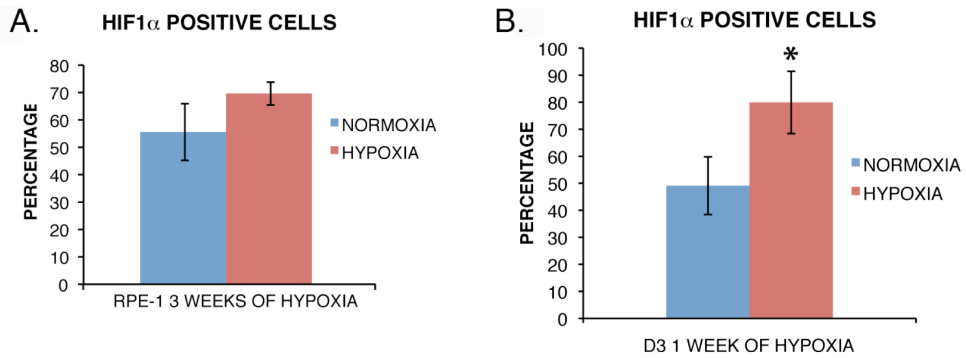


Figure 4.26. Hypoxia stabilizes Hif1 α . A. FACS analysis showing the percentage of Hif1 α positive cells in RPE-1 cells after 3 weeks of culture under normoxic (blue bar) and hypoxic (red bar) conditions. Graph corresponds to the mean of 3 independent experiments. B. Percentage of Hif1a positive cells in D3-mESC cells after 1 week of culture in presence of LIF under normoxic (blue bar) and hypoxic (red bar) conditions determined by FACS analysis. Graph corresponds to the mean of 3 independent experiments. * $p \leq 0.05$ was considered statistically significant.

4.6 Subretinal injection

To evaluate whether injected cells in suspension, generated using our protocol under hypoxia could survive and integrate *in vivo*, C57BL6/NCrl mice received unilateral subretinal injections of 75000 retinal cells differentiated until day 20 or medium alone (sham). The uninjected eye served as internal control for each animal. The PKH26 staining applied prior to transplantation was used to identify surviving retinal progenitors after 24 hours and 1 week of the injection (Figure 4.28A 4.28C, 4.28E, 4.29A, 4.29C 4.29E and 4.30A) The location of the main graft was subretinal in 7 eyes with a large cluster of transplanted cells localized between the host photoreceptors and the RPE layer.

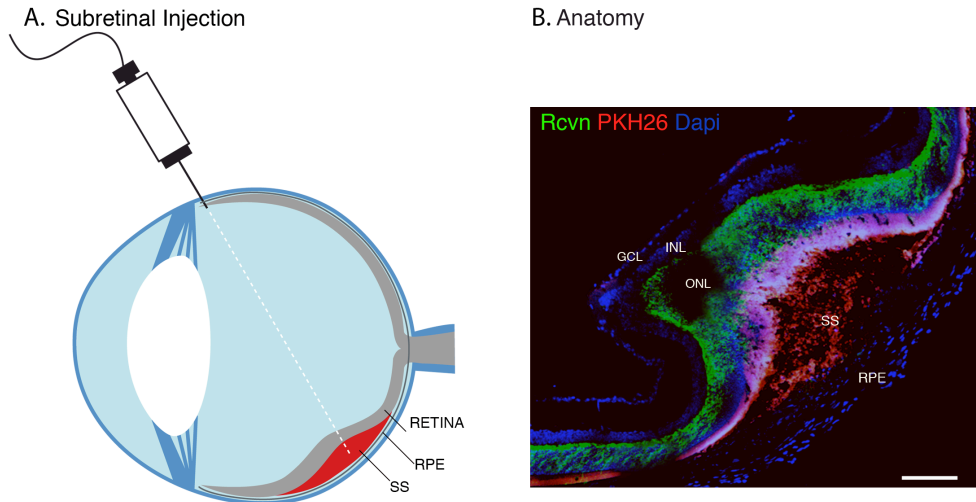


Figure 4.27 Transplanted cells were efficiently delivered into the subretinal space. A. Diagram showing the details of the subretinal injection used to deliver the transplanted cells. B. Immunohistochemical analysis showing the anatomy of the injection site after 24 hours of transplantation with Rcvn+ cells in green, transplanted PKH26+ cells in red and Dapi in blue.

EFFICIENCY OF DIFFERENTIATION

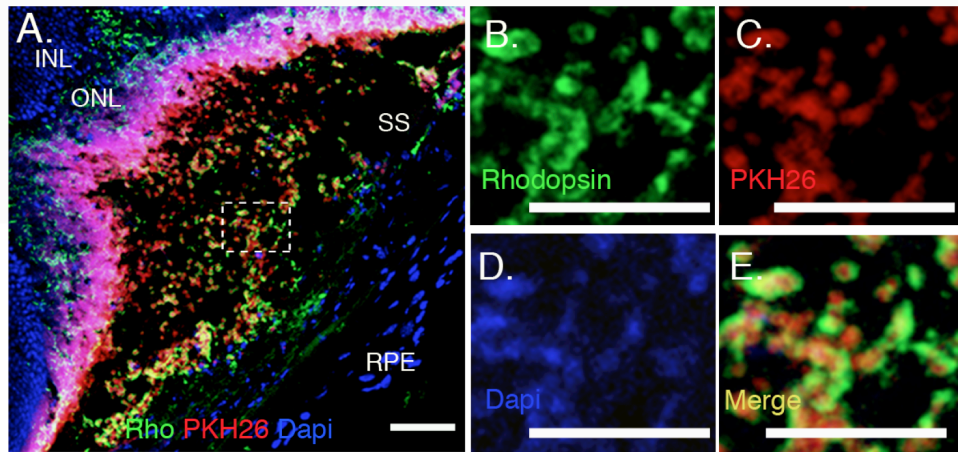


Figure 4.28 Transplanted photoreceptors derived from ESC under hypoxic conditions expressed Rhodopsin. A. Anatomy of the injection site and efficiency of differentiation 24 hours after transplantation. B. C. D and E. Higher magnification of inset in A. Coexpression of transplanted cells (red, PKH26), Rhodopsin+ (green) and DAPI (blue).

Immunohistochemical analysis demonstrated dispersed transplanted cells positive for Rhodopsin (Figure 4.28A, 4.28B, 4.28E and 4.30A) and Recoverin (Figure 4.29A, 4.29B and 4.29E) across the retina, singly or in small clusters. A large

number of donor cells injected in subretinal locations migrated and integrated in host retina.

EFFICIENCY OF DIFFERENTIATION

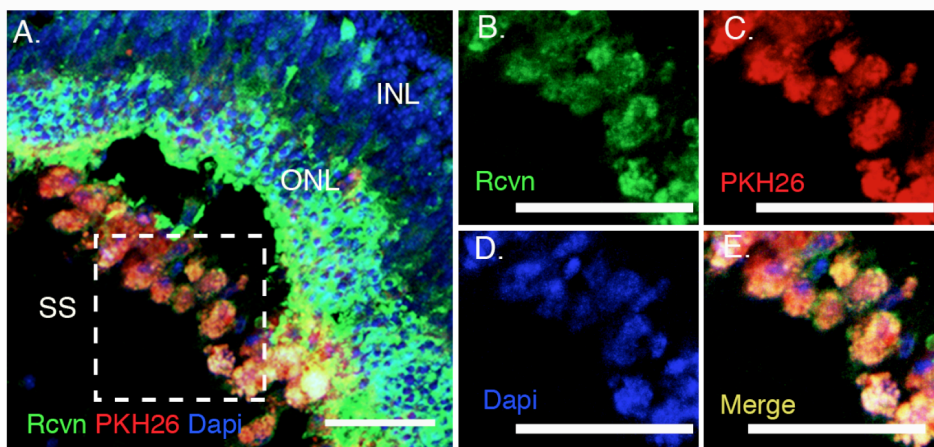


Figure 4.29 Transplanted photoreceptors derived from ESC under hypoxic conditions expressed Recoverin. A. The efficiency of differentiation towards photoreceptors 1 week after transplantation. B. C. D and E. Higher magnification of inset in A. showing transplanted cells (red, PKH26) colocalizing with Recoverin (Rcvn, green) and DAPI (blue).

Transplanted cells immunopositive for photoreceptor marker Rhodopsin and Recoverin were found in outer nuclear layer (ONL) and inner nuclear layer (INL) in the mice (Figure 4.30A).

MIGRATION / INTEGRATION

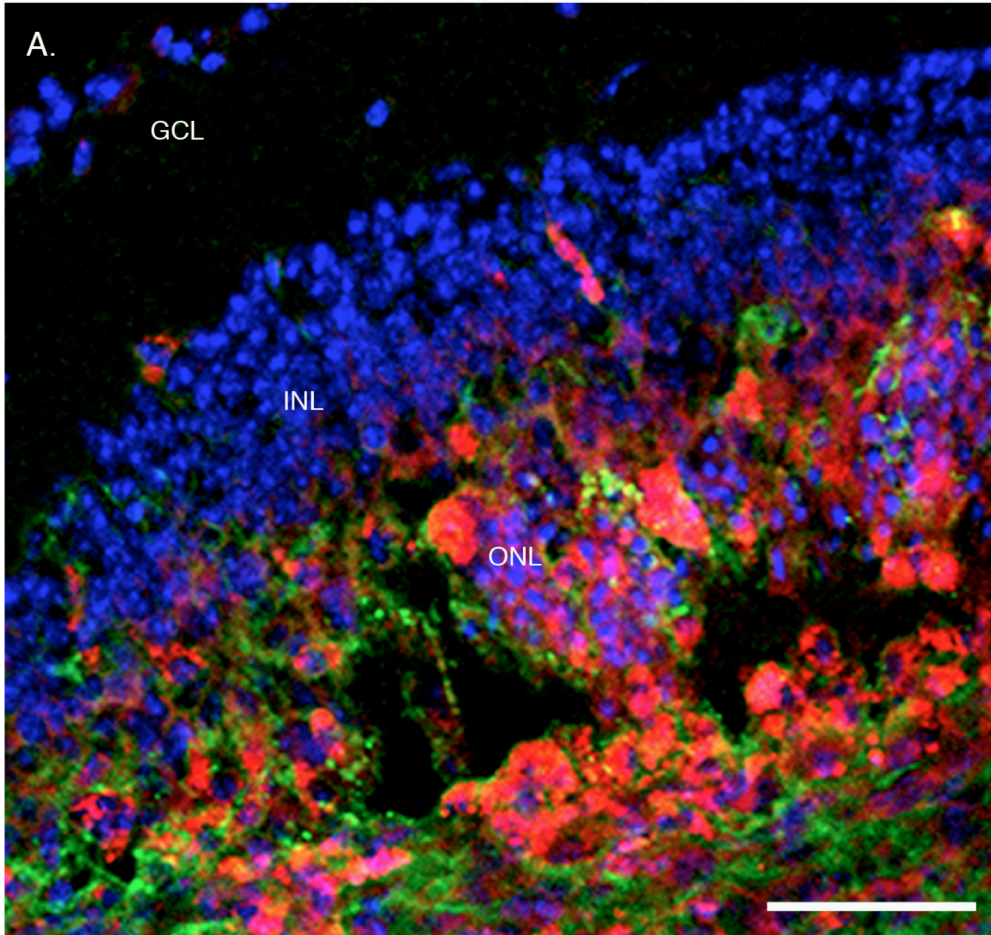


Figure 4.30 Transplanted photoreceptors migrated and integrated within the host retina. A. Colocalization of PKH26 and Rhodopsin in ONL 1 week after subretinal injection.

A much smaller fraction of transplanted cells was Opsin-S positive revealing the presence of cones.

There was no evidence of uncontrolled growth or tumor formation at any time, suggesting that donor cell proliferation might be regulated or balanced by cell death.

INTEGRATION OF TRANSPLANTED CELLS WITHIN THE HOST RETINA

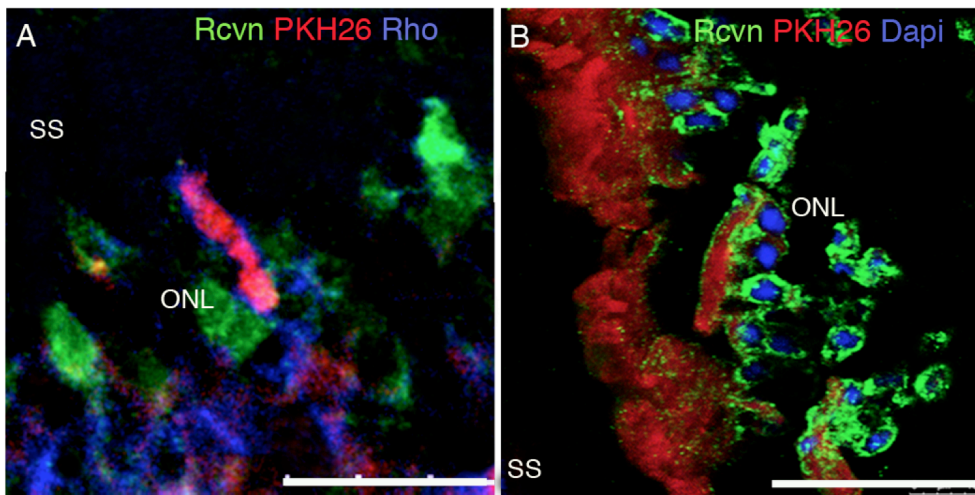


Figure 4.31. Integration of transplanted cells within the host retina. A. High magnification of immunofluorescence analysis performed in animals transplanted with retinal cells differentiated under hypoxia, showing integration in the outer nuclear layer (ONL) of cells positive for Rcvn and Rhodopsin. B. Integration of transplanted cells was confirmed when analysis for Dapi was performed and the transplanted cells were positive for Rcvn and were localized in the ONL among the other host photoreceptors. SS- subretinal space.

5. DISCUSSION

ESC offer an excellent *in vitro* tool to recapitulate mechanisms activated during early development. The efficient differentiation of retinal cells from ESC is a major challenge for the development of successful cell therapy, which can be applied in different retinal dystrophies such as Retinitis pigmentosa (RP) and age related macular disease (AMD). Although the early stages of development occur in a hypoxic environment, little is known about how low O₂ levels modulate the pluripotency and differentiation capacity of ESC. Our data demonstrate that mESC can be efficiently directed to retinal progenitors and other mature phenotypes, such as photoreceptors, by using a combination of small molecules and lowering O₂ tension can enhance this efficiency. Different protocols of generation of retinal phenotypes have been published and all of them have implied important advances in the field [17, 22]. Our main issue continues to be the accomplishment of a high yield of specific populations and the modeling of retinogenesis *in vitro*.

In the first phase of our protocol we used the combination of Dkk1, Activin A and LeftyA to direct mESC toward rostral neural progenitors, applying an approach used in a previous study [33]. This strategy is widely used to generate rostral neural progenitors [49-52]. Indeed, we observed high percentage of Rax⁺ and Otx2⁺, rostral neural progenitors and markers for early eye field. These two markers together with Pax6, Six3, Six6 and Lhx2 play an important role in the establishment of anterior neuroectodermal region which maintains high capacity for generation of future retinal progenitors [39, 53, 54]. Significant upregulation of some of these markers (Six3, Pax6 and Rax) was observed as early as by day 5 in culture. Large increase of the eye field markers coincided with rapid decrease of main pluripotency markers indicating high differentiation potential of our protocol. Our results have shown that lowering the O₂ tension near the physiological level is a more effective parameter for retinal differentiation, especially for photoreceptor precursors. It seems that the effects of this parameter are expressed very early in differentiation significantly increasing the expression of Six3, Pax6 and Rax (Fig. 3E, 3F and 3G).

The next phase in retinal specification *in vivo* occurs with the formation of the optic vesicle, determined mainly by the expression of *Mitf* and *Pax6* giving rise to multiple cell types of the functional retina. Cells coexpressing *Pax6* and *Chx10* give rise to neural retina only. Experiments using different vertebrates indicated that SHH and FGF signaling play a critical role in future specification of retinal cells [55-57]. Once neural retina progenitor phenotype has been acquired, it is necessary for further maturation of these cells. As shown by others, Notch signaling pathway needs to be blocked at this point to allow for an increase in the *Crx*⁺ cells [17]. For these reasons we supplemented the medium with the J-secretase inhibitor DAPT from day 10. Retinoic acid and taurine were added to obtain mature photoreceptors [17, 31]. Hence, further retinal specification included DAPT, FGFs, *Shh*, RA and Taurine. These conditions together with low O₂ tension significantly increase the population of cells coexpressing *Pax6* and *Chx10* as well as *Crx*⁺ cells compared to normoxia. High percentage of derived photoreceptors, reflected by coexpression of Rhodopsin and Recoverin for rods, and Opsin-S for cones, revealed that low O₂ tension promoted a photoreceptor fate of mESC. With regard to efficiency, the induction of rods, namely the yield of Rhodopsin⁺ cells, was higher when compared with other protocols using murine ESC [17, 31] or iPS [15, 58, 59]. The results in the present study, are consistent with the recently published study applying hypoxic condition [60], describing an increased yield of *Pax6* and *Chx10* positive cells. Here we further define the characterization of generated cells achieving a higher yield of mature retinal phenotypes as well as *in vivo* study. Our results have shown not only that the population of retinal cells can be increased under hypoxia in a way that mimics normal retinal development, but also, we demonstrate for the first time that, early rostral differentiation (*Otx2*), early eye field acquisition (*Six3*, *Rax* and *Pax6*) and mature retinal phenotypes (*Crx*, Opsin-S and Rhodopsin) are increased in yield under hypoxia. Furthermore, hypoxic condition seems to improve the timing of retinogenesis, as it has been observed by RT-PCR analysis. Eye

field transcription factors are highly expressed as early as by day 5 and the important suppression of *Mitf* by upregulation of *Chx10* occurred by day 16 instead of day 28 only when the cells differentiated under hypoxia. This allowed for a bigger population of neural retina progenitors earlier in the differentiation protocol that could mature into cells expressing photoreceptor markers, such as, *Crx*, *Nrl* and *Rhodopsin*, all three populations highly increased at the end of our protocol.

Our study went further showing efficient *in vivo* evaluation of generated retinal precursors. Previous studies have shown that photoreceptors taken from young animals efficiently incorporate in adult retina when transplanted in the sub-retinal space [25, 61]. We also successfully grafted *in vitro* generated retinal cells in the adult mouse retina, which resulted in cell survival. Interestingly, high percentage of transplanted cells expressed rod specific markers, such as *Rhodopsin* and *Recoverin*, though without known possible implication of local environment on further differentiation. It seems that specific retinal niche was preferable for mature differentiation of retinal progenitors.

This data demonstrates the viability of the cells and the robustness of our protocol. However, to fully validate the protocol further *in vivo* functional analyses have to be performed. Different studies on pluripotent stem cells have demonstrated improved differentiation when different hypoxic conditions were applied [60, 62]. The exact mechanism of hypoxia on retinal differentiation still remains to be elucidated. The primary transcriptional regulators of cellular hypoxic adaptation in mammals are hypoxia induced factors (HIFs). Hypoxic preconditioning was shown to stabilize HIF-1 α in the retina, further inducing the expression of target genes with neuroprotective properties like vascular endothelial growth factor (*Vegfa*) and erythropoietin (*Epo*) suggesting a link between HIF-1 α driven gene expression and neuroprotection [63, 64]. Little is known about the molecular effects of hypoxia on retinal differentiation. The published reports mainly correlate the expression of individual genes and hypoxia in different retinal functions [47].

For example, hypoxia increases vascular endothelial growth factor (Vegfa) expression in the retina [60, 63]. The identification of this gene together with p21 (Cdkn1a) is strongly suggestive of their role in general retinal neuroprotection [65, 66]. During our differentiation protocol under hypoxic conditions both genes were significantly upregulated when compared to normoxic conditions. This data suggests that hypoxia, through activation of HIF-1D, decreasing apoptosis and cytotoxicity, has influenced retinal differentiation [67, 68]. Although, the source of Vegfa could be RPE cells observed in retinal progenitors generated in hypoxic conditions [69] (data not shown), further investigation has to be performed to elucidate the origin of neuroprotective processes. Moreover, a significant decrease of proliferating cells at the end of our protocol in hypoxic conditions, suggests that hypoxia favors postmitotic cells, increasing therefore the number of photoreceptors (Figure 4.22 and Figure 4.21B). It is known that p21, increased in hypoxia (Figure 4.25B) not only provokes cell cycle arrest, necessary to start the differentiation process, but can also repress apoptosis [70] (Figure 4.24B). Therefore, we propose that hypoxia promotes retinal differentiation through activation of p21. Higher yield of early eye field markers in hypoxic condition suggests that hypoxia is preferable for EB formation and early differentiation. This data corroborates with earlier findings where an improved differentiation of human ES cells was observed in hypoxic conditions [71, 72]. We also observed a more compact structure of EBs in hypoxia (data not shown), which could have a consequence on further differentiation. For this to be confirmed, further detailed studies have to be performed.

5. CONCLUSIONS

“Lead us from darkness to light”

The discussion of the life force ‘Prana’,
Brihadaranyaka Upanishad (1.3.28) (800–2000 bc)

The present study provides the technical framework necessary for a highly efficient differentiation of mESC towards photoreceptors, which is important for advances in cell therapy and regenerative medicine. Given the results obtained with this work, we can conclude the following

We believe the application of a new modified protocol for differentiation of mESC reported here support the hypothesis that hypoxia is necessary to induce efficient differentiation of ESC towards a higher yield of retinal phenotypes. The timing of retinogenesis is also improved in hypoxic conditions, by decreasing the time to acquire an eye field phenotype and achieve mature population of photoreceptors *in vitro*. Purification of these specific retinal cells can allow us to define the conditions to expand a homogeneous population that will be further differentiated into fully mature photoreceptor cells. Further experimentation is required to elucidate the precise mechanism or mechanisms by which hypoxia exerts its effect on retinal differentiation. In summary, the novel findings of the work reported here are: 1. The most efficient protocol so far, for the differentiation of any kind of stem cells (mouse, human or induced-pluripotent cells), towards rod photoreceptor cells ($53\pm 1.5\%$). 2. The modeling of retinogenesis has been accomplished for the first time with mESC only under hypoxic conditions. 3. Photoreceptor precursors from mESC differentiate towards Rhodopsin/Recoverin double positive cells after transplantation in the retina, and a complete lack of tumor formation, demonstrates the importance of an efficient differentiation process and the loss of pluripotency of the transplanted cells.

7. FUTURE PERSPECTIVES

“The scientist is not a person who gives the right answers, he's one
who asks the right questions.”

— **Claude Lévi-Strauss**

model disease in vitro using our model

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9. ANNEXES

9.1 Glossary

9.2 Protocols of differentiation

9.3 Publications

9.4 Awards and honors

ANNEX 9.1 GLOSSARY

Adult stem cells (Somatic stem cells)

Stem cells found in different tissues of the developed, adult organism that remain in an undifferentiated state with a limited capacity for both self-renewal and differentiation. Such cells vary in their differentiation capacity, but it is usually limited to cell types in the organ of origin. This is an active area of investigation.

Allogeneic transplantation

Cell, tissue or organ transplants from one member of a species to a genetically different member of the same species.

Autologous transplantation

Cell, tissue or organ transplants from one individual back to the same individual. Such transplants do not induce an immune response and are not rejected.

Blastocyst

A very early embryo consisting of approximately 150 cells produced by cell division after fertilization. The blastocyst is a spherical cell mass produced by cleavage of the zygote. It contains a fluid-filled cavity, a cluster of cells called the inner cell mass and an outer layer of cells called the trophoblast, which forms the placenta).

Bone marrow stromal cell

Also known as mesenchymal stem cells, bone marrow stromal cells are a mixed population of cells derived from the non-blood forming fraction of bone marrow. Bone marrow stromal cells are capable of growth and differentiation into a number of different cell types including bone, cartilage and fat.

Cell culture

Growth of cells *in vitro* in an artificial medium for research or medical treatment.

Cell division

Method by which a single cell divides to create two cells. There are two main types of cell division depending on what happens to the chromosomes: mitosis and meiosis.

Cell line

Cells that can be maintained and grown in culture and display an immortal or indefinite life span.

Cell type

A specific subset of cells within the body, defined by their appearance, location and function.

i) **adipocyte**: the functional cell type of fat, or adipose tissue, that is found throughout the body, particularly under the skin. Adipocytes store and synthesize fat for energy, thermal regulation and cushioning against mechanical shock.

ii) cardiomyocytes: the functional muscle cell type of the heart that allows it to beat continuously and rhythmically.

iii) chondrocyte: the functional cell type that makes cartilage for joints, ear canals, trachea, epiglottis, larynx, the discs between vertebrae and the ends of ribs.

iv) fibroblast: a connective or support cell found within most tissues of the body. Fibroblasts provide an instructive support scaffold to help the functional cell types of a specific organ perform correctly.

v) hepatocyte: the functional cell type of the liver that makes enzymes for detoxifying metabolic waste, destroying red blood cells and reclaiming their constituents, and the synthesis of proteins for the blood plasma.

vi) hematopoietic cell: the functional cell type that makes blood. Hematopoietic cells are found within the bone marrow of adults. In the fetus, hematopoietic cells are found within the liver, spleen, bone marrow and support tissues surrounding the fetus in the womb.

vii) myocyte: the functional cell type of muscles.

viii) neuron: the functional cell type of the brain that is specialized in conducting impulses.

ix) osteoblast: the functional cell type responsible for making bone.

x) islet cell: the functional cell of the pancreas that is responsible for secreting insulin, glucagon, gastrin and somatostatin. Together, these molecules regulate a number of processes including carbohydrate and fat metabolism, blood glucose levels and acid secretions into the stomach.

Cell-based therapies

Treatment in which stem cells are induced to differentiate into the specific cell type required to repair damaged or destroyed cells or tissues.

Chromosome

A structure consisting of DNA and regulatory proteins found in the nucleus of the cell. The DNA in the nucleus is usually divided up among several chromosomes. The number of chromosomes in the nucleus varies depending on the species of the organism. Humans have 46 chromosomes.

Cloning

In biology, the process in which an organism produces one or more genetically identical copies of itself by asexual means.

The term also refers to creating multiple copies of a product such as a fragment of DNA.

1. In reference to DNA: To clone a gene, one finds the region where the gene resides on the DNA and copies that section of the DNA using laboratory techniques.
2. In reference to cells grown in a tissue culture dish: a clone is a line of cells that is genetically identical to the originating cell. This cloned line is produced by cell division (mitosis) of the original cell.
3. In reference to organisms: Many natural clones are produced by plants and (mostly invertebrate) animals. The term clone may also be used to refer to an animal produced by somatic cell nuclear transfer (SCNT) or parthenogenesis.

Culture medium

The liquid that covers cells in a culture dish and contains nutrients to nourish and support the cells. Culture medium may also include growth factors added to produce desired changes in the cells.

Differentiation

The process whereby an unspecialized embryonic cell acquires the features of a specialized cell such as a heart, liver, or muscle cell. Differentiation is controlled by the interaction of a cell's genes with the physical and chemical conditions outside the cell, usually through signaling pathways involving proteins embedded in the cell surface.

Directed differentiation

The manipulation of stem cell culture conditions to induce differentiation into a particular cell type.

Ectoderm

The outermost of three germ layers of the early embryo that gives rise in later development to the skin, cells of the amnion and chorion, nervous system, enamel of the teeth, lens of the eye and neural crest.

Embryo

The product of a fertilized egg, from the zygote until the end of the eighth week of gestation, when it is called a fetus.

Embryoid bodies

Spheroid colonies seen in culture produced by the growth of embryonic stem cells in suspension. Embryoid bodies are of mixed cell types, and the distribution and timing of the appearance of specific cell types corresponds to that observed within the embryo.

Embryonic germline cells

Embryonic germline cells, also called EG cells, are pluripotent stem cells derived from the primitive germline cells (those cells that give rise to eggs and sperm). Their properties are similar to those of embryonic stem cells.

Embryonic stem cell

Also called ES cells, embryonic stem cells are cells derived from the inner cell mass of developing blastocysts. An ES cell is self-renewing (can replicate itself), pluripotent (can form all cell types found in the body) and theoretically is immortal.

Endoderm

The inner of three germ layers of the early embryo that gives rise in later development to tissues such as the lungs, the intestine, the liver and the pancreas.

Epigenetic

Having to do with the process by which regulatory proteins can turn genes on or off in a way that can be passed on during cell division.

Fetus

The stage in development from the end of the embryonic stage, 7-8 weeks after fertilization, to developed organism that ends at birth.

Gamete

An egg (in the female) or sperm (in the male) cell.

Gastrulation

The process in which cells proliferate and migrate within the embryo to transform the inner cell mass of the blastocyst stage into an embryo containing all three primary germ layers.

Gene

A functional unit of heredity that is a segment of DNA found on chromosomes in the nucleus of a cell. Genes direct the formation of an enzyme or other protein.

Germ layers

The three germ layers are the endoderm, mesoderm and ectoderm and are the three precursory tissue layers of the early, primitive embryo (which form at approximately two weeks in the human) that give rise to all tissues of the body.

Heterologous

Not homologous or uniform. In the context of cells, heterologous is a mixed or divergent cell population or of a divergent origin.

Histocompatible

A tissue or organ from a donor (the person giving the organ or tissue) that will not be rejected by the recipient (the patient in whom the tissue or organ is transplanted). Rejection is caused because the immune system of the recipient sees the transplanted organ or tissue as foreign and tries to destroy it. Tissues from most people are not histocompatible with other people. In siblings, the probability of histocompatibility is higher, while identical twins are almost always histocompatible.

Homologous

Similar or uniform, often used in the context of genes and DNA sequences. In the context of stem cells, the term homologous recombination is a technique used to disable a gene in embryonic stem cells.

Homologous recombination

A technique used to inactivate a gene and determine its function in a living animal. The process of homologous recombination is more efficient in embryonic stem cells than in other cell types. It is achieved by introducing a stretch of DNA that is similar or identical (homologous) to part of a gene and to some of the DNA surrounding the gene, but different (not homologous) to a specific section of the gene. The DNA is then introduced into the stem cells and the stretch of homologous DNA will recognize the similar sequences of the gene within the cell, and replace it. But the cell is then left with a piece of DNA in the gene that has the wrong sequence and this interrupts the function of the gene. The gene is then said to be knocked out. From these embryonic stem cells, an entire mouse can be made by injecting the altered stem cells into a blastocyst, and implanting the blastocyst into a female mouse. This is one way to make genetically manipulated mice and other animals with altered gene function. These experiments are crucial to understand how specific genes work and interact in living animals.

Human embryonic stem cell

A stem cell that is derived from the inner cell mass of a blastocyst and can differentiate into several tissue types in a dish. They are similar to embryonic stem cells from the mouse; however, in the mouse, it is possible to inject those cells into a blastocyst, to make a new mouse, while this is not, and should not, be possible in humans for ethical reasons. Human embryonic stem cells are harder to grow than mouse embryonic stem cells.

Induced pluripotent stem cell (iPSC)

A type of pluripotent stem cell, similar to an embryonic stem cell, formed by the introduction of certain embryonic genes into a somatic cell.

Inner cell mass

A small group of cells attached to the wall of the blastocyst (the embryo at a very early stage of development that looks like a hollow ball). Embryonic stem cells are made by isolating and culturing the cells that make up the inner cell mass. In development, it is the inner cell mass that will eventually give rise to all the organs and tissues of the future embryo and fetus, but do not give rise to the extra-embryonic tissues, such as the placenta.

In vitro

Latin for "in glass"; in a laboratory dish or test tube; an artificial environment.

***In vitro* fertilization**

A procedure where an egg cell (the oocyte) and sperm cells are brought together in a dish (i.e. *in vitro*), so that a sperm cell can fertilize the egg. The resulting fertilized egg, called a zygote, will start dividing and after a several divisions, forms the embryo that can be implanted into the womb of a woman and give rise to pregnancy.

Mesenchymal stem cell

Also known as bone marrow stromal cells, mesenchymal stem cells are rare cells, mainly found in the bone marrow, that can give rise to a large

number of tissue types such as bone, cartilage (the lining of joints), fat tissue, and connective tissue (tissue that is in between organs and structures in the body).

Mesoderm

The middle of three germ layers that gives rise later in development to such tissues as muscle, bone, connective tissue, kidneys and blood.

Microenvironment

The molecules and compounds such as nutrients and growth factors in the fluid surrounding a cell in an organism or in the laboratory, which play an important role in determining the characteristics of the cell.

Mitosis

The type of cell division that allows a population of cells to increase its numbers or to maintain its numbers. The number of chromosomes remains the same in this type of cell division.

Morphology

Study of the shape and visual appearance of cells, tissues and organs.

Multipotent stem cells

Stem cells whose progeny are of multiple differentiated cell types, but all within a particular tissue, organ, or physiological system. For example, blood-forming (hematopoietic) stem cells are single multipotent cells that can produce all cell types that are normal components of the blood.

Neural stem cell

A type of stem cell that resides in the brain, which can make new nerve cells (called neurons) and other cells that support nerve cells (called glia). In the adult, neural stem cells can be found in very specific and very small areas of the brain where replacement of nerve cells is seen.

Neurons

Nerve cells, the principal functional units of the nervous system. A neuron consists of a cell body and its processes—an axon and one or more dendrites. Neurons transmit information to other neurons or cells by releasing neurotransmitters at synapses.

Nuclear transfer

A technique in which an egg has its original nucleus removed and exchanged for the nucleus of a donor cell. The egg now has the same nuclear DNA, or genetic material, as the donor cell. Nuclear transfer is also referred to as somatic cell nuclear transfer (SCNT), as the donor cell is usually a somatic cell (that is, any cell of the body except sperm and egg cells).

Nucleus

A part of the cell, situated more or less in the middle of the cell, that is surrounded by a specialized membrane and contains the DNA of the cell. This DNA is packaged into structures called chromosomes, which is the genetic, inherited material of cells.

Oligopotent progenitor cells

Progenitor cells that can produce more than one type of mature cell. An example is the myeloid progenitor cell which can give rise to mature blood cells, including blood granulocytes, monocytes, red blood cells, platelets, basophiles, eosinophiles and dendritic cells, but not T lymphocytes, B lymphocytes, or natural killer cells.

Parthenogenesis

A form of reproduction where an egg develops without the fusion of sperm with the egg cell. Parthenogenesis occurs commonly among insects and other arthropods. Artificially inducing parthenogenesis with human eggs may be a means to isolate stem cells from an embryo, without fertilization.

Passage

In cell culture, the process in which cells are disassociated, washed, and seeded into new culture vessels after a round of cell growth and proliferation. The number of passages a line of cultured cells has gone through is an indication of its age and expected stability.

Plasticity

A phenomenon used to describe a cell that is capable of becoming a specialized cell type of different tissue. For example, when the same stem cell can make both new blood cells and new muscle cells.

Phenotype

The description of the characteristics of a cell, a tissue or an animal; as black and white fur of a mouse are two phenotypes that can be found. The phenotype is determined by the genes (or the genotype) and by the environment. For example, short stature is a phenotype that can be genetically determined (and therefore inherited from the parents), but can also be caused by malnourishment during childhood (and therefore be caused by the environment).

Pluripotent

The state of a single cell that is capable of differentiating into all tissues of an organism, but not alone capable of sustaining full organismal development.

Scientists demonstrate pluripotency by providing evidence of stable developmental potential, even after prolonged culture, to form derivatives of all three embryonic germ layers from the progeny of a single cell and to generate a teratoma after injection into an immunosuppressed mouse.

Pluripotent stem cells

Stem cells that can become all the cell types that are found in an implanted embryo, fetus, or developed organism, but not embryonic components of the trophoblast and placenta (these are usually called extra-embryonic).

Post-implantation embryo

Implanted embryos in the early stages of development until the establishment of the body plan of a developed organism with identifiable tissues and organs.

Pre-implantation embryos

Fertilized eggs (zygotes) and all of the developmental stages up to, but not beyond, the blastocyst stage.

Progenitor cell

A progenitor cell, often confused with stem cell, is an early descendant of a stem cell that can only differentiate, but it cannot renew itself anymore. In contrast, a stem cell can renew itself (make more stem cells by cell division) or it can differentiate (divide and with each cell division evolve more and more into different types of cells). A progenitor cell is often more limited in the kinds of cells it can become than a stem cell. In scientific terms, it is said that progenitor cells are more differentiated than stem cells.

Proliferation

Expansion of the number of cells by the continuous division of single cells into two identical daughter cells.

Regenerative medicine

A field of medicine devoted to treatments in which stem cells are induced to differentiate into the specific cell type required to repair damaged or destroyed cell populations or tissues by aging or disease.

Reproductive cloning

The transfer into the uterus of an embryo derived by nuclear transfer with the intent to establish a pregnancy. Off-spring would be genetically identical to the donor of the transferred nucleus. A range of animals have been generated by reproductive cloning. The first mammal to be created by reproductive cloning was Dolly the sheep, born at the Roslin Institute in Scotland in 1996.

Signals

Internal and external factors that control changes in cell structure and function. They can be chemical or physical in nature.

Somatic cells

All the cells within the developing or developed organism with the exception of germline (egg and sperm) cells.

Somatic cell nuclear transfer (SCNT)

A technique that combines an enucleated egg and the nucleus of a somatic cell to make an embryo. SCNT can be used for therapeutic or reproductive purposes, but the initial stage that combines an enucleated egg and a somatic cell nucleus is the same.

Stem cells

Cells that have both the capacity to self-renew (make more stem cells by cell division) as well as to differentiate into mature, specialized cells.

Subculturing

Transferring cultured cells, with or without dilution, from one culture vessel to another.

Surface markers

Proteins on the outside surface of a cell that are unique to certain cell types and that can be visualized using antibodies or other detection methods.

Telomere

The end of a chromosome, associated with a characteristic DNA sequence that is replicated in a special way. A telomere counteracts the tendency of the chromosome to shorten with each round of replication.

Teratoma

A multi-layered benign tumor that grows from pluripotent cells injected into mice with a dysfunctional immune system. Scientists test whether they have established a human embryonic stem cell (hESC) line by injecting putative stem cells into such mice and verifying that the resulting teratomas contain cells derived from all three embryonic germ layers.

Therapeutic cloning

The generation of embryonic stem cells from an embryo derived by nuclear transfer for therapeutic purposes. The resultant cell line would be genetically identical to the donor of the transferred nucleus. In humans, the therapeutic potential includes research using patient- or disease-specific human embryonic stem cells to study the basis of disease or advance towards tissue replacement.

Totipotent stem cells

Stem cells that can give rise to all cell types that are found in an embryo, fetus, or developed organism, including the embryonic components of the trophoblast and placenta required to support development and birth. The zygote and the cells at the very early stages following fertilization (i.e., the 2-cell stage) are considered totipotent.

Transdifferentiation

The ability of a particular cell of one tissue, organ or system, including stem or progenitor cells, to differentiate into a cell type characteristic of another tissue, organ, or system.

Trophoblast

The tissue of the developing embryo responsible for implantation and formation of the placenta. In contrast to embryonic stem cells, the trophoblast does not come from the inner cell mass, but from cells surrounding it.

Unipotent stem cells

Stem cells that self-renew as well as give rise to a single mature cell type; e.g., spermatogenic stem cells.

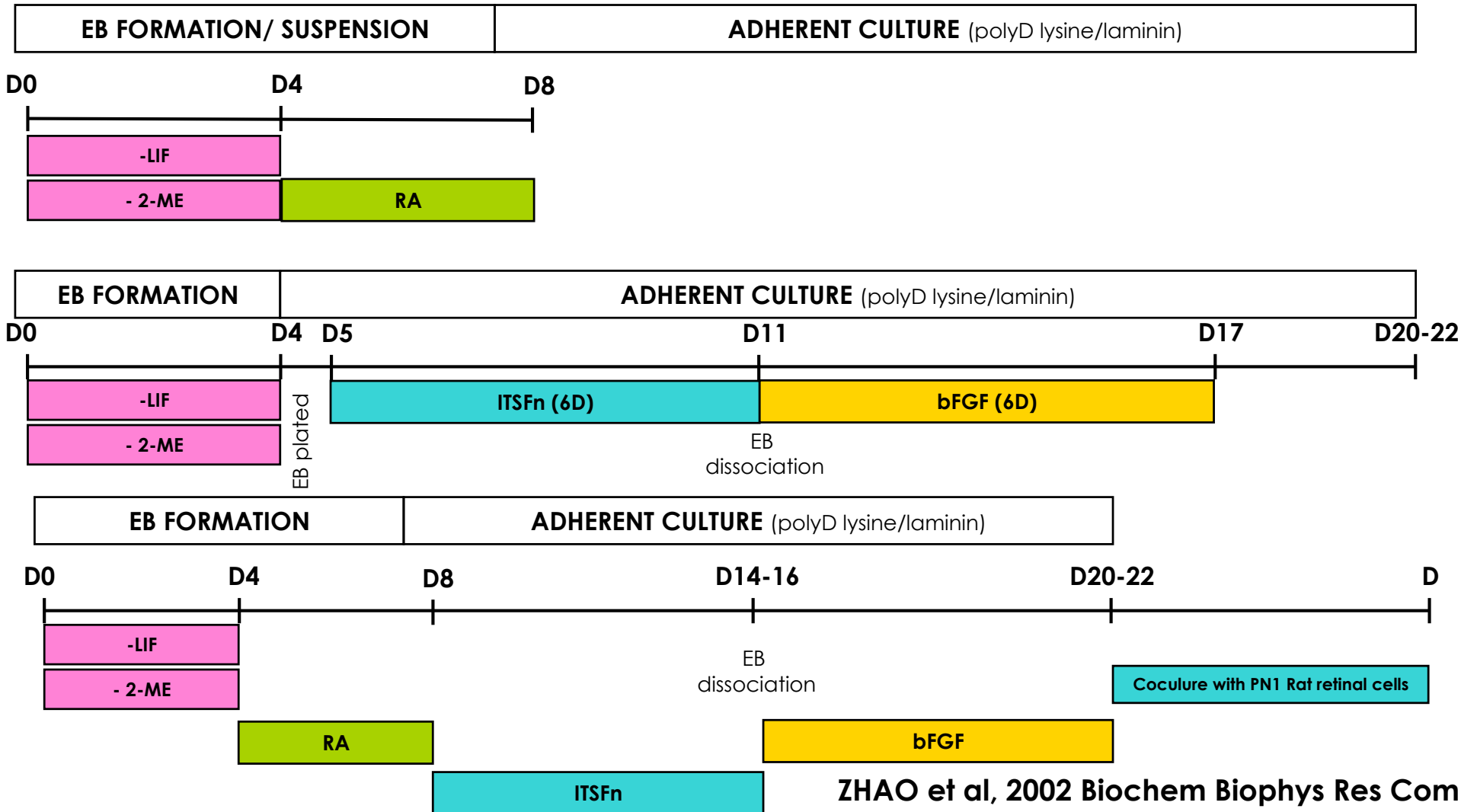
Zygote

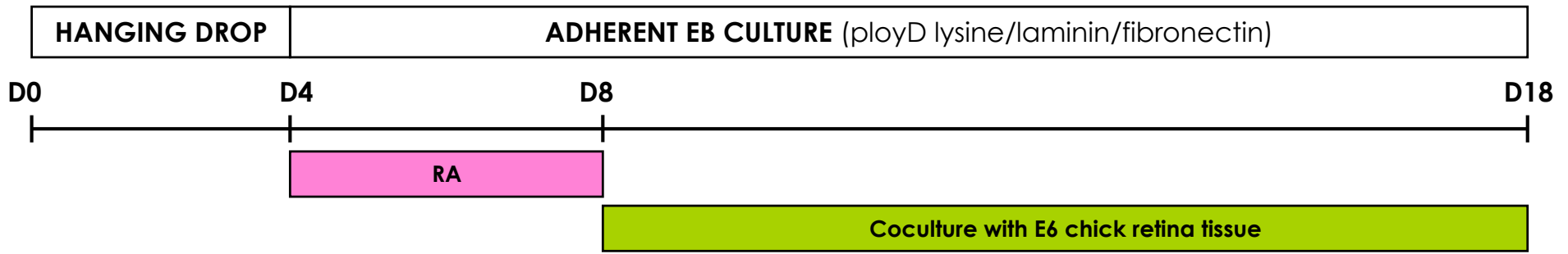
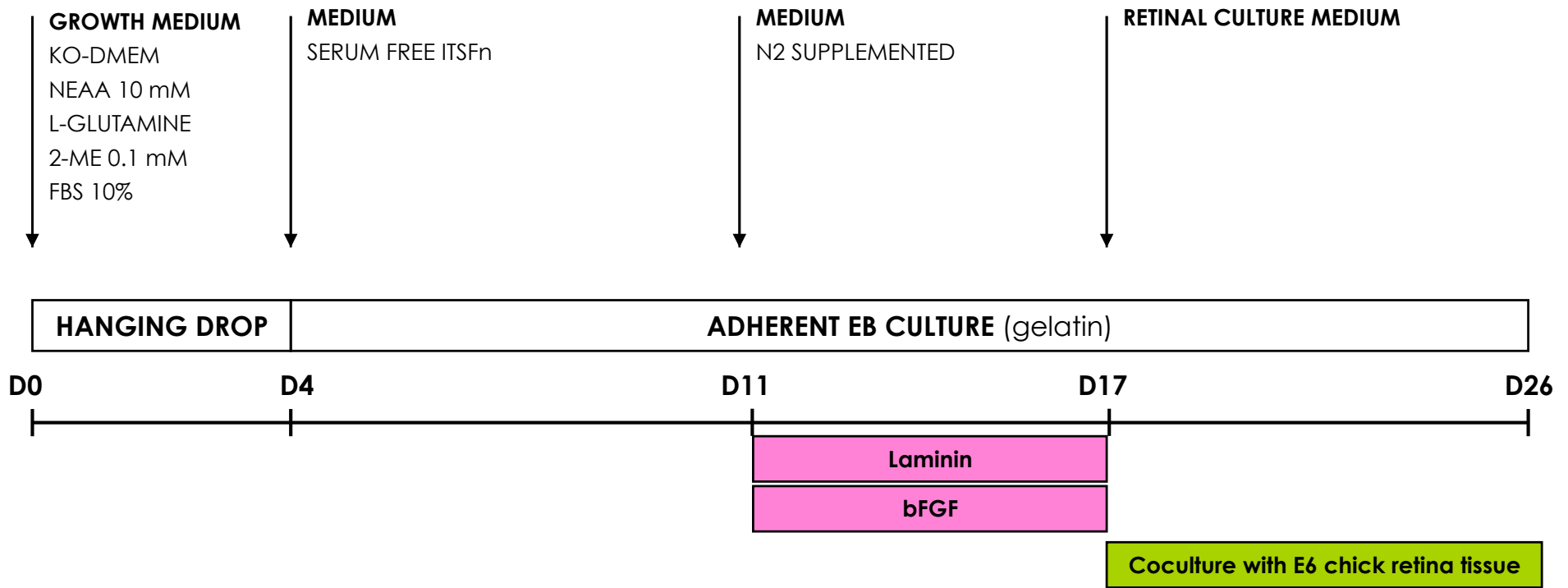
The cell that results from the union of sperm and egg during fertilization. Cell division begins after the zygote forms.

ANNEX 9. hk\ u\ #\ Oo

NEURAL INDUCTION MEDIUM

RETINAL DIFFERENTIATION MEDIUM



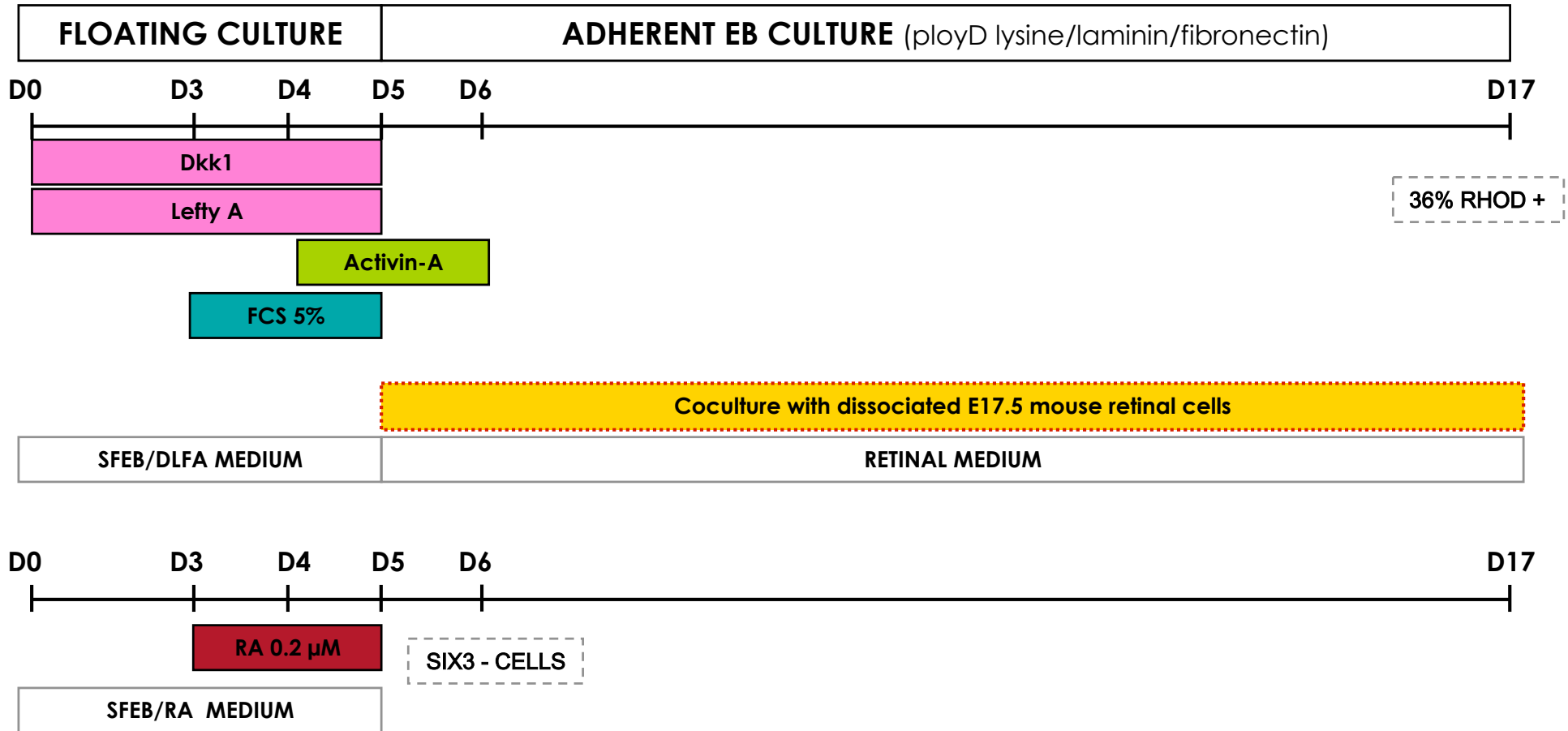


DIFFERENTIATION MEDIUM

GMEM
NEAA 0.1 mM
PYR 1 mM
2-ME 0.1 mM
KSR 5%

RETINAL CULTURE MEDIUM

MEM-E HEPES 66%
HBSS 33%
GLUCOSE 5.75 mg/ml
L-GLUTAMINE 200 µM
1% FCS SUPPLEMENTED WITH N2
PEN/STREP 100U/ml // 100 µg/ml

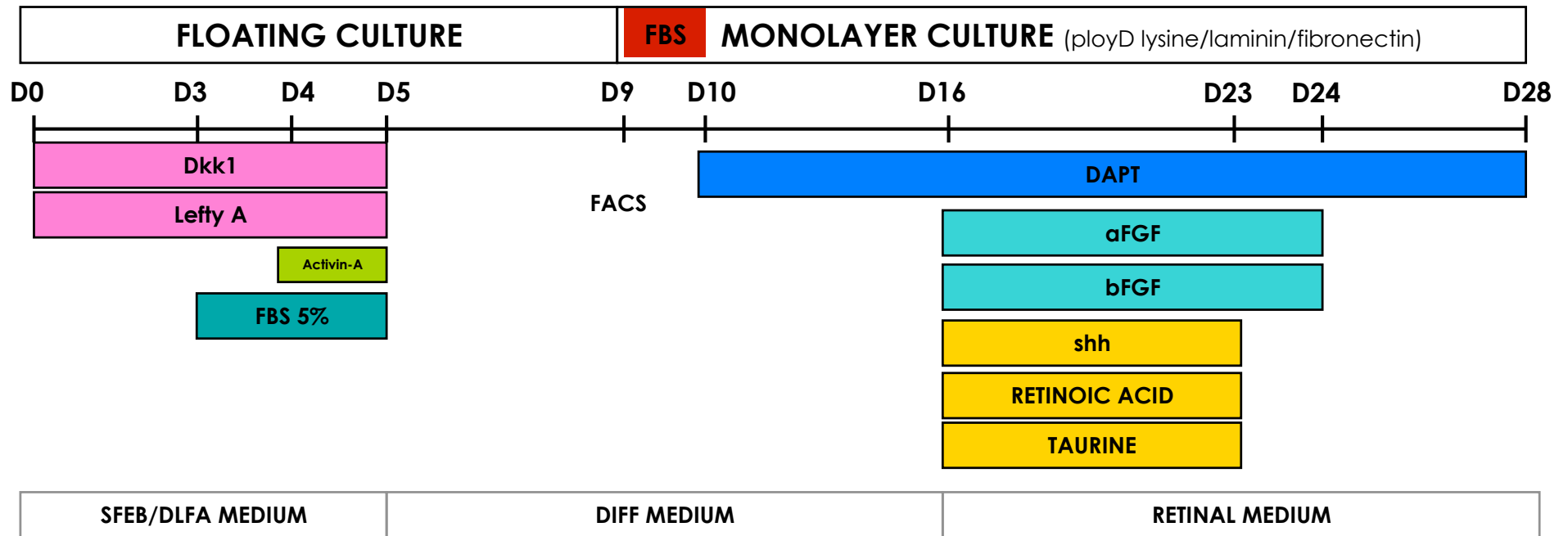


RETINAL PROGENITORS DIFFERENTIATION MEDIUM

GMEM
NEAA 0.1 mM
PYR 1 mM
2-ME 0.1 mM
KSR 5%

RETINAL CULTURE MEDIUM

MEM-E HEPES 66%
HBSS 33%
GLUCOSE 5.75 mg/ml
L-GLUTAMINE 200 µM
1% FCS SUPPLEMENTED WITH N2
PEN/STREP 100U/ml // 100 µg/ml

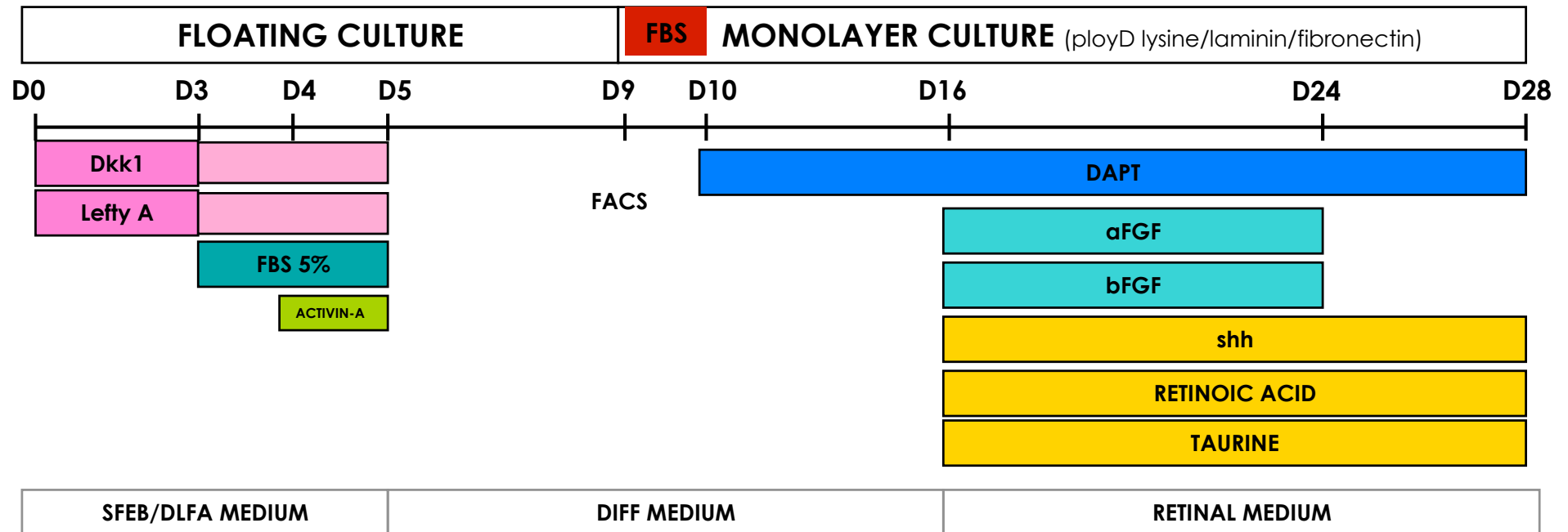


RETINAL PROGENITORS DIFFERENTIATION MEDIUM

GMEM
NEAA 0.1 mM
PYR 1 mM
2-ME 0.1 mM
KSR 5%

RETINAL DIFFERENTIATION MEDIUM

MEM-E HEPES 66%
HBSS 33%
GLUCOSE 5.75 mg/ml
L-GLUTAMINE 200 μ M
N2 SUPPLEMENT 1%
FBS 1%
PEN/STREP 25U/ml // 25 mg/ml

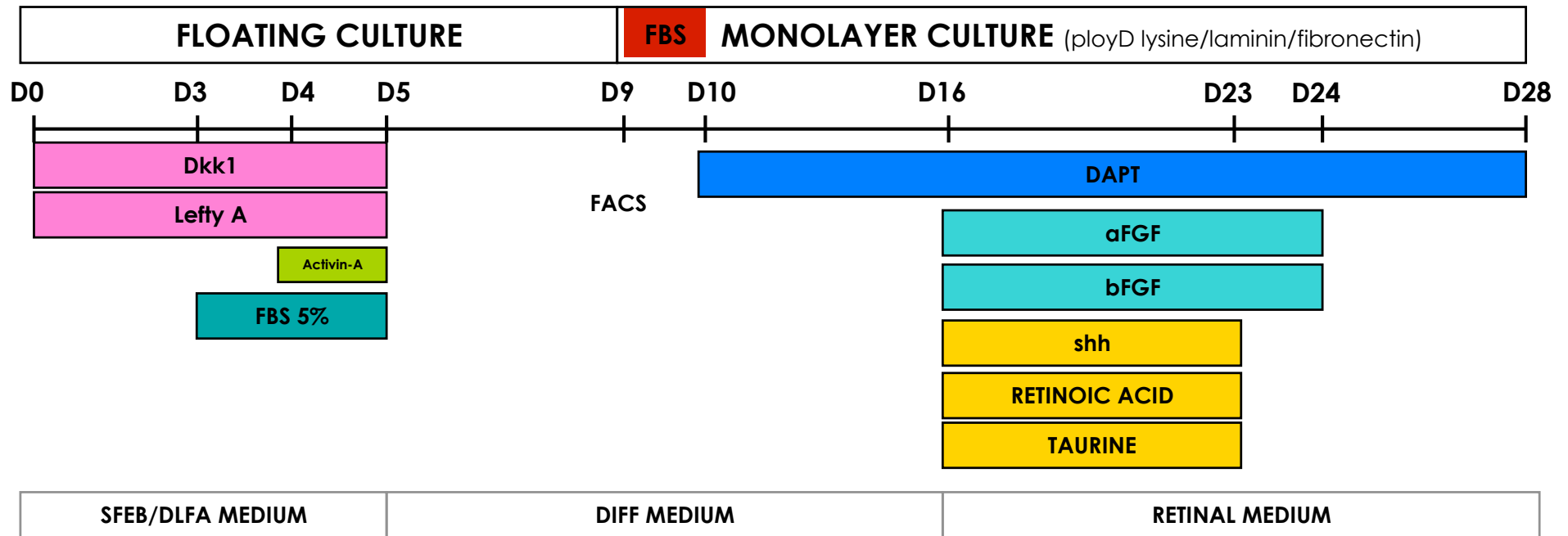


RETINAL PROGENITORS DIFFERENTIATION MEDIUM

GMEM
NEAA 0.1 mM
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RETINAL CULTURE MEDIUM

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HBSS 33%
GLUCOSE 5.75 mg/ml
L-GLUTAMINE 200 µM
1% FCS SUPPLEMENTED WITH N2
PEN/STREP 100U/ml // 100 µg/ml

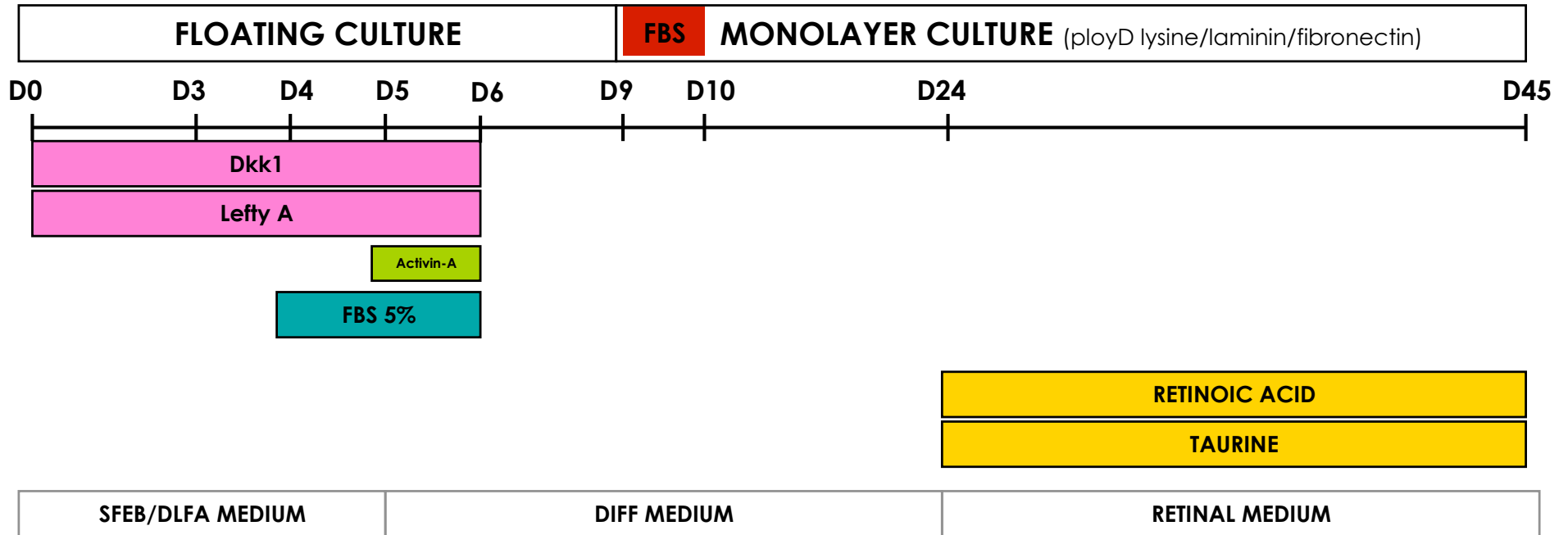


RETINAL PROGENITORS DIFFERENTIATION MEDIUM

GMEM
NEAA 0.1 mM
PYR 1 mM
2-ME 0.1 mM
KSR 5%

RETINAL CULTURE MEDIUM

MEM-E HEPES 66%
HBSS 33%
GLUCOSE 5.75 mg/ml
L-GLUTAMINE 200 µM
1% FCS SUPPLEMENTED WITH N2
PEN/STREP 100U/ml // 100 µg/ml



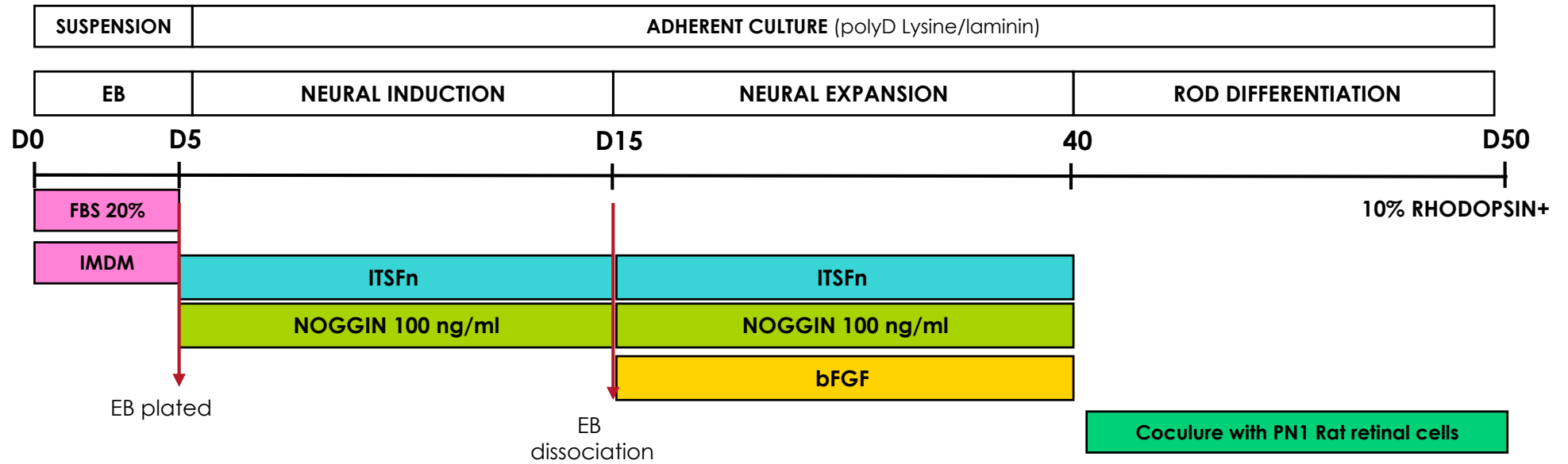
NEURAL INDUCTION MEDIUM

DMEM-F12
N2 supplement
Glutamine
B27

RETINAL DIFFERENTIATION MEDIUM

-LIF

- 2-ME



Annex 9.3 Publications

Garita-Hernández M, Diaz-Corrales F, Lukovic D, Gonzalez-Guede I, Díez-Lloret A, Sánchez-Valdés ML, Massalini S, Erceg S, Bhattacharya SS. **Hypoxia Increases the Yield of Photoreceptors Differentiating from Mouse Embryonic Stem Cells and Improves the Modeling of Retinogenesis in Vitro.** Stem Cells. 2013 Jan 29. doi: 10.1002/stem.1339. PMID: 23362204

Erceg S, Moreno-Manzano V, **Garita-Hernandez M**, Stojkovic M, Bhattacharya SS. **Concise review: stem cells for the treatment of cerebellar-related disorders.** Stem Cells. 2011 Apr;29(4):564-9. PMID: 21319272

Annex 9.4 Honors and Awards

Poster Presentations

ISSCR 9th Annual Meeting, June 15-18, 2011, Metro Toronto Convention Center, Toronto, CANADA.

Garita-Hernandez M., Massalini S., Díez-Llobet A., Krishna A., Erceg S., Bhattacharya S.S. **Hypoxia increases the yield of photoreceptors differentiating from mouse embryonic stem cells and improves the modeling of retinogenesis in vitro**

ISSCR 10th Annual Meeting, June 13-16, 2012, Pacific Yokohama, Yokohama, JAPAN.

Garita-Hernández M., Díaz-Corrales F., Lukovic D., Gonzalez-Guede I., Massalini S., Díez-Lloret A., Slaven Erceg, Bhattacharya S.S. **Retinal transplantation of photoreceptors derived from mouse embryonic stem cells under hypoxic conditions improved the efficiency of differentiation.**

Awards and Fellowships

EMBO Short Term Fellowship September 2012-March 2013. Institut de la Vision, Paris

Travel Award ISSCR 9th Annual Meeting, June 15-18, 2011, Metro Toronto Convention Center, Toronto, CANADA.

Hypoxia Increases the Yield of Photoreceptors Differentiating from Mouse Embryonic Stem Cells and Improves the Modeling of Retinogenesis *in Vitro*

Marcela Garita-Hernández¹, Francisco Diaz-Corrales¹, Dunja Lukovic¹, Irene González Guede¹, Andrea Diez Lloret¹, M^a Lourdes Valdés Sánchez¹, Simone Massalini¹, Slaven Erceg^{1*} and Shomi S. Bhattacharya^{1,2*}

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Key words. Cellular therapy • Differentiation • Embryoid bodies • Embryonic stem cells • Stem cell transplantation • Retina

ABSTRACT

Retinitis pigmentosa (RP), a genetically heterogeneous group of diseases together with age-related macular degeneration (AMD), are the leading causes of permanent blindness and are characterized by the progressive dysfunction and death of the light sensing photoreceptors of the retina. Due to the limited regeneration capacity of the mammalian retina the scientific community has invested significantly in trying to obtain retinal progenitor cells from embryonic stem cells (ESC). These represent an unlimited source of retinal cells, but it has not yet been possible to achieve specific populations, such as photoreceptors, efficiently enough to allow them to be used safely in the future as cell therapy of RP or AMD. In this study we generated a high yield of photoreceptors from directed differentiation of mouse ESC (mESC) by recapitulating crucial phases of retinal

development. We present a new protocol of differentiation, involving hypoxia and taking into account extrinsic and intrinsic cues. These include niche-specific conditions as well as the manipulation of the signaling pathways involved in retinal development. Our results show that hypoxia promotes and improves the differentiation of mESC towards photoreceptors. Different populations of retinal cells are increased in number under the hypoxic conditions applied, such as Crx positive cells, S-Opin positive cells and double positive cells for Rhodopsin and Recoverin, as shown by immunofluorescence analysis. For the first time this manuscript reports the high efficiency of differentiation *in vivo* and the expression of mature rod photoreceptor markers in a large number of differentiated cells, transplanted in the sub-retinal space of wild type mice.

INTRODUCTION

Stem cell therapy is a potential treatment for retinal dystrophies. Retinitis pigmentosa (RP)

and age-related macular degeneration (AMD), the leading causes of permanent blindness in humans are characterized by the progressive dysfunction and death of photoreceptor cells of

Author contributions: M.G.-H.: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript, other (perform experiments and graphic design); S.E.: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; F.D.-C.: other (perform experiments); D.L.: other (perform experiments); I.G.-G.: other (perform experiments); S.M.: other (perform experiments); L.V.: other (perform experiments); A.D.-L.: other (perform experiments); S.S.B.: Principal Investigator, Conception and design and final approval of manuscript.

*correspondence to: shomi.bhattacharya@cabimer.es and slaven.erceg@cabimer.es; Received August 07, 2012; accepted for publication November 23, 2012; 1066-5099/2013/\$30.00/0 doi: 10.1002/stem.1339

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the retina. For such diseases, the replenishment of functional photoreceptor precursors may be a good strategy for retinal regeneration, as gene therapy or growth factor supplement cannot regenerate dying photoreceptor cells. Retinal development is a multi-step process involving cell cycle exit, migration, and changes of cell morphology [1]. These changes result from a reciprocal relationship between tissue-tissue interaction and cell intrinsic factors [2]. Additionally, accumulating evidence suggests that other components of the niche, such as oxygen tension, play an important role in cell fate determination during the development of many tissues, including the nervous system and the retina [3, 4].

Mouse embryonic stem cells (mESC) allow us to recapitulate retinal development *in vitro*. These cells are derived from the early embryo and are characterized by their two unique features of pluripotency and self-renewal [5]. In particular, during implantation and fetal development, stem cells live at oxygen tensions between 2% to 8% [6].

Early embryonic formation during mammalian development occurs in a precise environment, where the O₂ tension plays a critical role [7]. In comparison to the atmospheric oxygen tension (20%) the uterus environment is hypoxic. Mammals, including rabbits (8.7% oxygen tension) and monkeys (1.5% oxygen tension [8]) as well as humans develop embryos under low oxygen tension. Up until the second trimester in humans this ranges from 0-3% [9, 10]. The retina is not an exception and recent studies have shown the important role that hypoxia may play in neuroprotection and development of the human retina [11]. This relative hypoxia or tissular normoxia is relatively low compared with traditional *in vitro* culture conditions (20% O₂ [12]). Once, the pluripotency agents such as Leukemia inhibitory factor (LIF) is removed, ESC spontaneously differentiate following a reproducible temporal pattern of development, that in many ways recapitulates early embryogenesis [13]. Due to these special

characteristics, ESC are considered an unlimited source for cell replacement therapies. The formation of embryoid bodies (EBs), which are three-dimensional aggregates of ESC, is the initial step in ESC differentiation. Therefore, EB culture has been widely utilized as a trigger for the *in vitro* differentiation of ESC. Numerous groups have recently demonstrated that ESC can be converted into cells that resemble retinal progenitors [14-16], photoreceptors [17] or RPE [18, 19]. Furthermore, Meyer and colleagues have very elegantly mimicked the early retinal development in a stepwise fashion typical of normal retinogenesis [20] and high yield of cells differentiated towards photoreceptors were achieved by Mellough and colleagues [21]. Others went further attempting to obtain 3D structures of early optic cup using scaffolds [22, 23]. However, evidence of fully characterized high yield populations of photoreceptors or mature RPE cells has not yet been accomplished. The main drawback of the differentiation methods available is the very low efficiency along with the lack of reproducibility. Most of the protocols published in recent years have been geared towards the induction of expression of retina-specific transcription factors, but very few publications have included information regarding *in vivo* integration of differentiated cells into the mouse retina, suggesting cell survival, migration and functionality of the grafted cells [24, 25]. In this study, we have optimized and fully characterized an original protocol of differentiation that allows us to obtain photoreceptors at a high efficiency in a reproducible way. Our approach involves the use of small molecules, growth factors and morphogenic drugs in specific growth medium to differentiate mESC using hypoxic conditions. Our work is based on the hypothesis that hypoxia influences stem cell characteristics *in vivo*, showing that lower oxygen tensions *in vitro* could mimic the microenvironment and improve the modeling of retinogenesis *in vitro*. To demonstrate the efficiency of our protocol *in vivo* we transplanted our cells after 20 days of differentiation in the subretinal space of wild type mice and found that they were able to

complete differentiation *in vivo*. More than 90% of the transplanted cells expressed mature rod specific markers such as Recoverin (Recvn) or Rhodopsin. Furthermore, no tumoral growth was observed in any of the animals transplanted, corresponding to the absence of proliferative cells in the grafted population due to a highly efficient differentiation process.

MATERIALS AND METHODS

Methods used to develop this work have been described elsewhere [26-28]. See supporting information for a detailed media description (Supplementary Table 1) and supplementary methods.

Maintenance of mESC pluripotent cultures. All experiments conducted in this study were carried out using ES-D3 cells (ATCC CRL 1934 [29]) passages 18-35. ESC were maintained modifying already published protocols [30, 31] and were incubated at 37°C under 20% Oxygen tension. Cultures were passaged every 4-7 days and grown at low confluence at a 1:1000 split ratio. Fresh medium was exchanged every 48 hours. Appropriate Master and Working Cell Banks were generated to allow all the experiments to be accomplished using early passages.

ES cell differentiation

One week prior to starting the differentiation protocol cells were incubated at 37°C in 5% CO₂ under either Normoxic (20% Oxygen tension) or Hypoxic (2% Oxygen tension) conditions in a Thermo Fisher incubator (CO₂/O₂ WJ IR Model 3141, Thermo Electron Corporation, Fisher Scientific). Both conditions were maintained during the whole time of differentiation. Oxygen tension control was monitored daily.

A. Spontaneous Differentiation: Model of Embryoid Bodies (EB)

EBs were generated following an optimized protocol of the hanging drop method described by Wobus and colleagues [32]. mESC were dissociated using 0.05% Trypsin for 4 min at 37°C. Trypsin was washed away adding EBs

medium. The cell suspension generated was spun down by centrifugation (Beckman coulter) and pelleted cells were resuspended in ES medium at the desired concentration (1000 cells per 30 µl). Hanging drops of 30 µl were plated onto the lid of a 150 mm² ultralow attachment plate (Soria Greiner) using a multichannel pipette (Eppendorf). ESC were allowed to aggregate in hanging drops for 3-4 days before transfer to a suspension culture. After 3 days identical spherical EBs were formed and each drop was collected individually with a 100 µl pipette and deposited into a 10-cm ultra-low-attachment dish (Soria Greiner) containing 10 ml of EB medium to a final concentration of 100 EBs per dish. The EBs were cultured for 5 and 7 days and the medium was changed every 2-3 days.

B. Directed Differentiation of mESC Towards Retinal Progenitors and Retinal Mature Phenotypes

Retinal differentiation of mESC was accomplished using an optimized protocol encompassing growth factors described in previously published protocols [15, 17, 33] (all R&D Systems, unless otherwise specified) combined with the manipulation of the microenvironment. EBs generated from mESC were induced to differentiate in Progenitors medium supplemented with 100 ng/ml Dickkopf-related protein 1 (Dkk1) and 500 ng/ml Lefty-A for 5 days at 37 °C with 5% CO₂ under Normoxic (20%) or Hypoxic (2%) conditions. Media was changed 72 hours later and fresh aliquots of the growth factors were added along with 5% fetal bovine serum (FBS) and 100 ng/ml Activin-A. On day 5, media was changed and EBs were cultured for 5 more days in Progenitors media without the addition of any growth factor. Fresh Progenitors medium was changed every 48 hours. On day 10, EBs were plated in 6-well plates or coverslips coated with human recombinant 30 µg/cm² Laminin (Sigma) and 150 µg/cm² Poly-L-Ornithine (Sigma) and cultured in Retinal medium supplemented with 10 µM N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT; Calbiochem) at 37 °C with 5% CO₂ for 48 hours.

Ideal cell density was established in 150 EBs per 9.6 cm². To allow better attachment of the EBs, 10% FBS was added to the medium for 48 hours. Retinal medium was exchanged every 2 days. On day 16 and until day 24, Retinal medium was supplemented with 10 μ M DAPT, 50 ng/ml acidic fibroblast growth factor (aFGF), 10 ng/ml basic fibroblast growth factor (bFGF, Millipore), 3 nM Sonic hedgehog homolog (Shh), 0.5 μ M retinoic acid (RA; Sigma) and 100 μ M Taurine (Sigma). From day 24 to day 28 Retinal medium was supplemented with 10 μ M DAPT, 3 nM Shh, 0.5 nM RA and 100 μ M Taurine. Samples were collected on day 0, day 5, day 10, day 16, and day 28 for molecular biology analysis and immunocytochemistry.

Methods of RNA isolation and quantitative RT-PCR, immunocytochemistry, apoptosis and cytotoxicity assays and FACS are described in Supplementary methods.

Statistical analyses

Data are the mean \pm standard error of mean (SEM) of at least three independent experiments, except for the immunocytochemistry, for which a representative image from three assays is depicted in the figures. Comparisons between values were analyzed using one-way analysis of variance (ANOVA); $p \leq 0.05$ was considered statistically significant.

Preparation of cells for transplantation

Cells, after 20 days of *in vitro* differentiation were trypsinized to obtain a single cell suspension. Harvested cells were labeled using a 2 μ M PKH26 solution (Sigma-Aldrich) and washed in DPBS (Gibco 14190). Stained cells were counted with a hemocytometer and the suspension to be transplanted was diluted to an appropriate cell density of 50,000 cells/ μ l.

Transplantation procedure

10-week old C57BL/6NCrl mice were used in this study. Animals were distributed in 2 groups of 7 animals each, according to the culture conditions of the cells, Normoxia or Hypoxia. All animal procedures were accomplished

following the guidelines of the local ethics committee of animal experimentation. Surgical procedures were performed under general anesthesia with 100 mg Ketamine and 5 mg diazepam per kilogram bodyweight. Additionally, the eye was topically anesthetized with 0.1% tetracaine and 0.4% oxybuprocaine. One drop of each 10% phenyleprine and 1% tropicamide were used to dilate the pupils. Following complete dilation, the anesthetized animal was placed in lateral recumbency under the SMZ-1 Nikon dissecting microscope and positioned with one hand holding mice. The mice fundus could be visualized with the application of a drop of 2.5% methylcellulose to the eye. The fundus observation served to evaluate the condition of the eye before injection and to compare with the postoperative condition of the retina. The needle with bevel up was advanced full thickness 1 mm posterior to the sclerocorneal limbus into the posterior chamber. At least 50% of the bevel was pushed through the choroid to produce a hole sufficiently large to insert the 33 gauge blunt needle (Hamilton Company, Reno, NV). The blunt needle tip was inserted through the choroidal puncture and advanced into the posterior chamber, avoiding trauma to ciliary body or lens. Subsequently, the needle shaft was aimed slightly nasally toward the posterior chamber and it was advanced toward the desired injection location in the posterior retina. A 10 μ l syringe (Hamilton, Switzerland) with a 33-gauge needle attached to an ultra-micropump (World Precision Instruments, Sarasota, FL) was used to inject 1,5 μ l of cell suspension (75,000 cells) slowly, at a rate of 0.05 μ l/second, into the subretinal space (SS). Immediately after injection, the fundus was examined and any animals with massive subretinal hemorrhage or vitreous hemorrhage were removed from the study. Injected animals developed a retinal detachment and small amount of bleeding in the same area of injection. Finally, a drop of antibiotic (0.3% ciprofloxacin) was administered on each eye and animals were kept on a 37°C pad until recovery from anesthesia.

Tissue preparation

Animals were sacrificed by cervical dislocation after 24 hours and 1 and 4 weeks of transplantation. Eyes were enucleated and immediately fixed overnight at 4°C in freshly prepared 4% paraformaldehyde solution. Eyes were then washed in PBS and transferred into 30% sucrose in PBS solution for at least 12 hours before inclusion in OCT and cryosectioning. Retinal sections (18 µm) were mounted in SuperFrost Ultra Plus® slides (MENZEL-GLÄSER, Braunschweig, Germany) and stored at room temperature (RT) until further processing.

Immunohistochemical Analysis

Sections were blocked in PBS containing 10% goat serum and 0.1% Triton for 1 hour at RT and incubated with primary antibodies overnight at 4°C. Primary antibodies used are listed in Supporting Information (Supplementary Table 3a). After incubation with primary antibodies, sections were washed with PBS containing 0.1% triton and incubated with secondary antibodies for 1 hour at room temperature. After successive washing in PBS, nuclei were counterstained with DAPI (4',6-diamino-2-phenylindole, dilactate; Invitrogen-Molecular Probes, Eugene, OR). Immunofluorescence was observed using a Leica DM 5500 microscope (Leica Microsystems, Wetzlar Germany) and a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar Germany).

RESULTS

Effect of Hypoxia on spontaneous differentiation

We wanted to assess whether hypoxic conditions could induce retinal fate of mESC. EBs were generated from ESC using the hanging drop method followed by culture in suspension under either normoxic or hypoxic conditions (Fig. 1A). Time course (day 0, day 5 and day 7) of spontaneous differentiation of EBs were analyzed for expression of pluripotency markers along with other markers of retinal commitment. FACS analysis showed significant differences in

the number of cells positive for pluripotency markers in hypoxic conditions when compared to the corresponding number under normoxia during the protocol (Fig. 1C). When the FACS analysis data in hypoxia was normalized against normoxia, data showed a significant decrease of Nanog⁺ (46±18%), Oct4⁺ (50±14%) and Sox2⁺ (42±17%) cells after 7 days under hypoxic conditions (Fig. 1C and 1D).

Besides, RT-PCR analysis demonstrated that retinal specific genes, such as Pax6 and Crx, were significantly upregulated (4.9±1.1 and 4.2±0.6 fold, respectively) when EBs were cultured under hypoxia for 7 days compared to normoxia (Fig. 1F). The upregulation of Pax6 and Crx genes in cells cultured under hypoxia corresponded with the downregulation of pluripotency genes (Fig. 1E) and the loss of pluripotency markers at a protein level as demonstrated by FACS analysis at this time point (Fig. 1C and 1D). Other retinal genes such as Six3, a well characterized eye field transcription factor [34], Chx10 [35], a neural retina marker and Nrl [36], a photoreceptor precursor marker, showed a slight but not significant increase under hypoxia condition (Fig. 1F). These results have shown that hypoxia significantly decreases pluripotency and increases the expression of different retinal genes in spontaneous differentiation.

Directed differentiation

The results observed with spontaneous differentiation led us to believe that hypoxic conditions could improve the differentiation of ESC towards a retinal fate, but obviously a more sophisticated protocol for retinal differentiation had to be applied. In order to demonstrate our hypothesis and corroborate whether we could obtain a higher yield of retinal cells, we have optimized a differentiation protocol that involves the culture of ESC under hypoxia (2% O₂), and the use of small molecules previously described by different authors [17, 33]. Our 3-step approach is a novelty in differentiation protocol. We included EB step where EBs were generated as a hanging drops because previously published

data show [37] that this is an efficient and scalable system which allows uniform distribution of culture parameters such as oxygen tension due to the homogeneous size of the generated EBs. Briefly, ES-cells characterized by the typical expression of Oct4, Nanog, Sox2 and SSEA-1 (Fig. 1B) were induced to differentiate into neural retina cells or Chx10 positive cells and more mature phenotypes of retinal commitment, such as Rhodopsin in the case of rod cells, in a sequential manner (Supplementary Fig. 1A). To induce *in vitro* retinal differentiation, ESC were cultured as hanging drops for 3 days in the presence of Dkk1 and Lefty A. Hanging drops containing the EBs were collected and allowed to differentiate in suspension for 7 more days in progenitors medium, after which, EBs were plated and allowed to further differentiate for 18 more days in adherent culture in Retinal Medium (Fig. 2B and Supplementary Fig. 1B). From D3 to D5 FBS and Activin A were added in culture. Different growth factors such as DAPT, Fibroblast growth factors (FGFs), Shh, RA and taurine were added to the protocol of differentiation as described in Materials and methods (Fig. 2B) to promote differentiation toward photoreceptors [17, 31, 33]. All results were compared to ESC differentiated under normoxic conditions (20% O₂). During the 28 days of differentiation the expression of various transcription factors associated to each major stage of retinogenesis was analyzed (Supplementary Fig. 1C).

Stemness, loss of pluripotency and eye field specification

Before the start of differentiation, undifferentiated ESC cultures were characterized. All cultures regardless of passage number showed normal morphology. ESC grew forming colonies and expressed pluripotency markers as expected; all colonies were positive for Oct4, Ssea-1, Nanog and Sox2 as determined by immunofluorescence (Fig. 1B) and the level of expression of these markers was determined by FACS each time to assure the purity and stemness of the culture prior to differentiation.

Nanog (98.4%) expression was higher than Oct4 (94.5%) and Sox2 (83.5%) but no levels lower than 80% were found in any passage of ESC used (18-35 passages, data not shown). To direct mESC toward rostral fate, the EBs were treated with Dkk1, an antagonist of Wnt/ β -catenin signaling, the nodal antagonist, LeftyA, and Activin A in presence of FBS. The loss of pluripotency, as a hallmark of ESC under differentiating pressure, was measured by RT-PCR. ESC rapidly lost expression of the pluripotency genes Oct4 and Nanog and even downregulated the neural induction marker Sox2 on day 5 (Fig. 2C). Sox2 levels of expression were higher than the levels of Oct4 and Nanog at all time points analyzed and its expression was upregulated on day 10, corresponding to differentiation efforts towards ectoderm (Fig. 2C). Indeed, we have observed a majority of cells expressing the anterior neural marker Otx2, and a greater number of Otx2 positive cells in hypoxia (Fig. 3B) which is consistent with a developmental *in vivo* study [38] (Fig. 3A).

Furthermore, ESC acquired the expression of transcription factors associated with eye field specification. RT-PCR analysis revealed that Six3, gene required for early eye field specification, as well as Pax6 and Rax expression was upregulated as early as on day 5 under hypoxia (Fig. 3E, 3F and 3G, respectively). Eye field cells are characterized by coexpression of Pax6 and Rax [39, 40] (Fig. 3H). As a matter of fact, on day 10 of the differentiation protocol high expression of Rax (Fig. 3G and 3H) coincided with the high expression of Pax6 (Fig. 3F and 3H). Not only the expression of these markers was upregulated earlier using our protocol but the levels of expression of each individual gene at their peak of expression were higher under hypoxia when compared to normoxia. Additionally, by day 16, hypoxia significantly increased the number of Rax⁺ cells (91% of total cells vs. 80% in normoxia; Fig. 3C and 3D). On day 10 the fold induction of Six3 in hypoxia was 6 times higher than in normoxia (8.2 \pm 2.9 against 2.1 \pm 0.91 fold change. Fig. 3E). RT-PCR analysis on day 5

revealed a difference of 14 fold change of Pax6 gene expression under hypoxia (16.4 ± 3.8 against 2.4 ± 0.2 ; Fig. 3G) and the expression of Rax was induced 4 times (6.6 ± 1.5 against 2.2 ± 0.6 ; Fig. 3G) above the level observed in normoxia on day 10. Our results showed that hypoxic conditions significantly increased the expression of the eye field genes.

Retinal commitment

To induce *in vitro* differentiation, ESC were cultured in suspension as EBs for 10 days in progenitors medium (Fig. 2B) after which EBs were plated and allowed to differentiate for another 18 days in adherent culture in Retinal Medium. The next stage of *in vivo* retinal specification occurs when optic vesicles from the paired eye fields are formed. At this stage all cells express the transcription factors Mitf and Pax6 and give rise to either neural retina or retinal pigment epithelium (RPE) [41]. Furthermore, the fact that Mitf positive cells destined to become neural retina derive from the subset of Rax positive cells [35, 42] was confirmed with coexpression of these two markers in culture (Fig. 4A). In fact, RT-PCR analysis revealed that Mitf expression was upregulated since day 5 coinciding with the peak of expression of Pax6 (Fig. 3F). During development, Mitf is downregulated in response to the onset of Chx10, a neural retina specific gene [35]. The dynamic expression of Mitf and Chx10 was examined over time and it was determined that neural retina phenotype was acquired as early as day 16 (Fig. 4E and 4F). Also, our cells at day 16 coexpressed Mitf and Rax (Fig. 4A), revealing RPE commitment [43], while the clusters (Fig. 4B, 4C and 4D) formed by day 16, of Chx10 positive cells maintaining expression of Pax6 revealed their commitment to retinal fate (Fig. 4C and 4D). This corresponded with the peak of expression of Chx10 as checked by RT-PCR (Fig. 4F).

Prolonged culture of Chx10⁺ and Pax6⁺ cells allowed for further maturation of these cells towards a photoreceptor phenotype. A crucial gene for rod specification is Nrl [44], highly

induced since day 16 under hypoxia (Fig. 5E) coinciding with high expression of Crx (Fig. 5C) as observed by RT-PCR analysis. These data was confirmed by immunocytochemistry analysis for these two markers at the end of the protocol where a high number of cells have shown to be positive for Crx (Fig. 5A and 5B) and Nrl (Fig. 5D). The importance of this finding is because during development Nrl interacts with Crx to induce the expression of rod specific genes such as Rhodopsin [45]. As it has been mentioned, the primitive cone and rod photoreceptor-specific transcription factor Crx was upregulated since day 16 as well (Fig. 5C), and the immunoreactive cells against Crx antibody accounted for 75% of the total cells ($75.1 \pm 1.9\%$ in hypoxia vs. $64.4 \pm 2.3\%$ in normoxia; Fig. 5B) at the end of the protocol.

Moreover, it seems that our differentiation protocol applying normoxia did not promote high yield of cone cells because from the Crx⁺ population, only about 8% ($8.1 \pm 5.5\%$) of cells were immunoreactive for the cone photoreceptor-specific protein Opsin-S (Fig. 5F and 5G). On the other hand, the percentage of Opsin-S⁺ cells was significantly increased to 32% of total cells under hypoxic conditions ($31.8 \pm 11.6\%$; Fig. 5F and 5G).

Consistently, differentiation of ESC in hypoxic conditions significantly increased their retinal commitment toward rod photoreceptors, as determined by coexpression of Rhodopsin and Recoverin at the end of the protocol (Fig. 6A, 6B, and 6D), when approximately 53% of total cells showed double positive staining for these markers (53% in hypoxia against 30% in normoxia. (52.9 ± 1.5 against 29.4 ± 3.5 ; Fig. 6E). The induction of Rhodopsin by hypoxia was confirmed by RT-PCR. The analysis showed significant increase in fold change of Rhodopsin since day 16 in hypoxia (28.5 ± 3.6 vs. 11.1 ± 2.9) compared to normoxia (Fig. 6C). Recoverin expression did not localize with Tuj1 indicating the presence of immature neurons and mature photoreceptors in our culture (Fig. 6G). This data

reveals that the generated photoreceptors are not immature neurons but mature retinal cells.

Furthermore, using hypoxic conditions, morphology typical of rods was observed in isolated cases, in sections of the culture where cell density allowed for the outgrowth of structures that suggest the formation of outer segments *in vitro* (Fig. 6B and data not shown).

To assess the proliferation capacity of differentiated cells, we stained the cells after 28 days of differentiation against the operational marker, Ki67 [46]. We have found that after 28 days of differentiation under hypoxic conditions, there was a significant decrease in the number of proliferating cells ($14.8 \pm 1.8\%$) against the amount of proliferating cells differentiated under normoxia over the same time (22.8 ± 1.7 , Fig. 6H and 5I). All cell phenotypes generated at the end of our protocol are depicted in Fig. 6F. To assess the possible mechanisms underlying the increased differentiation capacity of mESC in hypoxia, we observed that, a higher proliferation rate was not related with an increased yield of Rho⁺ cells. Hence, we did not observe any coexpression of Ki67 and Rho after 28 days of differentiation, neither under hypoxic or normoxic conditions (Supplementary Fig. 4A). Furthermore, this phenomena was also observed in other cellular models, RPE-1 cells showed a significant decrease in their proliferation capacity after 3 weeks of hypoxic culture, as determined by the percentage of Ki67⁺ cells in culture and compared to the number found in normoxic conditions (Supplementary Fig. 4B). To assess the safety of low oxygen tension in our cultures we performed cytotoxicity and apoptosis (presented by Caspase 3/7 activity) tests and found that, after 1 week of hypoxic culture, RPE-1 cells showed a significant decrease in cytotoxicity and Caspase 3/7 activity compared to normoxia (Supplementary Fig. 4C and 4D).

To correlate hypoxia and retinal development we followed the expression of Vegfa and Cdkn1a. The expression of these two genes is very well-known to be induced under hypoxia and recently

they have been directly related with retinal hypoxia [47, 48]. We observed that hypoxia significantly increased the fold induction of Vegfa (6.1 ± 1.0 in hypoxia vs. 4.6 ± 0.7 in normoxia; Supplementary Fig. 4E) and Cdkn1a (11.7 ± 1.0 in hypoxia vs. 5.5 ± 1.1 in normoxia; Supplementary Fig. 4F) at day 28 of differentiation, revealing possible mechanisms through which hypoxia promotes retinal development. The exposure of cells to hypoxia leads to the activation and stabilization of hypoxia inducible factor Hif1 α as determined by FACS analysis. The percentage of Hif1 α ⁺ cells in RPE-1 cells was higher after 3 weeks of hypoxia when compared to normoxia culture (69.6 ± 4.18 vs 55.6 ± 10.4 ; Supplementary Fig. 4G) and significantly higher in D3-mESC cells after 1 week of hypoxia in the presence of LIF (79.9 ± 11.5 vs. 49.1 ± 10.7 ; Supplementary Fig. 4H).

Subretinal injection

To evaluate whether injected cells in suspension, generated using our protocol under hypoxia could survive and integrate *in vivo*, C57BL6/NCrl mice received unilateral subretinal injections of 75000 retinal cells differentiated until day 20 or medium alone (sham). The uninjected eye served as internal control for each animal.

The PKH26 staining applied prior to transplantation was used to identify surviving retinal progenitors after 24 hours and 1 week of the injection (Fig. 7A, 7C, 7E, 7F, 7H, 7J and 7K). The location of the main graft was subretinal in 7 eyes with a large cluster of transplanted cells localized between the host photoreceptors and the RPE layer. Immunohistochemical analysis demonstrated dispersed transplanted cells positive for Rhodopsin (Fig. 7A, 7B, 7E and 7K) and Recoverin (Fig. 7F, 7G and 7J) across the retina, singly or in small clusters. A large number of donor cells injected in subretinal locations migrated and integrated in host retina. Transplanted cells immunopositive for photoreceptor marker Rhodopsin and Recoverin

were found in outer nuclear layer (ONL) and inner nuclear layer (INL) in the mice (Supplementary Fig. 7). A much smaller fraction of transplanted cells was Opsin-S positive revealing the presence of cones (Supplementary Fig. 6).

There was no evidence of uncontrolled growth or tumor formation at any time, suggesting that donor cell proliferation might be regulated or balanced by cell death.

DISCUSSION

ESC offer an excellent *in vitro* tool to recapitulate mechanisms activated during early development. The efficient differentiation of retinal cells from ESC is a major challenge for the development of successful cell therapy, which can be applied in different retinal dystrophies such as Retinitis pigmentosa (RP) and age related macular disease (AMD). Although the early stages of development occur in a hypoxic environment, little is known about how low O₂ levels modulate the pluripotency and differentiation capacity of ESC. Our data demonstrate that mESC can be efficiently directed to retinal progenitors and other mature phenotypes, such as photoreceptors, by using a combination of small molecules and lowering O₂ tension can enhance this efficiency. Different protocols of generation of retinal phenotypes have been published and all of them have implied important advances in the field [17, 22]. Our main issue continues to be the accomplishment of a high yield of specific populations and the modeling of retinogenesis *in vitro*.

In the first phase of our protocol we used the combination of Dkk1, Activin A and LeftyA to direct mESC toward rostral neural progenitors, applying an approach used in a previous study [33]. This strategy is widely used to generate rostral neural progenitors [49-52]. Indeed, we observed high percentage of Rax⁺ and Otx2⁺, rostral neural progenitors and markers for early eye field. These two markers together with Pax6,

Six3, Six6 and Lhx2 play an important role in the establishment of anterior neuroectodermal region which maintains high capacity for generation of future retinal progenitors [39, 53, 54]. Significant upregulation of some of these markers (Six3, Pax6 and Rax) was observed as early as by day 5 in culture. Large increase of the eye field markers coincided with rapid decrease of main pluripotency markers indicating high differentiation potential of our protocol. Our results have shown that lowering the O₂ tension near the physiological level is a more effective parameter for retinal differentiation, especially for photoreceptor precursors. It seems that the effects of this parameter are expressed very early in differentiation significantly increasing the expression of Six3, Pax6 and Rax (Fig. 3E, 3F and 3G).

The next phase in retinal specification *in vivo* occurs with the formation of the optic vesicle, determined mainly by the expression of Mitf and Pax6 giving rise to multiple cell types of the functional retina. Cells coexpressing Pax6 and Chx10 give rise to neural retina only. Experiments using different vertebrates indicated that SHH and FGF signaling play a critical role in future specification of retinal cells [55-57]. Once neural retina progenitor phenotype has been acquired, it is necessary for further maturation of these cells. As shown by others, Notch signaling pathway needs to be blocked at this point to allow for an increase in the Crx⁺ cells [17]. For these reasons we supplemented the medium with the γ -secretase inhibitor DAPT from day 10. Retinoic acid and taurine were added to obtain mature photoreceptors [17, 31]. Hence, further retinal specification included DAPT, FGFs, Shh, RA and Taurine. These conditions together with low O₂ tension significantly increase the population of cells coexpressing Pax6 and Chx10 as well as Crx⁺ cells compared to normoxia. High percentage of derived photoreceptors, reflected by coexpression of Rhodopsin and Recoverin for rods, and Opsin-S for cones, revealed that low O₂ tension promoted a photoreceptor fate of mESC. With regard to efficiency, the induction

of rods, namely the yield of Rhodopsin⁺ cells, was higher when compared with other protocols using murine ESC [17, 31] or iPS [15, 58, 59]. The results in the present study, are consistent with the recently published study applying hypoxic condition [60], describing an increased yield of Pax6 and Chx10 positive cells. Here we further define the characterization of generated cells achieving a higher yield of mature retinal phenotypes as well as *in vivo* study. Our results have shown not only that the population of retinal cells can be increased under hypoxia in a way that mimics normal retinal development, but also, we demonstrate for the first time that, early rostral differentiation (Otx2), early eye field acquisition (Six3, Rax and Pax6) and mature retinal phenotypes (Crx, Opsin-S and Rhodopsin) are increased in yield under hypoxia. Furthermore, hypoxic condition seems to improve the timing of retinogenesis, as it has been observed by RT-PCR analysis. Eye field transcription factors are highly expressed as early as by day 5 and the important suppression of Mitf by upregulation of Chx10 occurred by day 16 instead of day 28 only when the cells differentiated under hypoxia. This allowed for a bigger population of neural retina progenitors earlier in the differentiation protocol that could mature into cells expressing photoreceptor markers, such as, Crx, Nrl and Rhodopsin, all three populations highly increased at the end of our protocol.

Our study went further showing efficient *in vivo* evaluation of generated retinal precursors. Previous studies have shown that photoreceptors taken from young animals efficiently incorporate in adult retina when transplanted in the sub-retinal space [25, 61]. We also successfully grafted *in vitro* generated retinal cells in the adult mouse retina, which resulted in cell survival. Interestingly, high percentage of transplanted cells expressed rod specific markers, such as Rhodopsin and Recoverin, though without known possible implication of local environment on further differentiation. It seems that specific retinal niche was preferable for mature differentiation of retinal progenitors.

This data demonstrates the viability of the cells and the robustness of our protocol. However, to fully validate the protocol further *in vivo* functional analyses have to be performed.

Different studies on pluripotent stem cells have demonstrated improved differentiation when different hypoxic conditions were applied [60, 62]. The exact mechanism of hypoxia on retinal differentiation still remains to be elucidated. The primary transcriptional regulators of cellular hypoxic adaptation in mammals are hypoxia induced factors (HIFs). Hypoxic preconditioning was shown to stabilize HIF-1 α in the retina, further inducing the expression of target genes with neuroprotective properties like vascular endothelial growth factor (Vegfa) and erythropoietin (Epo) suggesting a link between HIF-1 α driven gene expression and neuroprotection [63, 64]. Little is known about the molecular effects of hypoxia on retinal differentiation. The published reports mainly correlate the expression of individual genes and hypoxia in different retinal functions [47]. For example, hypoxia increases vascular endothelial growth factor (Vegfa) expression in the retina [60, 63]. The identification of this gene together with p21 (Cdkn1a) is strongly suggestive of their role in general retinal neuroprotection [65, 66]. During our differentiation protocol under hypoxic conditions both genes were significantly upregulated when compared to normoxic conditions. This data suggests that hypoxia, through activation of HIF-1 α , decreasing apoptosis and cytotoxicity, has influenced retinal differentiation [67, 68]. Although, the source of Vegfa could be RPE cells observed in retinal progenitors generated in hypoxic conditions [69] (data not shown), further investigation has to be performed to elucidate the origin of neuroprotective processes. Moreover, a significant decrease of proliferating cells at the end of our protocol in hypoxic conditions, suggests that hypoxia favors postmitotic cells, increasing therefore the number of photoreceptors (Fig. 6F and 6I). It is known that p21, increased in hypoxia (Supplementary Fig. 4F), not only provokes cell cycle arrest,

necessary to start the differentiation process, but can also repress apoptosis [70] (Supplementary Fig. 4D). Therefore, we propose that hypoxia promotes retinal differentiation through activation of p21. Higher yield of early eye field markers in hypoxic condition suggests that hypoxia is preferable for EB formation and early differentiation. This data corroborates with earlier findings where an improved differentiation of human ES cells was observed in hypoxic conditions [71, 72]. We also observed a more compact structure of EBs in hypoxia (data not shown), which could have a consequence on further differentiation. For this to be confirmed, further detailed studies have to be performed.

Conclusion and future perspectives

We believe the application of a new modified protocol for differentiation of mESC reported here support the hypothesis that hypoxia is necessary to induce efficient differentiation of ESC towards a higher yield of retinal phenotypes. The timing of retinogenesis is also improved in hypoxic conditions, by decreasing the time to acquire an eye field phenotype and achieve mature population of photoreceptors *in vitro*. Purification of these specific retinal cells can allow us to define the conditions to expand a homogeneous population that will be further differentiated into fully mature photoreceptor cells. Further experimentation is required to elucidate the precise mechanism or mechanisms by which hypoxia exerts its effect on retinal

differentiation. In summary, the novel findings of the work reported here are: 1. The most efficient protocol so far, for the differentiation of any kind of stem cells (mouse, human or induced-pluripotent cells), towards rod photoreceptor cells (53±1.5%). 2. The modeling of retinogenesis has been accomplished for the first time with mESC only under hypoxic conditions. 3. Photoreceptor precursors from mESC differentiate towards Rhodopsin/Recoverin double positive cells after transplantation in the retina, and a complete lack of tumor formation, demonstrates the importance of an efficient differentiation process and the loss of pluripotency of the transplanted cells. We believe that our findings provide the technical framework necessary for a highly efficient differentiation of mESC towards photoreceptors, which is important for advances in cell therapy and regenerative medicine.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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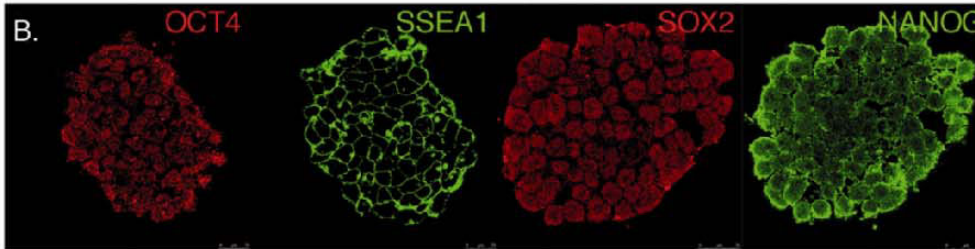
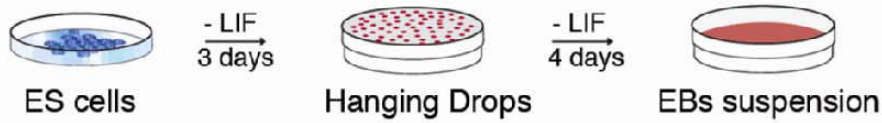
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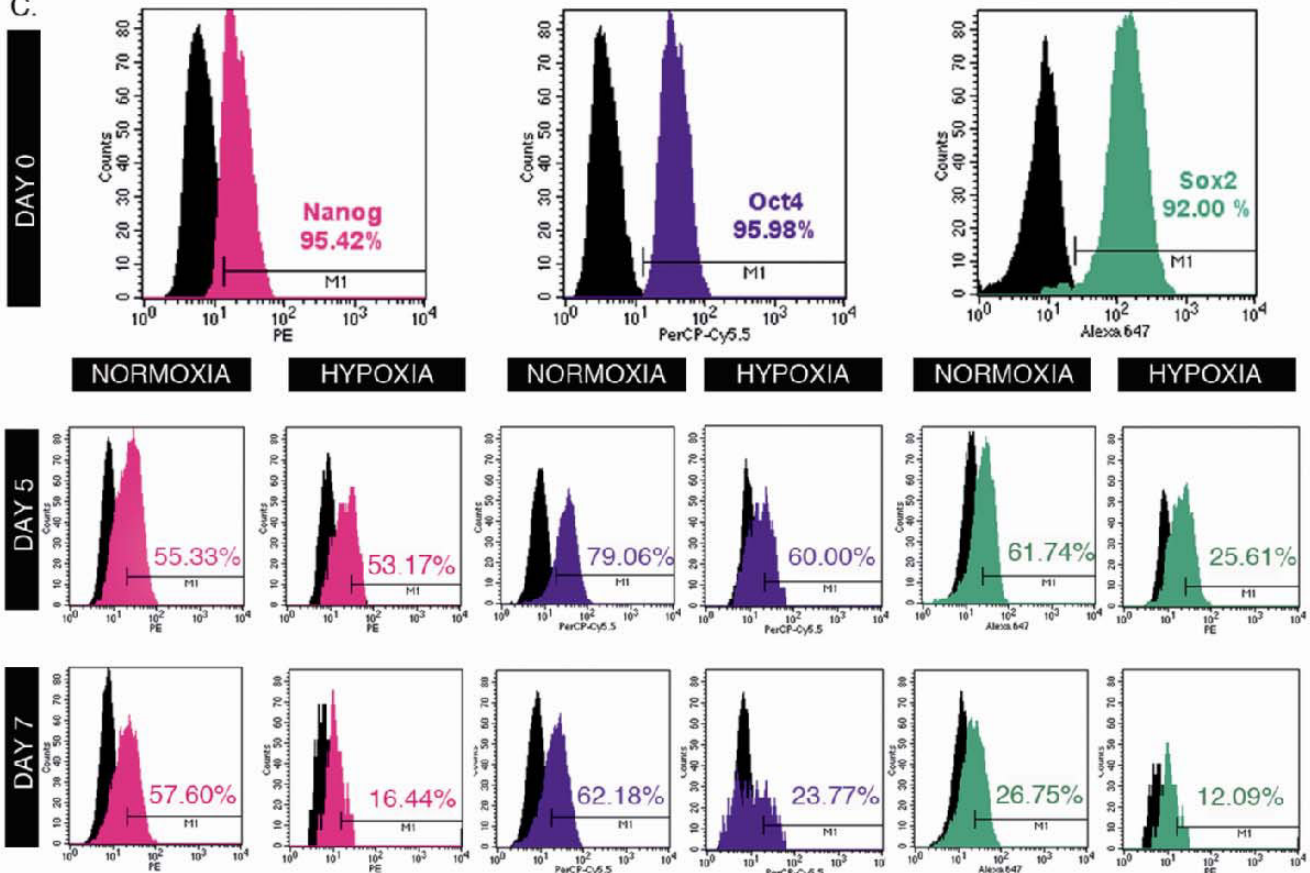
See www.StemCells.com for supporting information available online.

Figure 1. Effect of low oxygen tension during early spontaneous differentiation. **A.** Schematic of the generation of embryoid bodies from mESC. **B.** Immunofluorescence analysis against pluripotency markers; Oct4, Ssea-1, Sox2 and Nanog before differentiation. **C.** FACS analysis showing the loss of pluripotency markers is more efficient after 7 days of spontaneous differentiation in low oxygen when compared to normoxia. **D.** Decrease in number of positive cells for pluripotency markers under hypoxia when data was normalized against the normoxia values. **E.** qPCR analysis comparing the relative levels of expression of pluripotency marker genes after 7 days of spontaneous differentiation in normoxia (blue bar) and hypoxia (red bar). **F.** qPCR analysis comparing the relative levels of expression of retinal specific genes after 7 days of spontaneous differentiation in normoxia (blue bar) and hypoxia (red bar). * $p \leq 0.05$ or ** $p \leq 0.01$ was considered statistically significant.

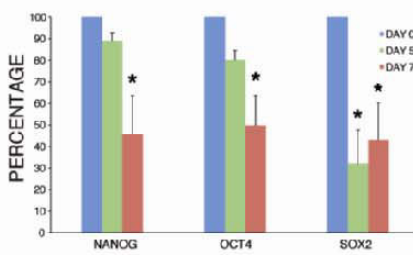
A. NORMOXIA / HYPOXIA



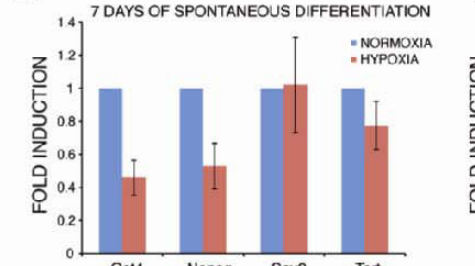
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D. DECREASE IN PLURIPOTENCY MARKERS



E. DOWNREGULATION OF PLURIPOTENCY MARKERS



F. UPREGULATION OF RETINAL MARKERS

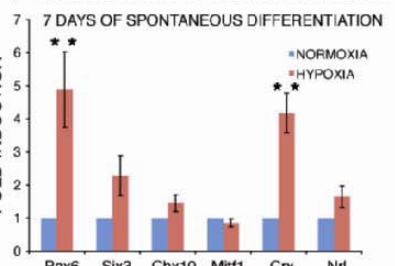


Figure 2. Protocol of differentiation and loss of pluripotency. **A.** Schematic diagram of the 3 step differentiation protocol used to generate retinal cells. **B.** Detail of the protocol of differentiation used to generate retinal cells. **C.** qPCR analysis showing efficient loss of pluripotency markers. * $p \leq 0.05$ was considered statistically significant.

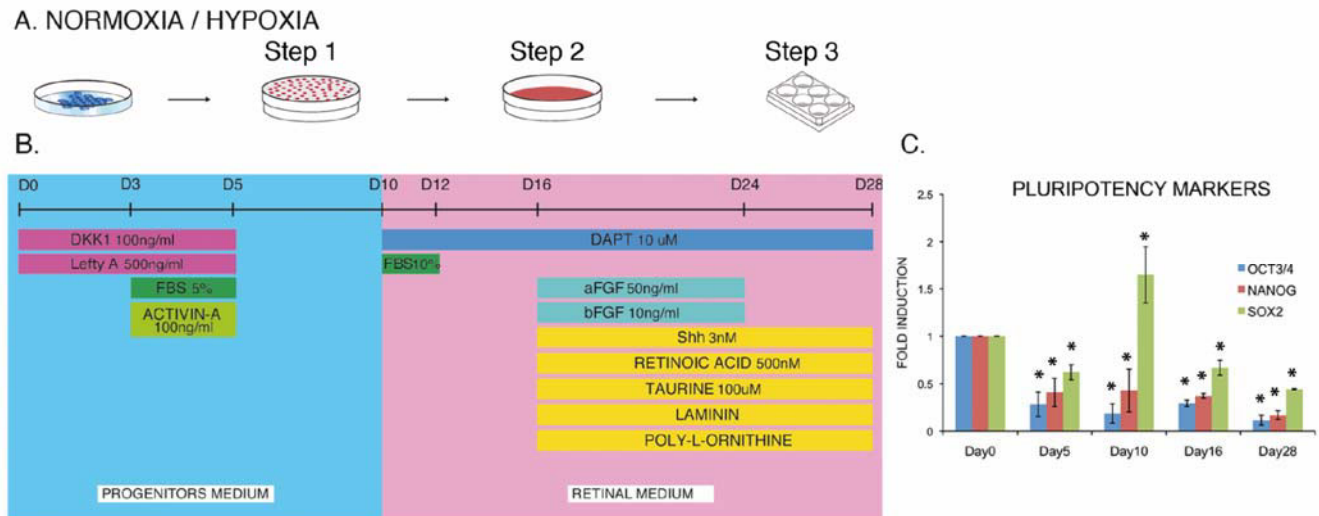


Figure 3. Hypoxia improves early retinogenesis. **A.** Schematic of early neural retina (purple) on embryonic day 14, where the differentiating cells are expressing the transcription factors Otx2 and Rax. **B.** Comparative immunofluorescence analysis showing positive cells for anterior neural specification marker Otx2 under hypoxia and normoxia. **C.** Comparative immunofluorescence analysis showing positive cells for the Eye Field Transcription Factor, Rax. **D.** Quantification of Rax positive cells derived under normoxic (blue bar), and hypoxic condition (red bar). **E.F.G.** The time course of expression of the eye field transcription factors, Six3, Pax6, and Rax respectively, by qPCR in normoxia (blue bar) and hypoxia (red bar). **H.** Immunofluorescence analysis showing Rax positive cells colocalized with Pax6 after 10 days of differentiation under hypoxia. * $p \leq 0.05$ was considered statistically significant.

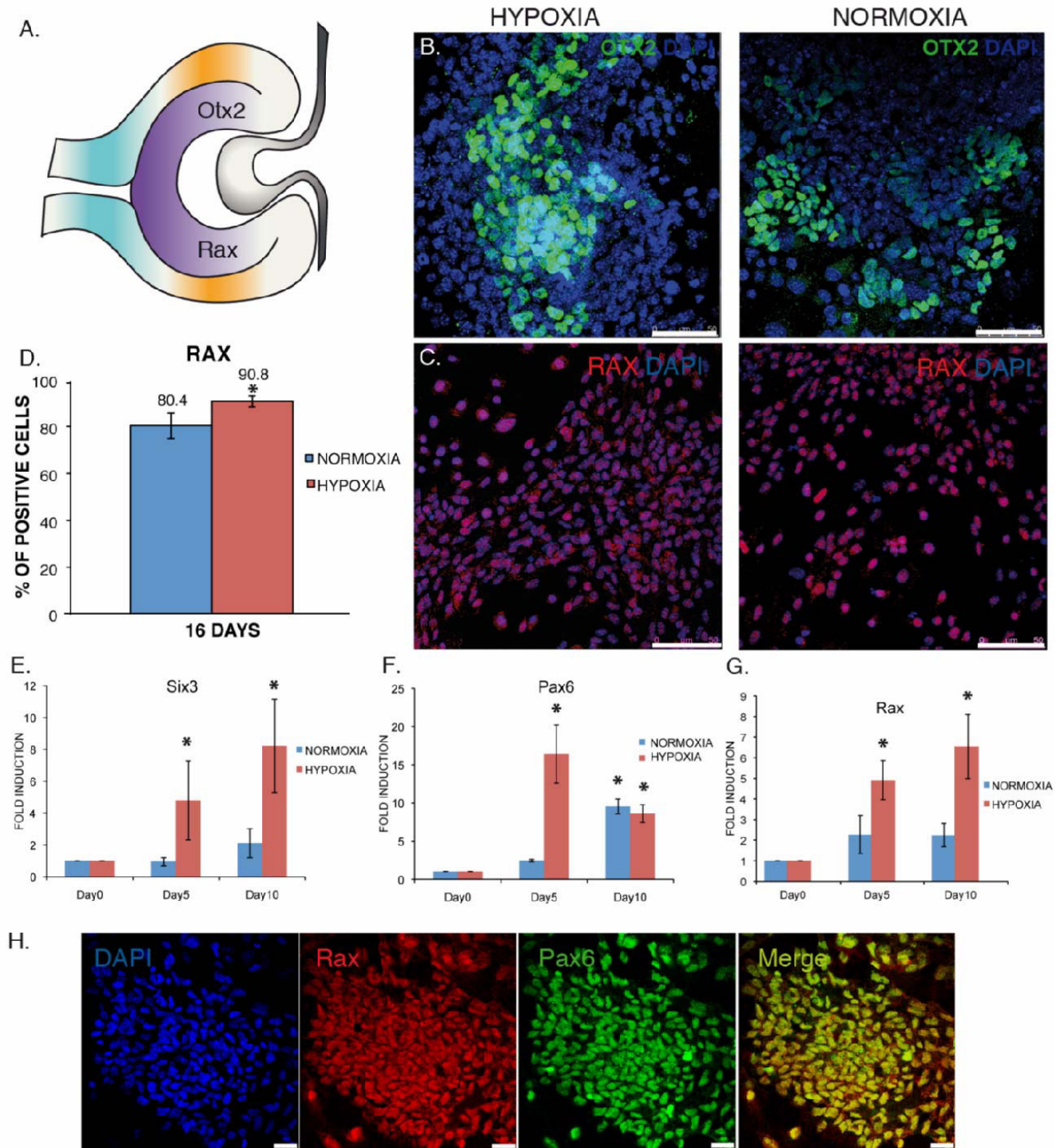


Figure 4. Hypoxia improves neural retina phenotype acquisition. **A.** Immunocytochemical analysis showing that all Rax positive cells were also positive for Mitf by day 16 under hypoxia. **B.** Photomicrograph showing clusters formed by day 16 of differentiation. **C.** Immunocytochemical analysis showed Chx10⁺/Pax6⁺ cells deriving radially away from the clusters by day 16 of differentiation. **D.** Higher magnification showing Chx10 positive cells were also positive for Pax6. **E.** **F.** The time course of expression of the neural retina transcription factors, Mitf and Chx10, respectively, by qPCR in normoxia (blue bar) and hypoxia (red bar). * $p \leq 0.05$ was considered statistically significant.

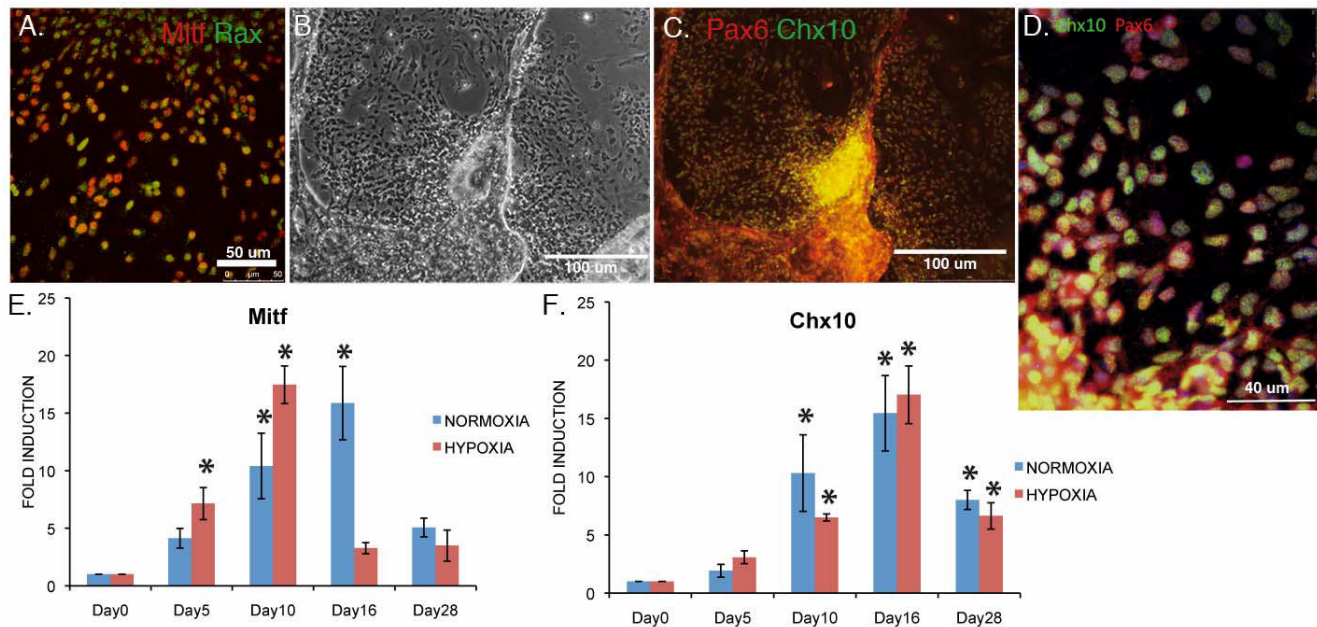


Figure 5. Hypoxia improves late retinogenesis. **A.** Immunocytochemical analysis showing Crx⁺ cells in hypoxic culture by day 28 of differentiation. **B.** Quantification of the percentage of Crx⁺ cells determined by 3 independent experiments under both conditions of differentiation. **C.** Comparative qPCR analysis of Crx gene expression over the 28 days of differentiation under normoxia (Blue bars) and hypoxia (red bars). **D.** Immunocytochemical analysis showing Nrl⁺ cells in hypoxic culture by day 28 of differentiation. **E.** Comparative qPCR analysis of Nrl gene expression during differentiation in normoxic (blue bar) and hypoxic (red bar) conditions. **F.** Quantification of the percentage of Opsin⁺ cells in normoxic (blue bar) and hypoxic (red bar) conditions. **G.** Comparative immunofluorescence analysis showing positive cells for cone specific marker Opsin-S after 28 days of differentiation under normoxia or hypoxia. * $p \leq 0.05$ was considered statistically significant.

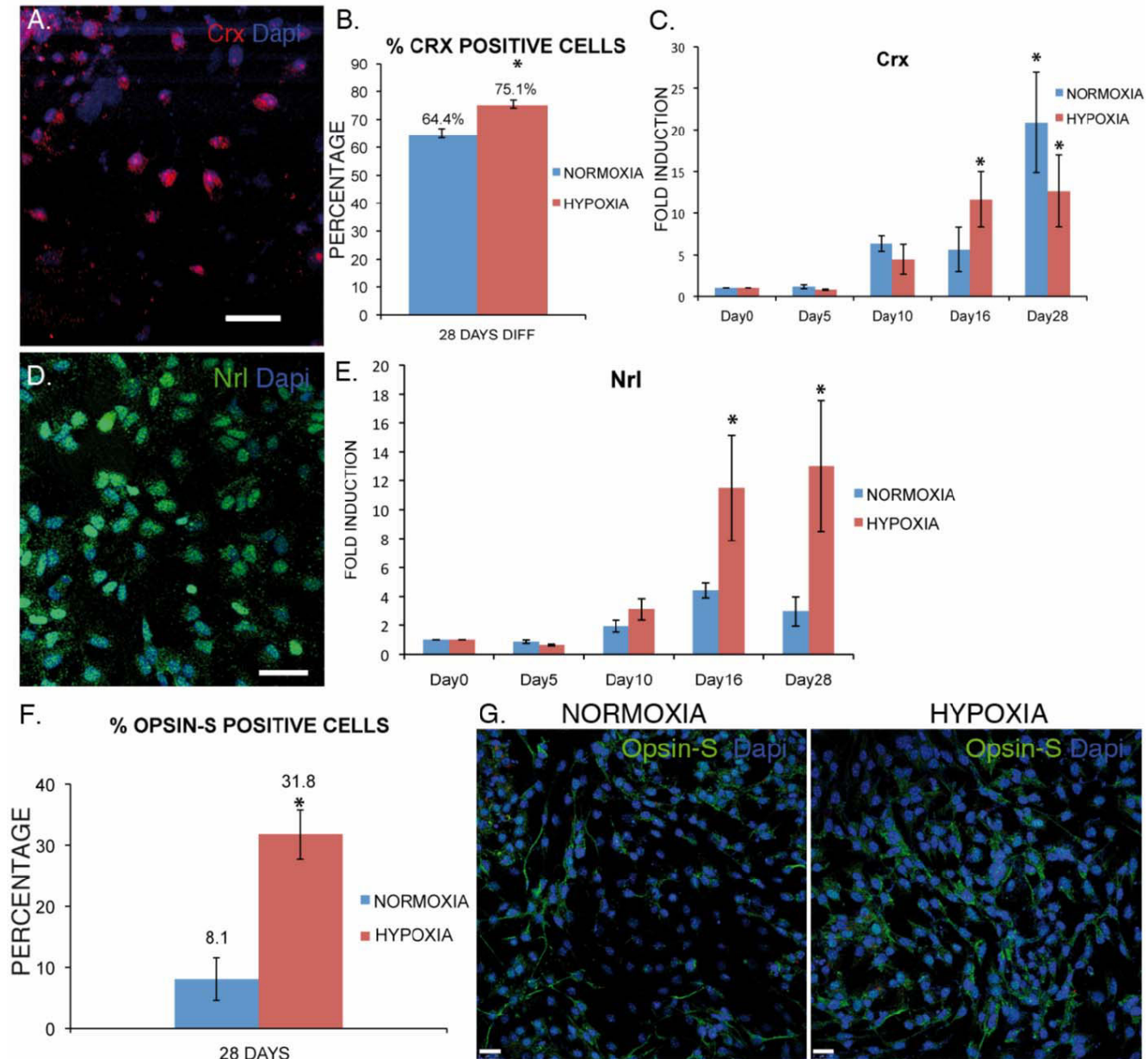


Figure 6. Hypoxia increase the yield of Rhodopsin positive cells. **A.** Immunocytochemical analysis showing the coexpression of rod photoreceptor markers Rhodopsin and Recoverin after 28 days in hypoxic culture conditions. **B.** A representative Rhodopsin⁺ cell in hypoxic conditions after 28 days in culture. **C.** Comparative qPCR analysis of Rhodopsin gene expression during the differentiation in normoxic (blue bars) and hypoxic conditions (red bars). **D.** Comparative immunofluorescence analysis of expression of Rhodopsin during the differentiation in hypoxic and normoxic conditions. **E.** Quantification of the percentage of Rhodopsin⁺ cells in normoxia (blue bar) and hypoxia (red bar). **F.** Schematic representation of obtained retinal cells. **G.** Immunofluorescence analysis of other cell types present after 28 days of differentiation Tuj1⁺/Recoverin⁻ cells. **H.** Comparative immunofluorescence analysis showing positive cells for the proliferation marker; Ki67 after 28 days of differentiation under hypoxia and normoxia. **I.** Quantification of the percentage of Ki67⁺ cells after 28 days of differentiation in normoxia (blue bar) and hypoxia (red bar). * $p \leq 0.05$ was considered statistically significant.

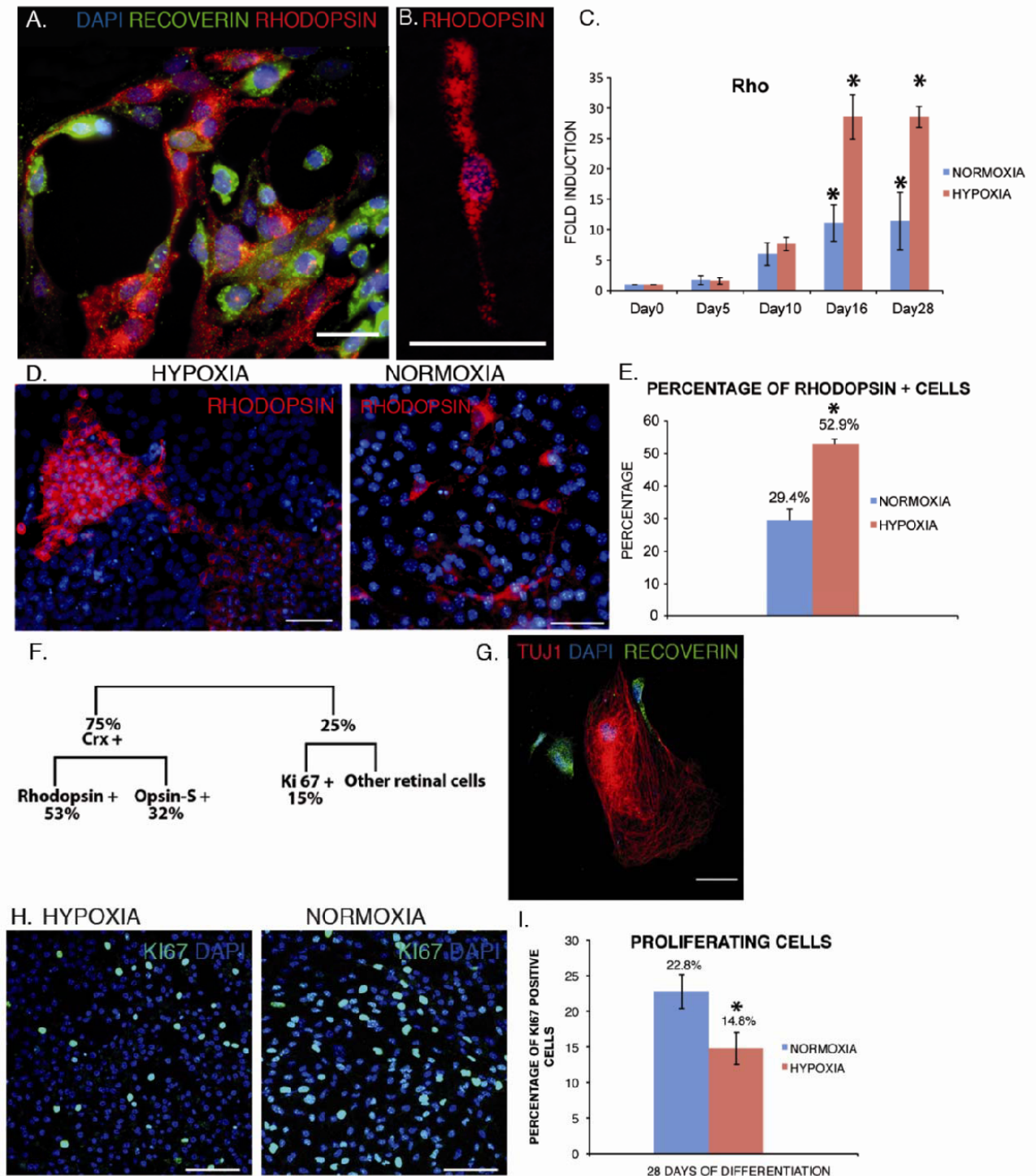


Figure 7. Transplantation of photoreceptors derived from ESC under hypoxic conditions.

A. Anatomy of the injection site and efficiency of differentiation 24 hours after transplantation. **B. C. D. E.** Higher magnification of inset in **A**. Coexpression of transplanted cells (red, PKH26), Rhodopsin⁺ (green) and DAPI (blue). **F.** The efficiency of differentiation towards photoreceptors 1 week after transplantation. **G. H. I. J.** Higher magnification of inset in **F**. showing transplanted cells (red, PKH26) colocalizing with Recoverin (Rcvn, green) and DAPI (blue). **K.** Colocalization of PKH26 and Rhodopsin in ONL 1 week after subretinal injection.

TRANSPLANTATION OF PHOTORECEPTORS DERIVED FROM MOUSE EMBRYONIC STEM CELLS

EFFICIENCY OF DIFFERENTIATION

MIGRATION / INTEGRATION

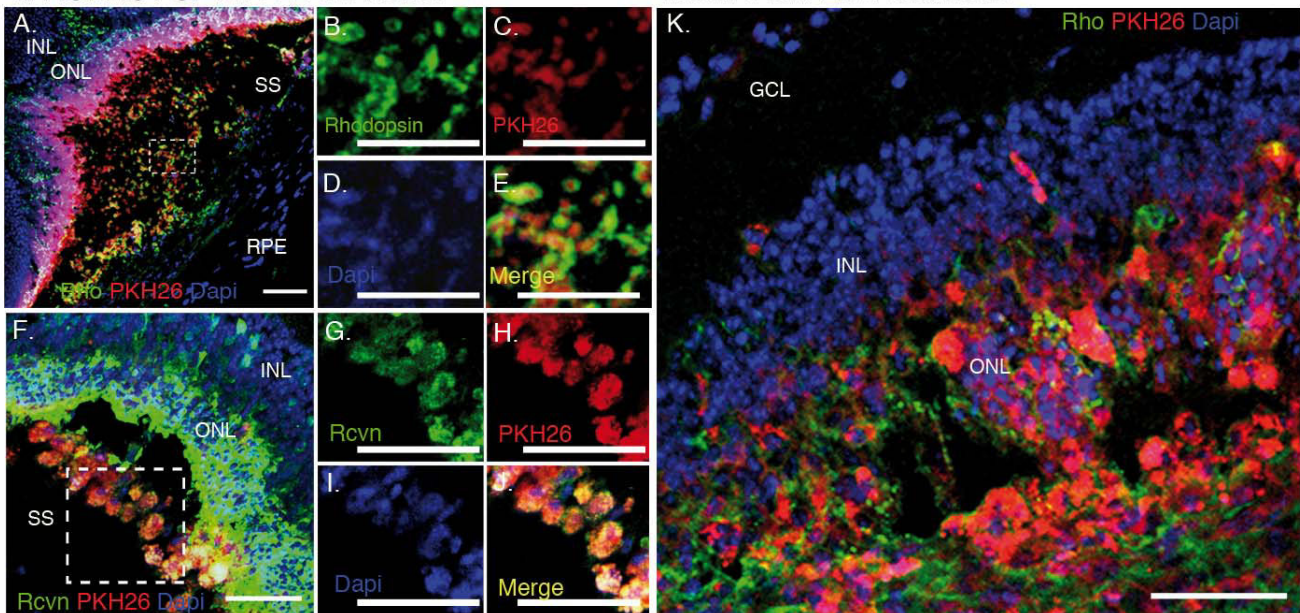


Table 3b. List of primary antibodies used for analysis of pluripotency and stemness.

Antibody	Type	Source	Dilution
SSEA-1	Mouse monoclonal	Cell Signaling	1:100
OCT-4	Rabbit monoclonal	Cell Signaling	1:100
Nanog	Goat monoclonal	R&D Systems	1:50
Sox2	Mouse monoclonal	R&D Systems	1:50
PE-Nanog	Mouse monoclonal	BD Pharmingen™	
PerCP-Cy5.5-Oct3/4	Mouse monoclonal	BD Pharmingen™	
Alexa Fluor® 647-Sox2	Mouse monoclonal	BD Pharmingen™	
PE-Mouse IgG1, κ	Mouse Isotype control	BD Pharmingen™	
PerCP-Cy5.5-Mouse IgG1, κ	Mouse Isotype control	BD Pharmingen™	
Alexa Fluor® 647-Mouse IgG2a, κ	Mouse Isotype control	BD Pharmingen™	

Supplementary Table 3a. List of primary antibodies used to study the retinal differentiation of embryonic stem cells

Antibody	Type	Source	Dilution
Ki67	Rabbit polyclonal	Abcam	1:200 (1:100 FACS)
Mitf	Mouse monoclonal	Abnova	1:500
Opsin-s	Rabbit polyclonal	Abcam	1:200
Otx2	Rabbit polyclonal	Millipore	1:500
Rhodopsin	Mouse monoclonal	Abcam	1:100
Recoverin	Rabbit polyclonal	Millipore	1:1000
RAX	Rabbit polyclonal	Abcam	1:300
ZO1	Rabbit polyclonal	Invitrogen	1:50
Crx	Mouse monoclonal	Novus Biologicals	1:100
RPE65	Mouse monoclonal	Abcam	1:250
Chx10	Sheep polyclonal	Exalpha	1:1000
Pax6	Mouse monoclonal	Hybridoma Bank	1:100
Nrl	Rabbit polyclonal	Gift	1:1000
Hif1a	Rabbit polyclonal	Santa cruz	1:50
Tuj1	Mouse monoclonal	Covance	1:500

Supplementary Table 2. PCR primers used to study the loss of pluripotency and the retinal differentiation

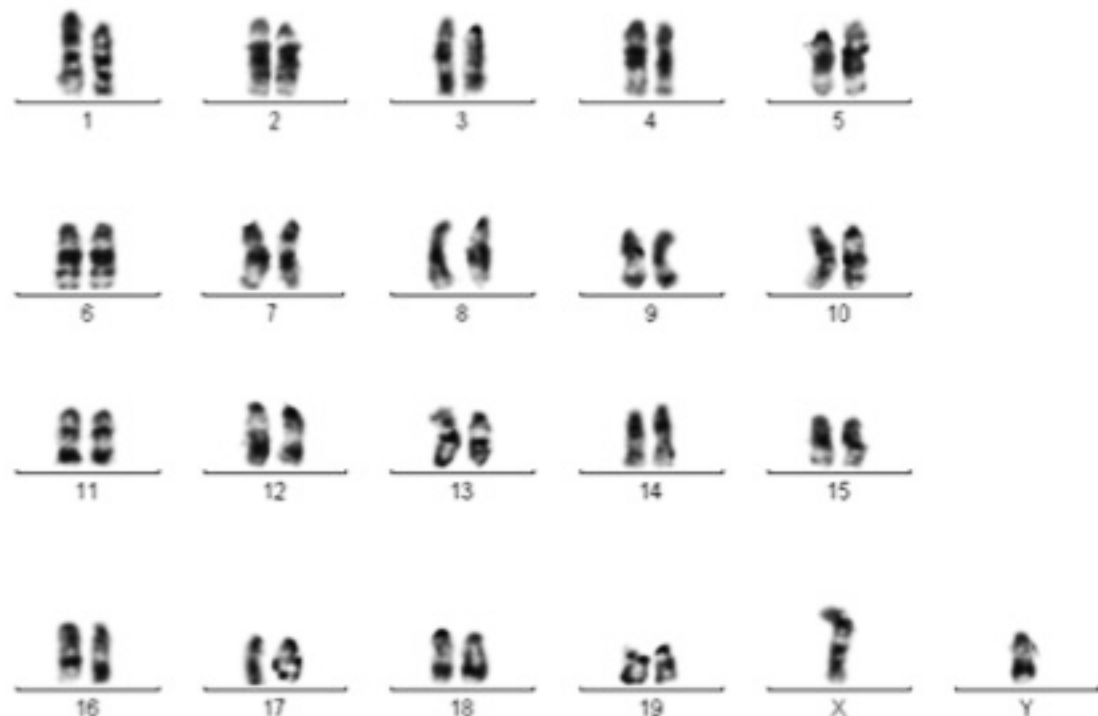
Gene name	Primer sequence	Ta (C°)	Product size (bp)	NCBI Accesion No.
B-Actin	F: TCCTGTGGCATCCACGAAACTACA R: ACCAGACAGCACTGTGTTGGCATA	60	93	NM_007393.3
Cdkn1a	F: TTGTCGCTGTCTTGCACTCTGGT R: AGACCAATCTGCGCTTGGAGTGAT	62	141	NM_007669.4
Vegfa	F: GGAGAGCAGAAGTCCCATGA R: ACTCCAGGGCTTCATCGTTA	60	184	NM_001025250
Pax6	QT01052786 Qiagen	60	120	
Chx10	QT00112056 Qiagen	60	134	
Nrl	QT00109298 Qiagen	60	71	
Crx	QT00115402 Qiagen	60	127	
Rax	QT01775193 Qiagen	60	88	NM_013833
Rhodopsin	QT00099022 Qiagen	60	78	
Nanog	QT01743679 Qiagen	60	190	
Sox2	QT00249347 Qiagen	60	103	
Tert	QT00104405 Qiagen	60	145	
Pou5f1	QT00109186 Qiagen	60	101	
Six3	QT01045219 Qiagen	60	117	X90871
Otx2	QT01079771 Qiagen	60	115	
Mitf	QT00131313 Qiagen	60	87	NM_001113198
ZO-1	QT00493899 Qiagen	60	150	NM_001163574

Supplementary Table 1. Formulations of media used.

Maintenance Medium (500mL)	EB Medium (500mL)	Progenitors Medium (500mL)	Retinal Medium (500 ml)
DMEM GLUTAMAX-I (GIBCO32430100) 459ml	DMEM GLUTAMAX-I (GIBCO32430100) 459ml	DMEM GLUTAMAX-I (GIBCO32430100) 459ml	MEM-HEPES 66% 330ml HBSS 33% 50 ml
FBS (Hyclone) 15% 75 ml	KSR (GIBCO 10828) 5% 25ml	NEAA (GIBCO 11140) 0.1mM 5ml	GLUCOSE SOL 2.88g Glucose in 115ml HBSS
NEAA (GIBCO 11140) 0.1mM 5ml	NEAA (GIBCO 11140) 0.1mM 5ml	ANTIBIOTICS (GIBCO 15140) 1% 5ml	L-GLUTAMINE (Sigma) 200 μ M 500 μ l
ANTIBIOTICS (GIBCO 15140) 1% 5ml	ANTIBIOTICS (GIBCO 15140) 1% 5ml	Na+ PYR (GIBCO 11360) 1mM 5ml	N2 SUPPLEMENT (GIBCO 17502) 1% 5mL
Na+ PYR (GIBCO 11360) 1mM 5ml	Na+ PYR (GIBCO 11360) 1mM 5ml	2-ME (GIBCO 31350-010) 0.1mM 1ml	FBS (Hyclone) 1% 5 ml
2-ME (GIBCO 31350-010) 0.1mM 1ml	2-ME (GIBCO 31350-010) 0.1mM 1ml	KSR (GIBCO 10828) 5% 25ml	ANTIBIOTICS (GIBCO 15140) 1% 5ml
LIF (Hyclone;			

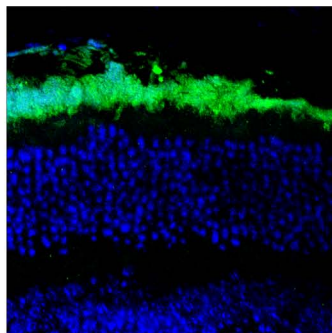
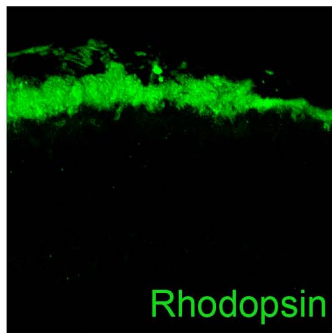
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Karyotype of mESC differentiated under hypoxia

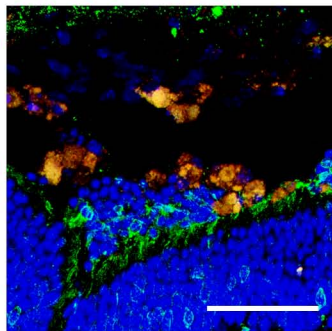
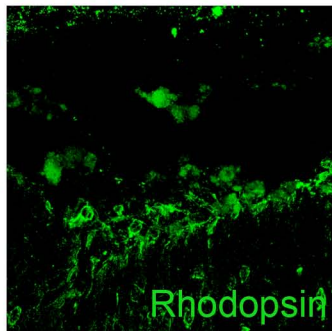
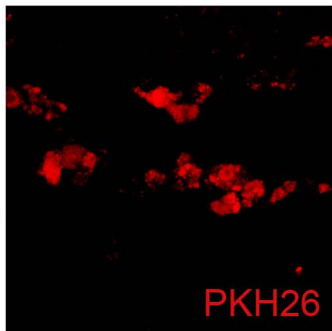


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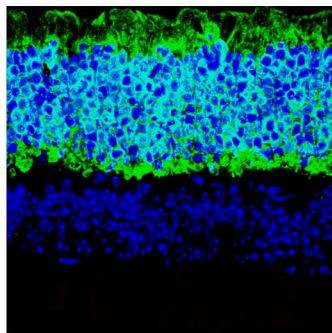
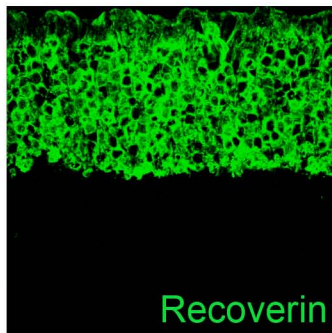
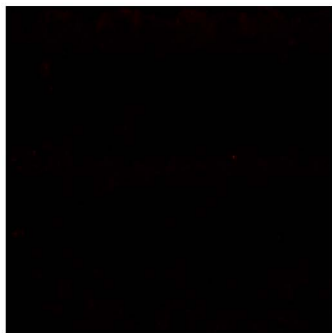
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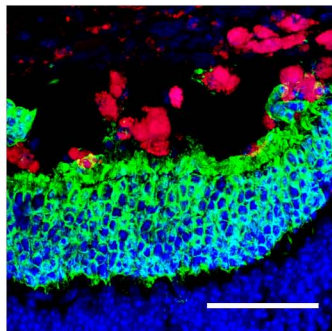
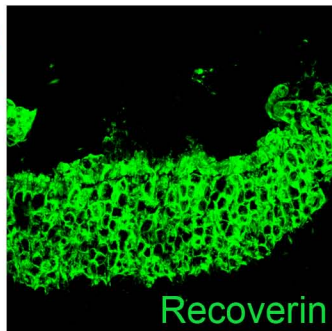
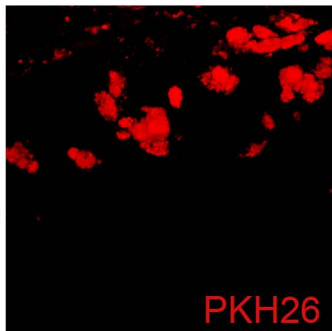
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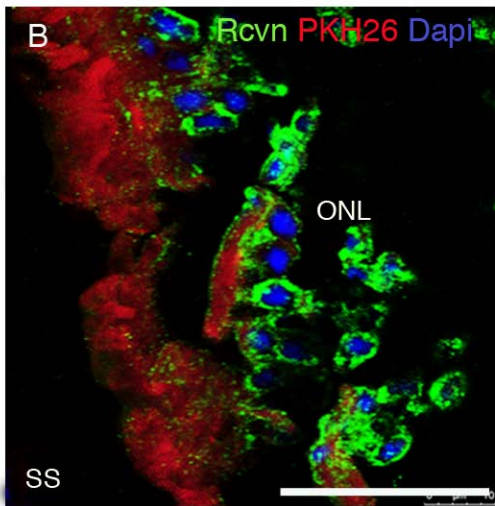
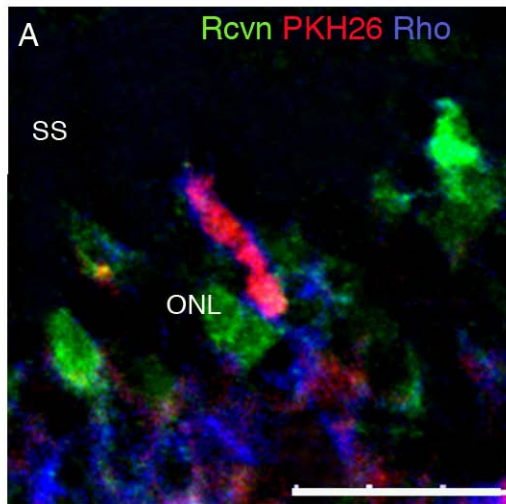
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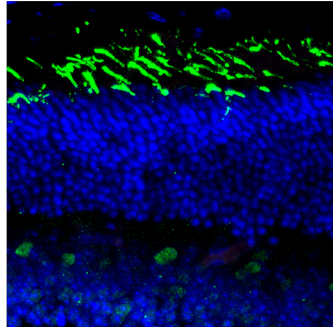
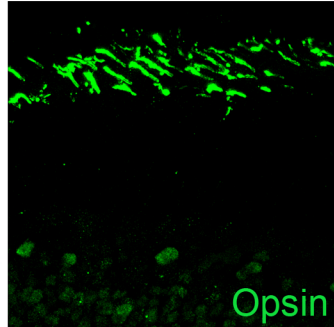
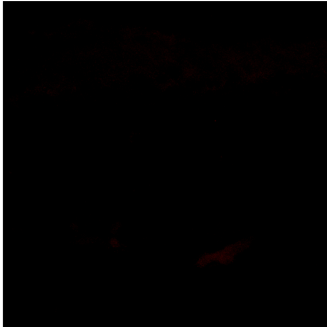
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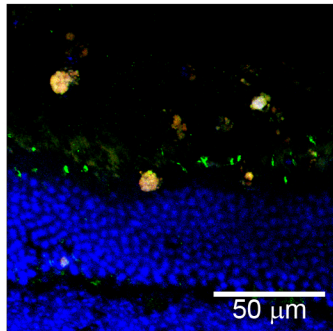
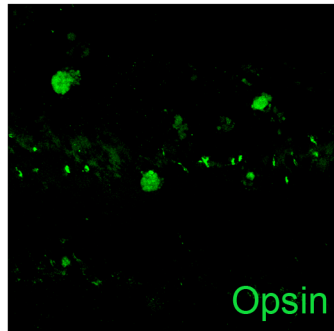
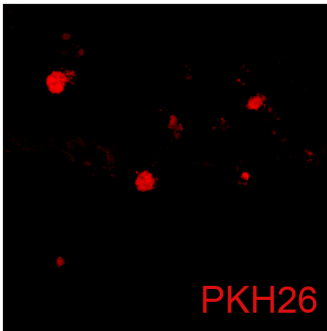
INTEGRATION OF TRANSPLANTED CELLS WITHIN THE HOST RETINA



Control



Injected



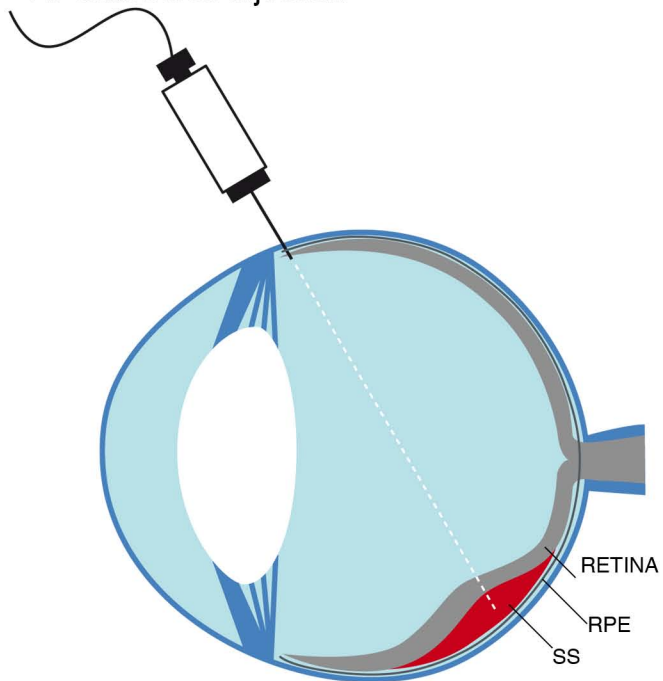
PKH26

Opsin

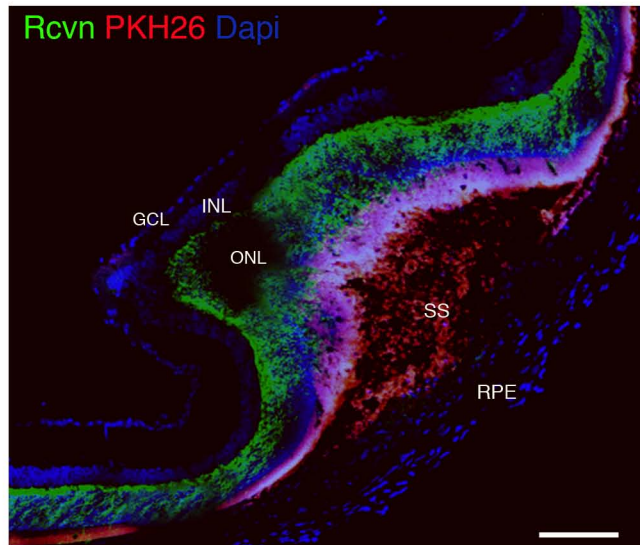
Opsin

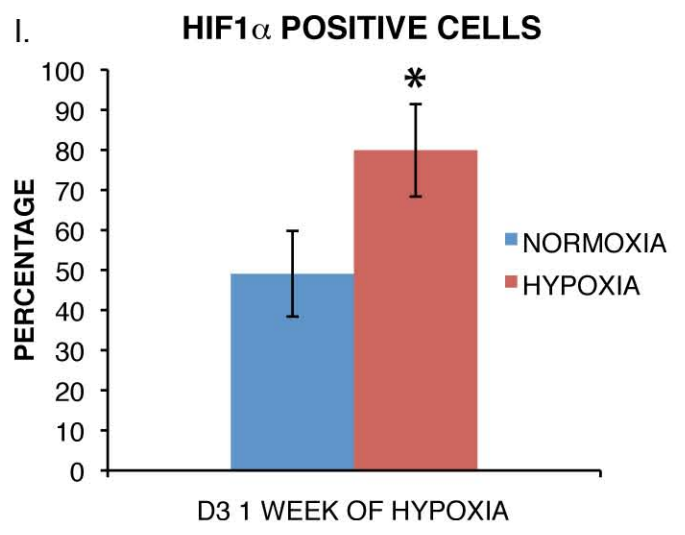
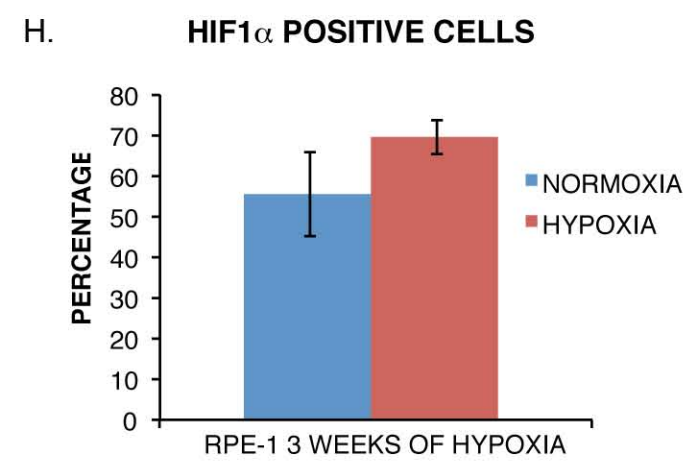
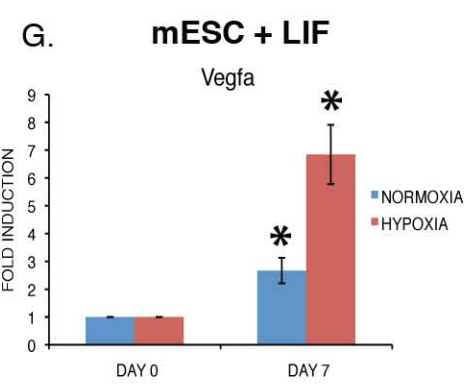
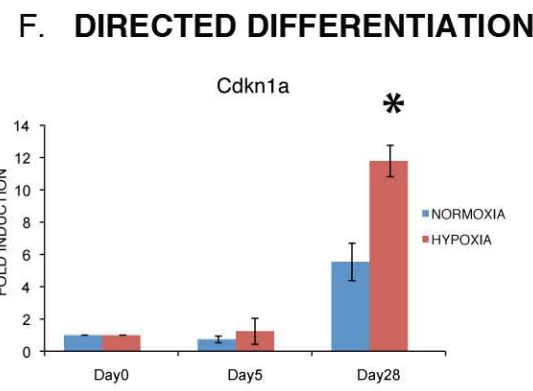
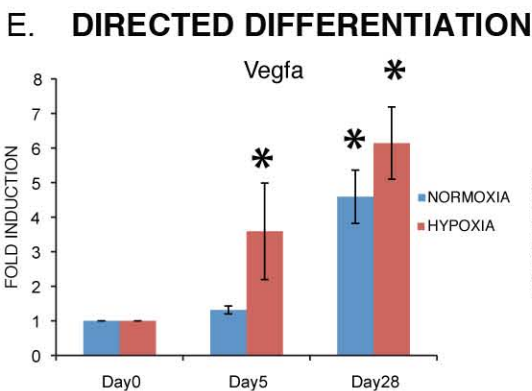
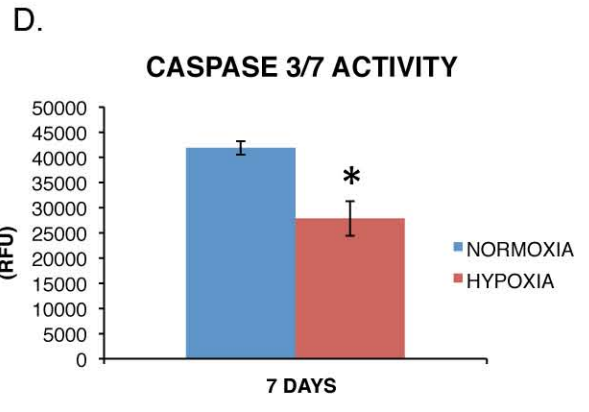
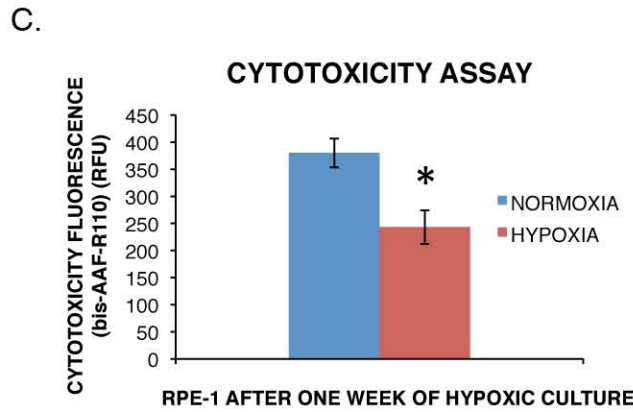
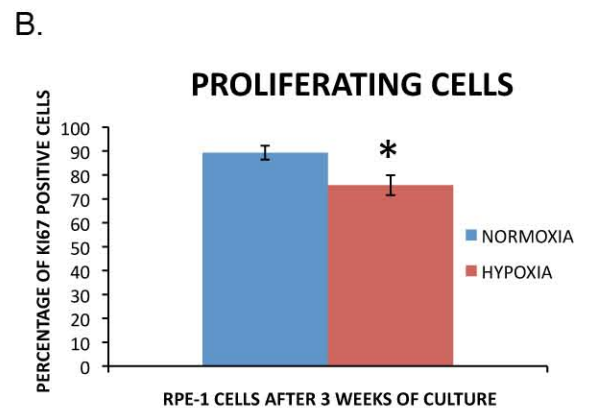
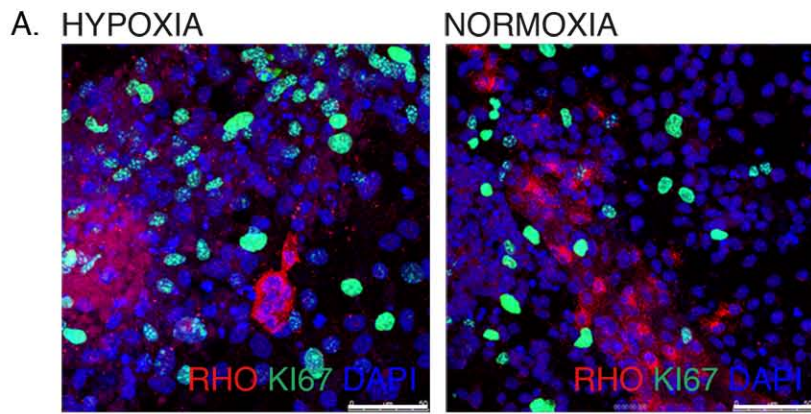
50 μ m

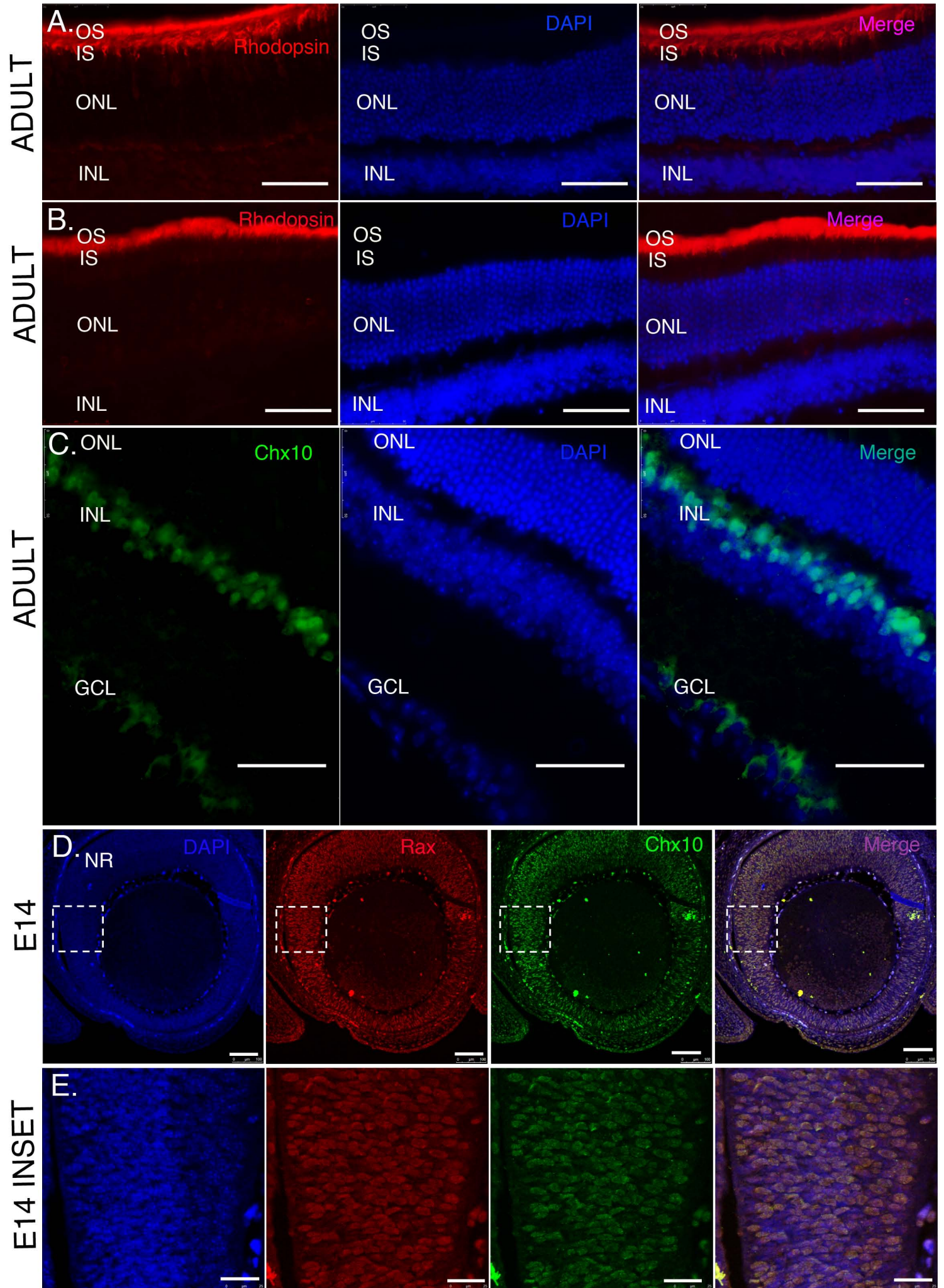
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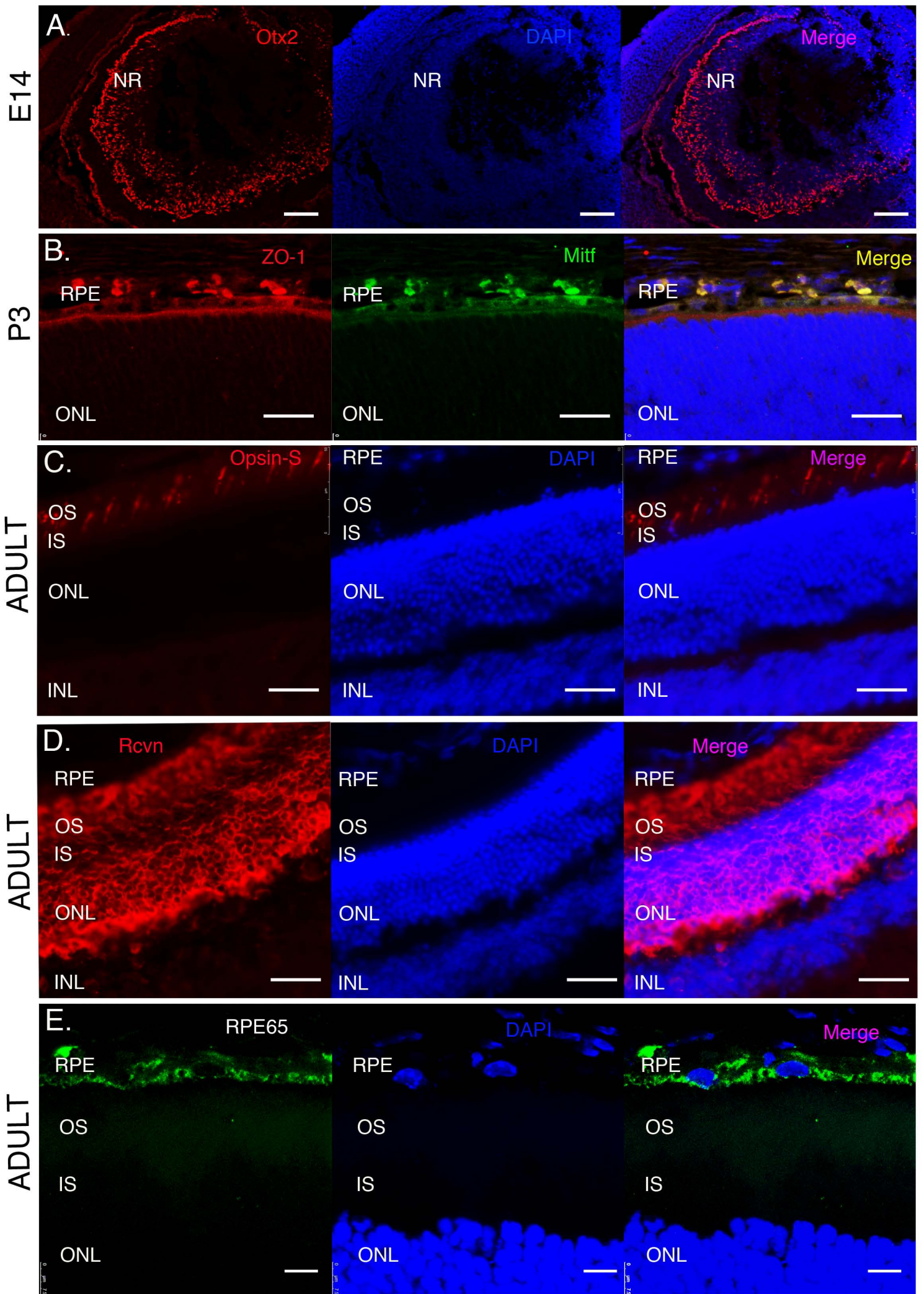


B. Anatomy

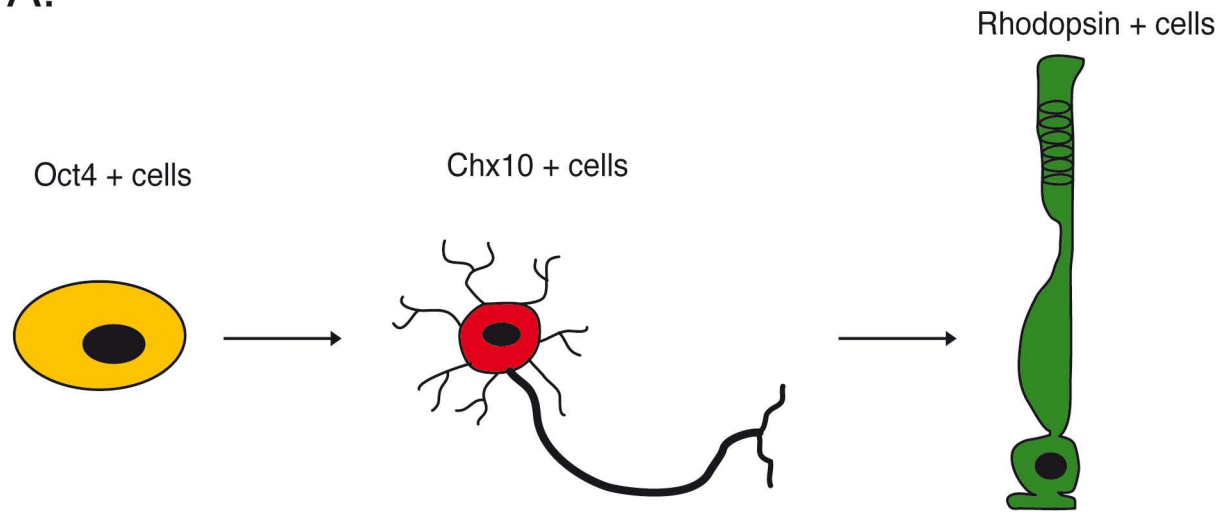




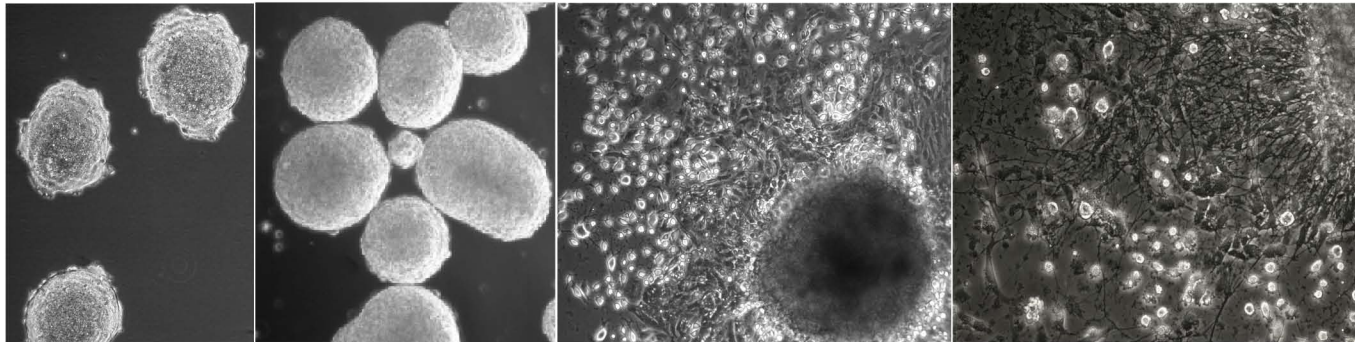
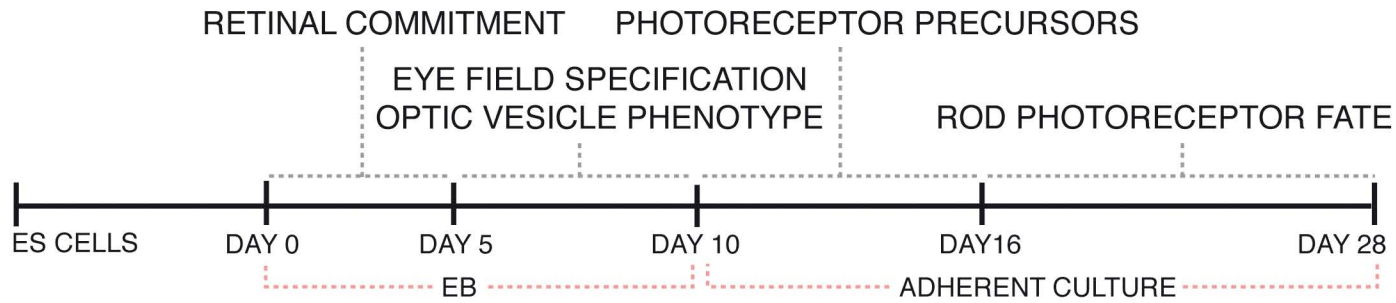




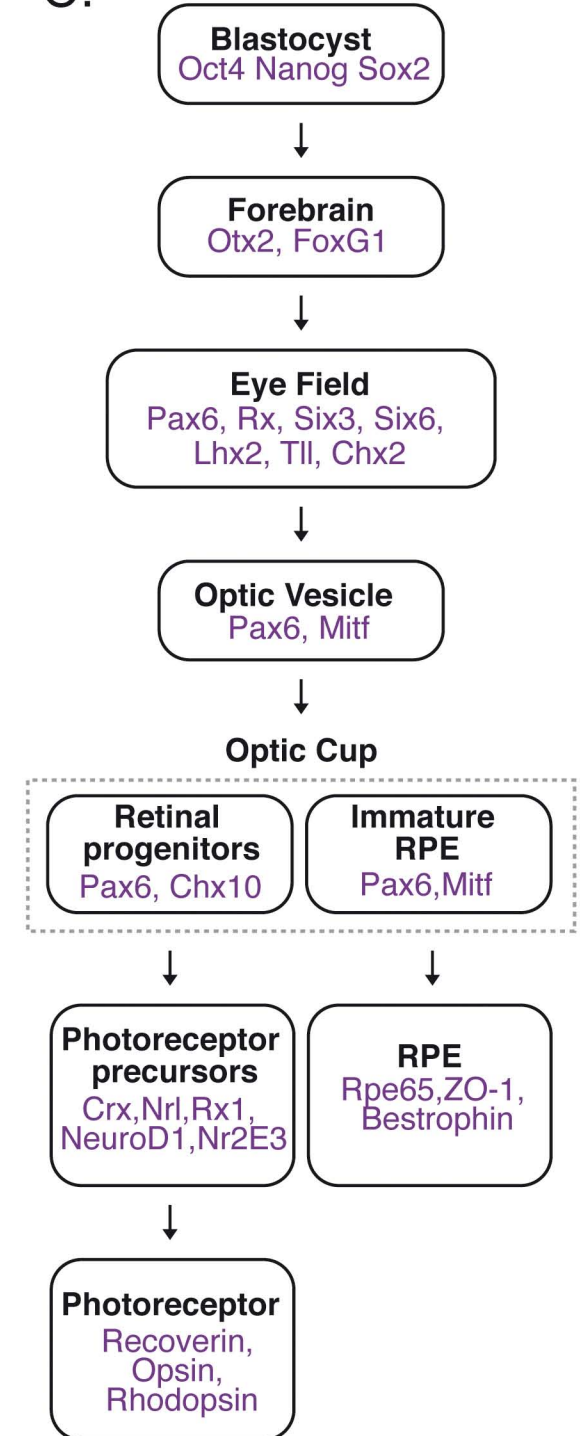
A.



B.



C.



Supplementary material and methods

RNA isolation and quantitative RT-PCR. Total RNA isolation was performed using Trizol® reagent (Invitrogen) or RNeasy kit (Qiagen), according to the manufacturer's instructions and followed by treatment with TURBO DNase™ (2 U/μl) (Ambion, Warrington, UK). RNA concentration and purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Thermo Scientific, Epsom, UK). The integrity of total RNA was qualitatively assessed on an Agilent 2100® Bioanalyser. Reverse transcription was carried out with 1 μg of total RNA using the Superscript III RT kit (Invitrogen). Quantitative PCR (qPCR) reactions were performed using SensiFAST™ SYBR No-ROX Kit (Bioline, London, UK). All samples were normalized against a housekeeping gene (β-actin). The primer sets, as well as the annealing temperatures are listed in Supporting Information (**Supplementary Table 2**). All qPCR reactions were run at 40 cycles and data analysis was done using the CFX Manager v2.1 software (BioRad) by the $\Delta\Delta C_T$ method.

Immunocytochemistry: Cell cultures were washed in PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (r.t.). Fixed cells were washed twice with PBS before staining. For nuclear staining, permeabilization within ice methanol was accomplished for 30 min at -20°C. After permeabilization, cells were blocked with 3% Donkey serum, 3% Goat serum in 0.5% Triton-PBS for 30 min at r.t. Immunostaining was performed overnight at 4°C in 0.5% Triton-PBS using the antibodies listed in Supporting Information (**Supplementary Table 3a and 3b**). Cells were counter-stained with 300 nM DAPI for 10 min at r.t. For negative controls, primary antibodies were omitted and the same staining procedure was carried out. Positive cells were detected using either Alexa488-, Alexa594-, Alexa633- (Invitrogen) or Dylight549- conjugated secondary antibodies in a Leica DM 5500 microscope (Leica

Microsystems, Wetzlar Germany) and a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar Germany). Specificity of each antibody was determined in mouse embryonic and adult retinal tissue (**Supplementary Figure 2 and 3**).

Image analyses

The immunocytochemistry experiments were repeated at least 3 times. For Image analysis at least 8 microscopic fields from each sample were taken randomly using a 40X lens objective in a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar Germany). To reduce human bias, a semi-automated image analysis system was used to determine the percentage of immunoreactive cells from digital images using the MetaMorph NX ® v 7.5.1.0 Software (Molecular Devices, Downingtown, PA). First, cells and processes of interest were outlined to exclude adjacent cells or areas of non-specific immunoreactivity.

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EBs were washed twice with DPBS (Gibco 14190) and enzymatically digested by incubation with the Embryoid Body Dissociation Kit from Miltenyi Biotech (Bergisch Gladbach, Germany) for 15 minutes at 37°C. The single cell suspension obtained was washed in DPBS and fixed with 4% paraformaldehyde in PBS for 20 min at r.t. Fixed cells were washed twice with PBS before staining. Cells were then immunostained using the BD Stemflow™ Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit (BD Biosciences) following the manufacturer's instructions. Appropriate isotype controls provided with the kit were used to immunostain the negative populations. At least 10,000 events were analyzed in each experiment using FACSCalibur system (BD Biosciences). Results correspond to at least 6 individual runs and the number of positive cells within the gated population was analyzed using CellQuest™ Pro (BD Biosciences) software.

RPE-1 and D3 cells were washed twice with PBS and enzymatically digested by incubation with Trypsin for 4 minutes at 37°C. The single cell suspension obtained was washed in PBS and fixed with 4% paraformaldehyde in PBS for 20 min at r.t. Fixed cells were washed twice with PBS before staining. Cells were then permeabilized with 1X Perm Wash Buffer (BD Biosciences) at r.t. for 10 min and incubated overnight with the Rabbit Polyclonal Hif1 α primary antibody (1:50 dilution) and the corresponding IgG Isotype control in staining buffer containing 1% FBS in PBS. Immunostaining was completed with Goat anti rabbit Alexa488-conjugated secondary antibody (1:500). 10.000 events were analysed in each experiment (n=3) using FACS Calibur system (BD Biosciences). The number of positive cells within the gated population was analysed using Cell Quest™ Pro (BD Biosciences) software.

ApoTox-Glo™ Triplex Assay

This kit combines three assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. In the first part of the assay, it measures two protease activities simultaneously; one being a marker of cell viability and the other being a marker of cytotoxicity. Peptide substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. Peptide substrate (bis-alanylalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Bis-AAF-R110 is not cell-permeable, so no signal from this substrate is generated by intact, viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected

simultaneously. In the second part of the assay, the Caspase-Glo[®] 3/7 Reagent, added in an "add-mix-measure" format, results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal produced by luciferase.

RPE-1 cells were seeded in a flat 96-well micro-plate (approximately 500/well) (Nunc) as triplicates. Three different types of controls, namely: positive, untreated, and negative controls were used throughout the study. Positive control had cells with culture medium exposed to 10 μ M MG132 for 16 hours to induce apoptosis. Control cell cultures contained cells untreated with MG132. Negative control (background) contained only culture medium without cells.

After 7 days of exposure to either normoxic or hypoxic conditions, 20 μ l of Viability/Cytotoxicity reagent containing both GF-AFC and bis-AAF-R110 substrates was added to each well, and briefly mixed by orbital shaking at 500 rpm for 30 seconds and then incubated at 37°C for 30 minutes. Fluorescence was measured at 400_{Ex}/505_{Em} (Viability) and 485_{Ex}/520_{Em} (Cytotoxicity) by using Thermo Scientific Varioskan[®] Flash Spectral Scanning Multimode Reader. After that 100 μ l of Caspase-Glo 3/7 reagent was added to each well, and briefly mixed by orbital shaking at 500 rpm for 30 seconds and then incubated at room temperature for 30. Luminescence was measured using a Thermo Scientific Varioskan[®] Flash Spectral Scanning Multimode plate reader. Luminescence RFUs are proportional to the amount of caspase activity present.

RPE-1 cell cultures

Telomerase-immortalized human retinal pigment epithelia 1 (RPE-1), cells were cultured in DMEM-Ham's F12 supplemented with 10% fetal bovine serum (FBS) and L-Glutamine. RPE-1 cells were maintained at 37° C in a humidified 5% CO₂ environment under either Normoxic (20% oxygen Tension) or Hypoxic (2% Oxygen

tension) conditions in a thermo Fisher incubator (CO₂/O₂ WJ IR Model 3141, Thermo Electron Co, Fisher Scientific). Cell cultures were passaged every 7 days to 1:10 split ratio and fresh medium was exchanged every 48 hours.