Stem Cells and the Retina – Challenges for Regenerative Medicine

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1. Introduction

The retina, as one of the most accessible parts of the nervous system, has been extensively studied in different animal models. These studies have highlighted a remarkable conservation of the molecular mechanisms and gene regulatory networks involved in eye development and retinal differentiation. The initial phases of retinal specification see the activation and cooperation of a network of transcription factors (the Eye Field Transcription Factors, EFTFs) that allow the generation of a morphogenetic eye field in the anterior neural plate. The development of the Vertebrate eye then continues with a series of morphogenetic events, concomitant with the closure of the neural tube, that include evagination of the optic vesicles from the ventral forebrain and the subsequent formation of lens and retina. The retina comprises two juxtaposed parts: the neural retina represents the structure lining the inner surface of the eye and is involved in the reception and first elaboration of light stimuli. It presents a precise and stratified architecture based on seven different cell types: six types of neurons (cones, rods, amacrine cells, horizontal cells, bipolar cells and ganglion cells) and one type of glial cells (Müller glia). The Retinal Pigmented Epithelium is generated by the outer part of the neural retina and is fundamental for the correct functioning of retinal photoreceptors. Different molecular pathways that will be described are involved in retinal cell type specification.

As most adult organs, the adult eye has an endogenous population of stem cells – the retinal stem cells – localized in different anatomical structures (Ciliary Marginal Zone in amphibians and fish; Adult Ciliary Epithelial cells in Mammals). These cells remain multipotent throughout the lifetime of the organism, but in Mammals, especially in humans, they have lost the capability of reactivating after injury, thus being unable to repair the damaged retina. Identifying the molecular cues necessary to reactivate *in vivo* mammalian retinal stem cells is one of the strategies under study to repair damaged retinae, the other being the transplantation of differentiated cells. In the last years, many efforts have been devoted to the possibility to drive embryonic stem cells to become retinal cells. The aim is to initially generate retinal progenitors and subsequently mature retinal neurons of the types lost in degenerative diseases, such as photoreceptors and ganglion cells. Currently, only few protocols show promising results: we will describe and discuss them, analyzing their efficiency and capability of generating different mature retinal neuronal types. We will also discuss to which extent *in vitro* differentiation protocols recapitulate the developmental steps of embryonic development, and whether a more reliable representation of *in vivo*

processes could ameliorate the *in vitro* protocols. This type of analysis aims to elucidate the differentiation of retinal precursors into mature retinal cells and to give important suggestions to reproduce these processes *in vitro*. One important clue derives from our group, as we showed the capability of *Xenopus laevis* stem cells (Animal Cap Embryonic Stem cells, or ACES) to originate a functional retina when overexpressing Noggin, a secreted factor normally involved in the specification of the neural tissue during embryogenesis (Lan et al., 2009).

The last part of the chapter will describe the current applications and future perspectives of *in vitro* differentiated retinal cells. Retinal degenerative diseases affect many individuals and are the principal cause of irreversible and invalidating blindness in the western world. Cell replacement therapies represent one of the possible strategies to cure retinal dystrophies with regenerative medicine. We will describe what has been accomplished up to now in terms of stem cells transplantation in the retina, in animal models. Moreover, an emerging field of application of stem cell differentiation, especially since the generation of induced pluripotent stem cells, is the possibility to create patient-specific cellular models of retinal diseases. These will have great value for understanding the molecular mechanisms of the disease, screening for drug candidates that can prevent or slow down degeneration, understanding the variation in response of different patients.

2. Molecular regulation of Vertebrate eye development

The retina is an excellent model to study complex features of the nervous system, such as cell lineage, cell migration and connectivity. For these reasons, the eye represents one of the most important systems analysed during central nervous system development.

Genetic and molecular information acquired in eye and retinal development show that genes controlling retinal cell-fate are remarkably conserved among vertebrate species. These genes encode for different types of proteins, such as signalling molecules and transcription factors, and contribute both to retinal precursor specification as to their final differentiation. In the last years, the increasing know-how in transplantation techniques and more in general in regenerative medicine have cast a new interest on the genes and mechanisms involved in retina and eye formation, with the perspective of setting up cell replacement strategies to cure to retinal degenerative diseases (Lamba et al., 2009a).

2.1 Initial specification of retinal fate: the eye field

Most of the experimental approaches targeted to understand the initial phases of specification of retinal precursors were conducted in *Xenopus* embryos, which represents an accessible vertebrate animal model to study the role of cell-fate determinants, lineage effects and cell-cell interactions during stages prior to gastrulation (Sive et al., 2000). In *Xenopus*, lineage analyses showed that each retina originates from nine specific blastomeres at the 32-cell stage even if their contribution is not retina specific, as they participate in the formation of all three germ layers (Zaghloul et al., 2005). In this context, the first positional information is given by the expression of maternal factors such as VegT and Veg1, which are known to promote endo-mesodermal fates. The expression of these genes at the vegetative pole contributes to the localization of blastomeres competent to become retina in the animal pole of the *Xenopus* embryos (Yan & Moody, 2007).

A retina-specific presumptive territory becomes well defined only at neurula stages. This region, the "eye field", is localized in the most anterior part of the neural plate, and will

subsequently split into two lateral domains that evaginate (optic vesicles). The eye field is composed by a non-homogeneous population of cells having different potentialities, constituted by early Retinal Progenitors Cells (RPCs) (Agathocleous & Harris, 2009). The RPCs comprise two groups of cells: multipotent progenitors, that are able to produce all retinal subtypes, and progenitors that produce only the retinal cells belonging to a single layer (Andreazzoli, 2009) (Fig.1).

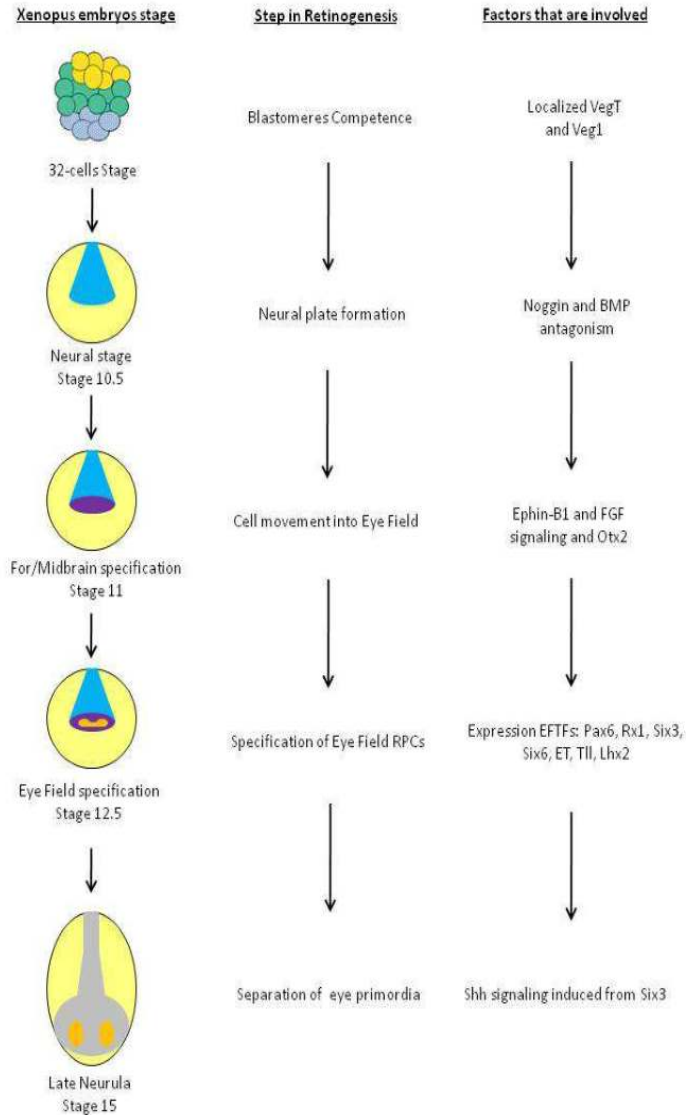| Xenopus embryos stage	Step in Retinogenesis	Factors that are involved
32-cells Stage	Blastomeres Competence	Localized VegT and Veg1
Neural stage Stage 10.5	Neural plate formation	Noggin and BMP antagonism
For/Midbrain specification Stage 11	Cell movement into Eye Field	Ephin-B1 and FGF signaling and Otx2
Eye Field specification Stage 12.5	Specification of Eye Field RPCs	Expression EFTFs: Pax6, Rx1, Six3, Six6, ET, Tll, Lhx2
Late Neurula Stage 15	Separation of eye primordia	Shh signaling induced from Six3

Fig. 1. Eye field and eye primordia development in *Xenopus* embryos, retinal specification steps and main known factors involved in each.

The first processes that are involved in the generation of the eye field are due to cell movements and rearrangements that start during gastrulation, continue with the identification of the two lateral retina primordia and end with the formation of the optic cup. In *Xenopus*, cells fated to form the anterior neural plate and the eye field show early cellular dispersal movement. This behaviour is promoted by Ephrin-B1 (Eph-B1) and inhibited by Fibroblast Growth Factors (FGFs), which modulate Eph-B1 intracellular ligands and down-regulate its signalling cascade (Moore et al., 2004). Other important factors involved are the inhibition of Wnt posteriorizing signaling and the inhibition of the Bone Morphogenetic Proteins (BMPs) cascade (Pera et al., 2001; Moore et al., 2004; Delaune et al., 2005). Moreover, our recent results suggest an important role of the secreted factor Noggin, a BMP antagonist, that is able to induce retinal fate in ectodermal explants (animal caps) of *Xenopus* embryos in a dose-dependent manner (Lan et al., 2009).

In all vertebrate species, the eye field and RPCs can be identified by the expression of several transcription factors. This set of homeobox transcription factors are named Eye Field Transcription Factors (EFTFs) and they are Pax6, Rx1, Six3, Optx2, ET, tll, Lhx2. They act as a self-regulating feedback network that specifies the eye field territory, and their over-expression is sufficient to induce ectopic eyes in *Xenopus* embryos (Zuber et al., 2003). The evidences about their relevance are shown by the fact that mutations in each of the EFTFs produce alterations of vertebrate eye development. For example, over-expression of Pax6 generates ectopic eyes in frogs while its loss-of-function leads to the "small-eye" or aniridia phenotypes in both mouse and humans (Chow et al., 1999, Hanson & Van Heyningen, 1995). Rx1/Rax is involved in eye field and pineal gland specification (Casarosa et al., 1997; Mathers et al., 1997). Overexpression of Rx1 in *Xenopus* produces an enlargement of the eye and ectopic retinal pigmented epithelium (RPE), while Rax knock-out mice present anophtalmia (Mathers et al., 1997; Voronina et al., 2004). Overexpression of Six3 in mice generates ectopic retinal tissue and a lack of it causes the absence of optical structures (Carl et al., 2002). ET/Tbx3, a T-box protein involved in the block of Rx1 transcriptional activity, is expressed in eye and cement gland primordia (Zuber et al., 2003). Moreover, both Six3 and ET are involved in Sonic hedgehog (Shh) signaling activity during the formation of the two bilateral eye fields from the initially single one (Takabatake et al., 2002; Geng et al., 2008). Lhx2, a LIM-homeodomain protein, is recruited in EFTFs transcriptional complexes, but it is involved in later events such as optic cup and lens formation (Yun et al., 2009). Moreover, Lhx2 promotes retinal proliferation *in vitro* (Tetreault et al., 2009). In the the eye field, the activation of the EFTFs and in particular of Rx1, that is fundamental for eye progenitors specification and proliferation, represses the anterior brain marker Otx2. This transcription factor is required for the initial specification of the eye field, but not for later stages of retinal development (Andreazzoli et al., 2003; Zuber et al., 2003).

2.2 Morphogenesis of the optic vesicle

As the neural tube closes, the two bilateral eye fields give rise to the optic vesicles, that evaginate from the lateral walls of the diencephalon. The optic vesicles come into contact with the epidermis and induce it to thicken and form the lens placode. As the lens differentiates, it invaginates until it pinches off from the epidermis. The lens acts as an inducer back to the optic vesicle to transform it into the optic cup and back to the epidermis to transform it into the cornea. The connection between the optic cup and the brain will form the optic nerve, while the optic cup delaminates into two layers: the outer layer of optic cup evolves in retinal pigmented epithelium (RPE) and the inner layer differentiates in neural retina (NR).

Optic cup development requires different signals deriving from surrounding tissues, such as: Shh, TGFß/BMP and FGF.

Shh is a secreted molecule, that as we said is involved in the formation of the two bilateral eye domains. Shh also activates the transcription of several factors that mediate the transition of the optic vesicle to optic stalk, RPE and NR, and enhances the differential identity of the ocular tissues. Shh binds its intramembrane receptor Patched. The binding produces a conformational change in Patched that fails to block Smoothened. The activation of Smoothened activates a transcriptional cascade that drives Gli family protein to transcribe specific transcription factors. At the end of this cascade: the proximal portion of each optic primordium, including optic stalk and ventral retina, starts to express Pax2 and Vax family homeoproteins (Liu et al., 2008; Vitorino et al., 2009); the distal portion, including RPE and dorsal retina, express Pax6 and Rx1 (Mathers et al., 1997; Futukawa et al., 1997). Shh persistence in pre-RPE tissue results in the down-regulation of Pax6 and in the transcription of Mitf (a RPE specific marker) and, transiently, of Otx2. The synergistic activity of Mitf and Otx2 converts pre-RPE in mature RPE (Martinez-Morales et al., 2001). In retina primordium, Pax6 and Rx1 expression persist. In conclusion, the cross-repression between Pax2 and Pax6 seems to form a boundary between optic stalk/ventral retina and RPE/dorsal retina (Yang, 2004).

TGFß/BMP factors are involved in dorso-ventral patterning of the neural tube and in neuronal differentiation. They also play a role in the morphogenesis of the optic vesicle. TGFß/BMP proteins in fact represent an important signal in later stages of eye development, when the optic cup evolves in the three ocular tissues (optic stalk, RPE and NR). In chick and mouse, BMP4 is expressed in distal optic vesicle structures and in dorsal retina and ventral RPE and, subsequently, in the peripheral margin of the differentiating retina (Sakuta et al., 2001). BMP4 expression, in chick, is able to inhibit Vax and Pax2 and to promote Tbx5, a gene expressed only in dorsal retina (Koshiba-Takeuchi et al., 2000), permitting to speculate on a competition among Vax and Pax2 (Shh effectors) in the ventral region, and Tbx5 (BMP4 effector) in the dorsal side to establish a correct dorso-ventral axis in eye structures. The important involvement of BMPs in eye development is shown by BMP7 null mice that frequently show an eyeless phenotype. In mouse, BMP7 is expressed in the surface ectoderm (pre-lens tissue) contacted by the optic vesicle. Many evidences suggest that the formation of the double-layered optic cup requires signals deriving from the pre-lens ectoderm (Dudley & Robertson., 1997; Hyer et al., 2003). Indeed, Activin, a TGFß factor secreted from the extraocular mesenchyme surrounding the optic vesicle, seems involved in RPE specification, repressing the neural retina specific markers Pax6, Six6 and Chx10 and promoting Mitf expression (Fuhrmann et al., 2000).

FGF signalling presents a regionalization function in optic cup morphogenesis. This family of Growth Factors is prevalently involved in neural retina development. In chick, mouse and *Xenopus* embryos, FGF8b is able to convert RPE in NR, while FGF2 treatments block differentiation of retinal layers and do not affect RPE. These data support the idea of a role of different FGFs in the organization of the double-layered optic cup (Pittack et al., 1997; Martinez-Morales et al., 2005).

The different factors that are involved in optic vesicle morphogenesis also contribute to the maturation of the three optic tissues that are formed during lamination processes (optic stalk, RPE and NR). The optic stalk evolves in the optic nerve when the choroidal fissure closes and retinal nerve fibers fill it. The RPE becomes pigmented and starts to produce trophic factors that are involved in NR successive lamination and maintenance and forms a barrier with blood vessels. NR precursors will give rise to a stratified neural retina.

2.3 Determination of retinal cells fates

Each retinal layer is characterized by the presence of specific classes of retinal neurons. A variety of basic helix-loop-helix (bHLH) transcription factors are expressed by a subset of retinal progenitors cells or by their post-mitotic progeny (Ohsawa & Kageyama, 2008). One of the first genes to be transcribed is Math5/Ath5 involved in ganglion cell precursors specification (Yang et al., 2003). Subsequently, in ganglion cell precursors are activated Brn3b and Isl-1, that confer a final differentiating stimulus, and Math5 is down-regulated (Pan et al., 2008; Qiu et al., 2008).

The specification of amacrine and horizontal cells requires the transcriptional activation of Foxn4 that mediates the activation of Pt1fa, transcriptional regulator of Prox1 (Li et al., 2004). Precursors in which Prox1 expression persists, become horizontal cells (Dyers et al., 2003). On the contrary, precursors in which are also expressed NeuroD and Math3 become amacrine cells. Different types of amacrine cells are generated under the activation of downstream factors, such as Barhl2 (glycinergic amacrine cells), BhlhB5 (GABA-ergic subtypes) and Isl-1 (cholinergic amacrine cells) (Elshatory et al., 2007) (Fig.2).
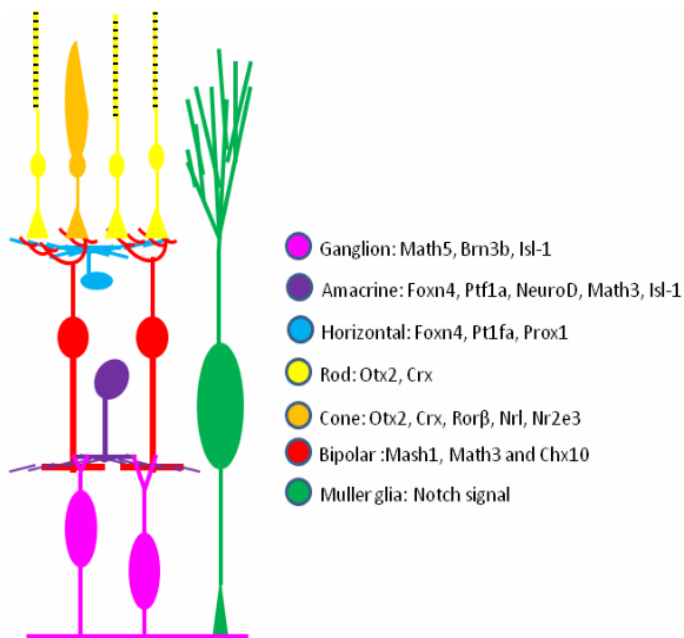

Fig. 2. Principal bHLH transcription factors involved in neural retina cells specification.

Otx2 is fundamental for photoreceptor fate. Otx2 acts through Crx, a cone-rod specific factor, involved in differentiation of photoreceptors (Reese, 2010). Crx activates the transcription of Rorβ, which in turn activates Nrl. Finally, Nrl activates Nr2e3. This transcriptional cascade switches the photoreceptor phenotype to rod fate (Corbo & Cepko, 2005; Oh et al., 2007).

Bipolar cells production requires the activation of Mash1 and Math3 that cooperate with the homeobox transcription factor Chx10. Down-regulation of only one of these genes produce Müller glia cells instead of bipolar cells (Tomita et al., 2000).

Müller glia cells are the last retinal cells produced during eye development. During the last division, the two daughter cells differentiate one into a bipolar cell or a rod photoreceptor and the other in a Müller glia cell. Notch signalling seems to repress proneural genes activation and contribute to the production of Müller cells (Jadhav et al., 2009).

In retina, an evolutionarily conserved aspect in the generation of cell diversity is represented by symmetric vs. asymmetric cell divisions. Symmetric divisions occur when a mother cell generates two equivalent daughter cells and asymmetric when it divides unequally (Huttner & Kosodo, 2005). Data acquired in transgenic zebrafish, in which GFP expression is under control of the Ath5 promoter (an essential proneural inducer of ganglion cells), suggest that retinal cells divide either symmetrically or asymmetrically. In fact, early retinal progenitors have a central-peripheral division and late progenitors a circumferential division (Poggi et al., 2005). Symmetric and asymmetric division occurs in relation to different cell fate determinants, such as Numb, an asymmetrical signal in mammalian retina. During division, Numb segregates to the apical part of daughter cells in asymmetric apico-basal divisions and it is equally distributed in both daughter cell following symmetric planar divisions. (Cayauette & Raff, 2003). Other information on retinal fates specification derives from positional information. Del Bene and collaborators (Del Bene et al., 2008) showed that signaling molecules, such as Notch, play a crucial role in determining retinal fate during interkinetic nuclear migration. Interkinetic nuclear migration is a process in which the nuclei of retinal progenitors, that generally contact the apical and basal surfaces of the retina, migrate along this apico-basal axis in different phases of cell cycle. This process is different in each type of early retinal progenitor and seems to be involved in the specification of a heterogeneous final retinal population. Cells with a reduced basal migration remain apical and proliferative, while cells that migrate to the basal side generate daughter cells that will become postmitotic (Andreazzoli, 2009).

The specification of retinal cell fates is also closely related to the retinal clock. The first evidences for a retinal clock are shown in the fruit fly *Drosophila Melanogaster* in which a complex of four factors Hunchback-Kruppel-Pdm-Castor seems to be involved in neuroblast competence. In fact, these neuroblasts divide asymmetrically and produce ganglion mother cells (GMCs) that express each of the genes of the complex transiently during different phases of GMCs divisions. Hunchback and Kruppel are necessary and sufficient to control early-born neuroblasts, while Pdm and Castor are expressed with further progression of the cell cycle. These observations allow to conclude that a cell cycle clock is involved in the timing of the generation of specific cell types (Isshiki et al., 2001). Recently, a similar mechanism has been shown in Mammals with the identification of Ikaros, a Hunchbach homologue, and Castor, both expressed in the mouse retina. Ikaros is expressed in early progenitors and its expression is switched off during cell cycle progression and retinal fates acquisition, suggesting a role for Ikaros in early retinal progenitors manteinance (Blackshaw et al., 2004; Elliot et al., 2008).

The length of cell cycle clock represents a crucial point to establish the transition between early and late progenitors. In fact, the first ones have a short cell cycle characterized by a great number of divisions, while the second ones have a long cell cycle, probably, due to the requirement of the transcription of specific factors that identify specific cell types (Decembrini et al., 2006). Many evidences suggest a role of Shh as regulator of cell cycle kinetics in retinal precursors (Decembrini et al., 2009) (Fig.3).
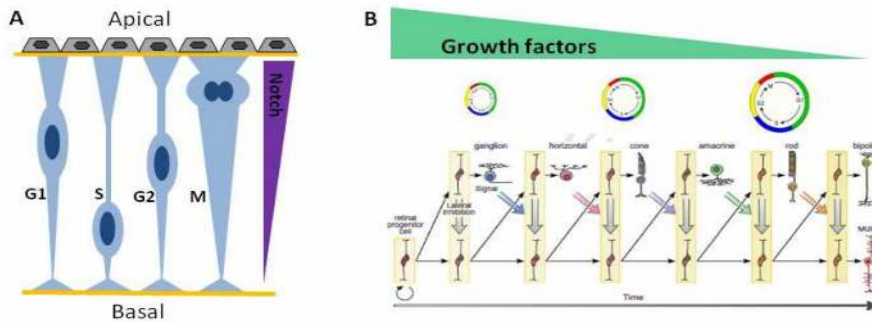
Fig. 3. Different processes involved in retinal cells fate specification. A) Interkinetic nuclear migration; B) Retinal clock in which Growth Factors, such as Shh, influence cell cycle elongation and retinal cell fates specification (*Andreazzoli, 2009*).

3. Retinal stem cells

Many evidences suggest that a population of stem cells, the retinal stem cells, persists in specific regions of the vertebrate retina such as the Ciliary Marginal Zone (CMZ) in amphibians and in Mammals, also called Circumferential Germinal Zone (CGZ) in fishes (Locker et al., 2009). In the last years, many other studies in fact suggest that retinal stem cells represent only a little group of cells with stemness properties in mature and adult retina. In fact, data support the idea that different mature cell types, such as Müller glia and retinal pigmented epithelium cells, if suitably stimulated, are able to transdifferentiate in other retinal specific subtypes.

3.1 Fish and amphibians

A first evidence of the existence of retinal stem cells in vertebrates derived from autoradiography experiments in fishes and amphibians. During the period between 1971 and 1977, Straznicky and Gaze and successively Johns showed that the retina of *Xenopus laevis* and *Carassius auratus* (goldfish) is constantly growing to fit to the growth of the animal body. They were able to identify the region in which proliferation was taking place and named it Ciliary Marginal Zone (CMZ) (Straznicky & Gaze, 1971; Johns, 1977). The CMZ is a circumferential region located at the periphery of the retina. Stem cells are found in the most peripheral part of the CMZ are able to give rise to both neural retina and pigmented epithelium cells, being thus equivalent to cells that form the earlier eye field (Harris & Perron, 1998; Locker et al, 2009). As they divide, their daughter cells move toward the central portion of the CMZ. These cells are mitotic but not stem cells and they are called retinoblasts. Retinoblasts differentiate in all the cell types that form the retina (Harris & Perron, 1998). The most internal part of the CMZ contains cells that have generally left the cell cycle and are committed to became specific retinal subtypes. The final fate of these cells is determined by extracellular interactions with the neighboring cell types and final localization of undifferentiated cell. The mentioned mechanism recapitulates the processes that occur during retinal development, in which, after differentiation in specific retinal neuronal subtypes, the newly formed cells inhibit the other to choose the same fate (Waid & Mcloon, 1998; Harris & Perron, 1998).

Specification of CMZ cells, in the three different regions, is under the control of the same factors that specify the eye primordia during embryonic development. Six3, Pax6, Rx1 and the other EFTFs are also expressed in the most peripheral part of CMZ in which are localized retinal stem cells in fish and *Xenopus* (Raymond et al., 2006; Locker et al., 2009). Moreover, components of the Shh pathway (Gli2, Gli3, X-Smoothened), and of Notch-Delta signaling, (XHairy1 and XHairy2, the respective orthologs of human Hes1 and Hes4), are expressed in the peripheral CMZ cells of *Xenopus laevis* supporting the idea of their stemness properties (Raymond et al., 2006; Locker et al, 2009). The central zone of *Xenopus* CMZ (in which are mitotic cells but not stem cells) expresses Xash1, a gene that drives stem cells to become neural and susceptible to differentiate in a specific neural subtype. In this region, the expression of athonal homologs (Xath5 and NeuroD) stimulates the generation of retinal precursors (Harris & Perron, 1998). During the progression toward the most central region, CMZ cells switch off the previously described genes and turn on specific retinal fate genes, such as Brn3, that promotes differentiation and survival of ganglion cells (Gan et al., 1996).

In the last years, analysis in fishes and other vertebrates showed that, in response to injury, Müller glia cells are able to start proliferating again and subsequently transdifferentiate in other retinal neuronal subtypes. This evidence suggests that Müller glia cells can represent another source of pluripotent stem cells in the adult eye (Locker et al., 2009). Retinal pigmented epithelium represents another source of mature retinal cells that can transdifferentiate in neural retina cells. In fact, the treatment of RPE cells with specific growth factors, such as FGFs, is able to convert pigmented epithelium in proliferative cells that can acquire a neural phenotype (Martinez-Morales et al., 2005).

3.2 Mammals

Ten years ago, evidence of a potential population of retinal stem cells is obtained in Mammals, even if it is not possible to identify a proliferating CMZ as in fishes and amphibians (Amato et al., 2004). In Mammals, two different types of cells are present in the adult eye that represent potential retinal stem cells: cells of the ciliary body (ciliary margin) and of the iris epithelium. The cells of ciliary body derive from epithelial cells and in particular conditions, such as damage, are induced to display retinal stem cell properties. Moreover, if cultured *in vitro*, they are able to generate retinal neurons. Similarly, the iris epithelium cells, which derive from the inner layer of iris tissue, express nestin (stem cells marker) and possess the ability to differentiate in multiple neuronal types including retinal neurons (Bi et al; 2009). Analyses in Mammals reveal that ciliary body and iris stem cells express Pax6, Rax, Chx10 and Six3, thus supporting the idea of retinal precursors properties and a conserved molecular mechanism in retinal stem cells generation among vertebrates (Locker et al., 2009).

The first evidences of the capacity of some mature retinal cell types to transdifferentiate in other types derive from studies in Mammals. In 2004, Ooto and colleagues proved the capacity of Muller glia cells to generate retinal neurons. Successively, an accurate analysis of gene expression in retinal precursors and Muller glia cells confirms an overlap between the profile of these two cell types and the potentiality of Muller glia cells as neural stem cells (Ooto et al., 2004; Bi et al., 2009). Transdifferentition of RPE cells into neural retina has been demonstrated in embryonic rat after the treatment with growth factors such as basic FGFs (Ballios & Van der Kooy, 2010).

4. Differentiation of stem cells into retinal cells: do *in vitro* protocols recapitulate embryonic development?

The retina is affected by a broad range of pathologies (collectively called retinopathies) often sharing a heterogenic genetic background and leading to cell depletion through a degenerative process. Commonly, photoreceptors represent the retinal cell population primarily affected by the degeneration: depending upon which type of photoreceptor dies first, we distinguish rod-cone degenerations from cone-rod degenerations. After the loss of most photoreceptors, the other retinal cell types show dendrite atrophy or are involved in a considerable remodeling of the entire circuitry (Strettoi et al., 2003; also reviewed in Marc et al., 2003). As retinal pigmented epithelium is fundamental for photoreceptors activity, also the impairment of its function may lead to retinal degeneration.

Among retinopathies, inherited retinal degenerations (such as Retinitis Pigmentosa) are a major cause of visual impairment in the juvenile-to-young adult population. As reviewed in Shintani et al., 2009, various approaches have been assessed for this type of retinopathies, but the results are often disaccording. For Retinitis Pigmentosa, it has been postulated that the assumption of nutritional supplements such as Vitamin A palmitate, Lutein and Docosohexanoic acid (DHA) can reduce the rate of the degeneration and preserve photoreceptors viability. Unfortunately, the effects seem to last for short periods and in some situations toxic side effects have been postulated.

Gene therapy is another potential approach and it has been carried out in three ways:

- through replacement of the mutated gene with a normal one, using vectors such as adenoviruses;
- using a ribozyme therapy aimed at disrupting the production of the aberrant protein;
- through RNA interference, for the same aim as the ribozyme.

However, the long-term safety of the procedure and the concrete effectiveness must both be assessed.

The common pharmaceutical strategies exert their positive effect in the presence of fully functional tissues, but they often fail where the target is mostly compromised. Like the previous approaches, the pharmaceutical one is actually unable to enhance cell viability, but it tries to slow down the course of the degeneration. The treatment with basic Fibroblast Growth Factor (bFGF) and Ciliary Neurotrophic Factors (CNTFs) seems to ameliorate the condition of rat models of Retinitis pigmentosa, by reducing the loss of photoreceptors. Retina pigmented epithelial (RPE) cells genetically modified to produce CNTFs can be encapsulated and implanted in the affected eye. This therapy is in phase I of clinical trial and it has been shown increasing the visual acuity of the patients. Antiapoptotic drugs used for Parkinson's Disease are currently under investigation: it is the case of Zelapar™ (Valeant Pharmaceuticals, Swindon, Wiltshire, United Kingdom).

The overall situation suggests that an efficient solution is still lacking, although it seems that embryonic stem cells and the newly described induced pluripotent stem cells (iPS), once correctly differentiated, may become an useful (yet challenging) tool to approach diseases having a degenerative substrate - a strategy generally referred to as cell-based substitutive therapy (see section 4 for the application in the retina). Embryonic stem cells may be differentiated either towards neural retina cells or towards retinal pigmented epithelium cells. A certain number of protocols have been described for the differentiation of mouse (Zhao et al., 2002; Hirano et al., 2003; Ikeda et al., 2005; Sugie et al., 2005; Osakada et al., 2008; Osakada et al., 2009a), human (Banin et al., 2006; Lamba et al., 2006; Osakada et al.,

2008; Aoki et al., 2009; Meyer et al., 2009; Osakada et al., 2009a; Osakada et al., 2009b; Nistor et al., 2010) and primate (Osakada et al., 2008) embryonic stem cells into retinal cells, and some of these have been demonstrated to work also on mouse (Hirami et al., 2009; Parameswaran et al., 2010) and human (Hirami et al., 2009; Meyer et al., 2009; Lamba et al., 2010) induced pluripotent stem cells (iPS cells). In a similar way, protocols of differentiation into retinal pigmented epithelium cells have been described for mouse (Kawasaki et al., 2002), human (Klimanskaya et al., 2004; Osakada et al., 2008; Idelson et al., 2009; Meyer et al., 2009; Osakada et al., 2009a) and primate (Yue et al., 2006; Osakada et al., 2008) embryonic stem cells, as well as for human (Buchholz et al., 2009) induced pluripotent stem cells.

Generally, it is believed that *in vitro* differentiating cells requires the simulation of the molecular environment to which the cell type of interest is exposed during embryogenesis. This simulation should lead the cultured cells to recapitulate the morphogenetic modifications also observed *in vivo* during the embryonic development. Regarding the retina, this means that the cells must be instructed to express in the right succession the combination of genes responsible for the specification of the structures we have seen previously (see Molecular regulation of Vertebrate eye development):

• eye field
• optic vesicles
• retinal progenitor cells
• immature photoreceptors
• photoreceptors

while activating neural genes and repressing the expression of genes related to a pluripotent condition. All the protocols aim to reproduce these conditions, as this represents the most reliable thread to follow.

4.1 Current *in vitro* protocols

Mouse embryonic stem cells are maintained and propagated in culture with different types of media depending on the cell line, but two key elements are always present: Leukemia Inhibitory Factor (LIF) and serum. Leukemia inhibitory factor can be either provided by a layer of mitotically-inactivated mouse embryonic fibroblasts (MEF), on which stem cells are cultured, or added in the medium if fibroblasts are not provided. Bone morphogenetic factor 4 (BMP-4) contained in Fetal Bovine Serum acts synergistically with LIF to inhibit differentiation and promote proliferation (Ying et al., 2003). Regarding human embryonic stem cells, the pathway activated by LIF seems to have no effect on the maintainance of pluripotency (Daheron et al., 2004; Humphrey et al., 2004). Instead, a role for basic Fibroblast Growth Factor and Activin-A has been described (Amit et al., 2000; Beattie et al., 2005; Dvorak et al., 2005; Vallier et al., 2005; Levenstein et al., 2006). The first published protocols about retinal differentiation *in vitro* describe a procedure to obtain retinal progenitors and photoreceptors from mouse embryonic stem cells by means of co-culture systems (Zhao et al., 2002; Hirano et al., 2003; Ikeda et al., 2005; Sugie et al., 2005).

Zhao and colleagues promote the formation of embryoid bodies from mouse stem cells by removal of LIF (Zhao et al., 2002). These embryoid bodies are three-dimensional structures consisting of few hundreds cells and they are often used to begin the differentiation simulating an embryonic-like environment (Doetschman et al., 1985). The formation of neural progenitors cells is obtained exposing embryoid bodies to Retinoic Acid (RA) or

plating them in the presence of a medium containing Insulin, Transferrin, Selenium, Fibronectin (ITSFn) and basic Fibroblast Growth Factor. In either ways, cells are then dissociated and expanded with basic Fibroblast Growth Factor. At this point, they are in active proliferation and they have acquired neural features, as demonstrated by the incorporation of Bromodeoxiuridine (BrdU) and the expression of Nestin, which is a marker of neuroectodermal stem cells (Lendahl et al., 1990). Moreover, the neural progenitors obtained in either way are mostly positive for Pax6 and Notch1, which are respectively involved in eye development and spatial-temporal organization of retinal neurons. When cultured with postnatal day 1 rat retinal cells, these neural progenitors start expressing genes involved in the specification of photoreceptors, such as Rx, Crx, Nrl and NeuroD. Prolonged time of co-culture leads to the expression of Rhodopsin in 6% of the overall population of cells but this evidence does not correlate with the acquisition of typical photoreceptor morphology.

A similar experiment has been conducted using chick retina harvested on embryonic day 6 for co-culture (Sugie et al., 2005). Mouse embryonic stem cells are first induced to acquire a neural phenotype using two possible protocols (Bain et al., 1995; Okabe et al., 1996), then they are cultured for 10 days on chick retinal tissue in the presence of the medium described previously (Zhao et al., 2002). In these conditions, 21.5% of total cells results positive for Rhodopsin when one protocol of neuralization has been used (Okabe et al., 1996) and 10% is positive following the other neuralization protocol (Bain et al., 1995). Crx, Interphotoreceptor Binding Protein (IRBP) and Recoverin (markers of both cones and rods) expression is detected by PCR during co-culture starting from day 4, 7 and 10 respectively.

Structures resembling eyes and containing photoreceptors positive for Rhodopsin and Recoverin are obtained also co-culturing mouse embryonic stem cells with the stromal cell line PA6 in the presence of basic Fibroblast Growth Factor, Dexamethasone and Cholera Toxin (Hirano et al., 2003). However, it seems that only a small fraction of cells produces these eye-like structures, thus a much smaller percentage of cells is thought to become photoreceptors. Almost the same result is observed when applying this protocol to human embryonic stem cells (Aoki et al., 2009).

Co-culture with retinal pigmented epithelium is also used to induce the formation of neural retina cells (Chiou et al., 2005). Human bone marrow stem cells can be induced towards a neural phenotype by treating them with Insulin, Progesterone, Putrescein, Selenium Chloride, Transferrin, Epidermal Growth Factor and basic Fibroblast Growth Factor. After 2 weeks they form spheroid aggregates and express Nestin (75.8% of total cells). When these spheroid structures are co-cultured with mitotically inactivated human pigmented cells, they give rise to neural cells expressing Opsin (29.6% of bone marrow stem – derived cells).

Sasai's group starting from 2005 has pursued a different strategy (Watanabe et al., 2005; Ikeda et al., 2005). Mouse embryonic stem cells are kept in suspension for 9 days in a medium containing KnockOut™ Serum Replacement (KSR, Euroclone) in order to form serum-free embryoid body-like aggregates (SFEB). Once treated with the anti-Wnt reagent Dickkopf-1 (Dkk-1) and the anti-Nodal reagent Lefty-A, these floating aggregates are plated and produce a good percentage (35%) of telencephalic precursors expressing the transcription factor Bf1 (Watanabe et al., 2005). Moreover, most of the floating bodies express Six3, which is the first eye field transcription factor expressed during the embryogenesis (Ikeda et al., 2005). These observations led the authors to think that the serum-free, floating culture of embryoid body-like aggregates could be used to obtain retinal neurons. Thus, in order to enhance the transcription of both Rx and Pax6, whose co-

expression identifies retinal progenitor cells, Fetal Calf Serum and Activin-A are added to the suspension culture. Once plated, 15% of total cells express Rx and 6.4% results positive for both Rx and Pax6 (Ikeda et al., 2005). Eventually, the co-culture with dissociated E17.5 mouse retinal cells leads 36% of differentiated cells to express Recoverin and Rhodopsin, but only when embryonic stem-derived cells and true photoreceptors are in close proximity.

When the procedure to obtain retinal progenitors is applied on mouse Rx-GFP knock-in cell line, cells expressing Rx can be isolated from the aggregates and subsequently sorted to produce an almost pure culture (Osakada et al., 2008; Osakada et al., 2009a). The treatment with the γ-secretase inhibitor DAPT from day 10 of differentiation induces the expression of Crx in 22.4% of total cells, although the percentage decreases significantly when a different cell line is used. The consecutive addition of acidic Fibroblast Growth Factor, basic Fibroblast Growth Factor, Taurine, Sonic Hedgehog (SHH) and Retinoic Acid then leads to the co-expression of Recoverin and Rhodopsin in 17.2% of total cells, but it has no effect on the production of Red/Green opsin+ and Blue opsin+ cones. Also in this case, the yield differs consistently depending on the cell line.

Monkey embryonic stem cells can be directed to retinal differentiation following almost the same procedure. Similarly, human embryonic stem cells can be used to obtain floating aggregates to be treated for 20 days with Dickkopf-1, Lefty-A. Y-27632, a Rho kinase inhibitor introduced to improve viability, is added to the medium. 15.8% of colonies originating from the aggregates results positive for both Rx and Pax6. As they are further treated with Retinoic Acid, Taurine and N2 supplement starting from day 90, 19.6% of total cells become Crx+. These eventually produce Rhodopsin+/Recoverin+ rods (8.5% of total cells), Red/Green opsin+ cones (8.9%) and Blue opsin+ cones (9.4%). To a different extent, the procedure has effect also on other human embryonic stem cell lines.

More recently, this protocol has been applied also on mouse and human induced pluripotent stem cells with some adjustments (Hirami et al., 2009). On day 120 of differentiation, nearly 3.5% of total cells (human iPS cells) expresses Recoverin and 50% of them is also positive for Rhodopsin.

In a therapeutic perspective, the differentiation of human stem cells obtained with compounds produced by animal cells or *E. coli* could lead to infection or immune rejection. For this reason, Osakada and colleagues have also described a second protocol having the same pipeline as before, but substituting factors such as Dickkopf-1 and Lefty-A with chemical compounds sharing equivalent effects: respectively, Casein Kinase I Inhibitor (CKI-7) and SB-431542 (Osakada et al., 2009b). Again, Y-27632 is used to improve cell viability. After 10 days from the replating of aggregates, 25.4% of colonies are Rx+, 79.2% are Pax6+. Moreover, 22.8% of total cells is Mitf+: 10 days later, 18.1% of total cells are pigmented and display a squamous, hexagonal morphology. These cells eventually form tight junctions (ZO-1+) after a whole 100 days of culture. As for photoreceptor induction, the treatment with Taurine and Retinoic Acid by day 140 produces Rhodopsin+ cells (nearly 6.5% of total cells) that don't acquire the morphology of mature rods. This population contains also cells positive for Recoverin, Phosphodiesterase 6b and 6c (respectively specific for rods and cones) and other markers correlated with phototransduction, indicating that these cells may respond to light stimuli.

A recent modification of Osakada's protocol (Osakada et al., 2008; Osakada et al., 2009a) has been applied on human embryonic stem cells to produce a three - dimensional tissue structure (Nistor et al., 2010). Differentiation is promoted in adhesion with B27 supplement, Insulin–Selenite–Transferrin (IST), triiodothyronine (T3), Taurine, Hyaluronic Acid (HA), Dickkopf-1,

LeftyA and Fibroblast Growth Factor. At day 7 cells are trypsinized and replated to promote aggregates formation. For the following 7 days, cells are cultured in a medium with B27 supplement, IST, T3, Taurine, HA, FGF and Retinoic Acid, which is added from day 10 to 13. At day 42, patches of retinal pigmented epithelium are isolated and replated to promote their expansion in a medium with B27 supplement, IST, T3, Taurine, HA, and Fetal Bovine Serum. Then, at day 50-60, neural cultures are replated onto the seeded RPE cells. With this procedure the production of a three – dimensional tissue is promoted, where it is possible to find cells expressing retinal markers such as Rx, Pax6, NeuroD, Mitf, Crx and Nrl. However, it is not clear what percentage of cells acquire each retinal phenotype.

Banin and colleagues obtain photoreceptors starting from human embryonic stem cells (Banin et al., 2006). The differentiation into neural precursors is enhanced by culture on feeders for 8 days in a medium containing Fetal Calf Serum and Noggin, which is a renowned neuralizing factor (Smith & Harland, 1992), and in the same medium without Noggin for an additional 5 days. Then, small patches of cells are explanted and replated in a serum – free medium containing B27 supplement, Epidermal Growth Factor and basic Fibroblast Growth Factor. Under these conditions, there is the development of floating aggregates that can be subcultured for 4 weeks (Reubinoff et al., 2001). At this point, nearly 98% of cells within the aggregates is positive for Nestin. Moreover, they express the common eye field and photoreceptors markers. Once plated, these aggregates give rise to a population of neurons that keep on expressing the above-mentioned markers but fail to produce the related proteins. This problem is partially overcome when the neural precursors are engrafted into rat retinas. Although the mature morphology is never acquired, nearly 1.5% of engrafted cells are positive for Rhodopsin protein.

An alternative approach is the one described by Lamba and colleagues (Lamba et al., 2006). Starting with human embryonic stem cells, the formation of embryoid bodies is promoted in a medium containing KnockOut™ Serum Replacement, B27 supplement, Dickkopf-1, Insulin-like Growth Factor 1 (IGF-1), whose overexpression in *Xenopus* embryos leads to the formation of ectopic eyes (Pera et al., 2001), and Noggin. Embryoid bodies are then collected and plated in a medium containing B27 supplement, N2 supplement, Dickkopf-1, Insulin-like Growth Factor 1, Noggin and basic Fibroblast Growth Factor. At the end of this procedure, nearly 12% of total cells expresses Crx, 5.75% expresses Nrl but only small percentages (less than 0.01%) of total cells express mature photoreceptors-specific markers, such as S-opsin and Rhodopsin. Similarly to Ikeda and colleagues, Lamba's group manages to co-culture their retinal progenitors with adult mice retinal explants. Interestingly, photoreceptor differentiation of these progenitors is seen only when retinal explants derive from mice models of photoreceptor degeneration.

The same protocol with some modifications has been recently applied on human induced pluripotent stem cells (Lamba et al., 2010). In this case, instead of promoting the formation of embryoid bodies, iPS cells are directly cultured with Noggin, Dickkopf-1 and Insulin-like Growth Factor 1 at lower concentrations. After 3 days, their concentration is the same as in Lamba et al., 2006 and the cells are kept in this medium for 3 weeks. Terminal differentiation is enhanced in a N2 - B27 containing medium for some months. After 2 months of induction, compatibly with the results of the previous protocol, nearly 12% of total cells expresses Crx. Late markers such as Recoverin, Rhodopsin and S-opsin are expressed in less than 1% of cells. However, better effects may be obtained when an IRBP – GFP cell line is used and Fluorescence Activated Cell Sorting (FACS) is performed to isolate a pure population of IRBP+/Crx+ cells.

Meyer and colleagues have recently described one more method, which is applied on both human stem cells and human induced pluripotent stem cells (Meyer et al., 2009). Stem cells are grown in suspension to allow for the formation of aggregates that are eventually transferred in a medium containing N2 supplement and Heparin. In the following days, the aggregates are harvested and plated onto Laminin – coated dishes, where they soon form neural rosettes. On day 16 of differentiation, the medium is converted in a B27 supplement – containing one. Rosettes are then explanted and put in suspension in order to produce floating neurospheres. At this point, there is a consistent expression of markers belonging to a general neural lineage (Sox1, Sox2) but also of markers of the eye field (Rx, Pax6, Lhx2, Six3, Six6), whose co-expression has been assessed in some cases. Western blot and qPCR analysis confirm the endogenous production of both Noggin and Dkk-1 in this culture and the use of antagonists suppresses the expression of Pax6 and Rx. Since this procedure allows for the production of eye field cells, the authors proceed to describe how to reach the acquisition of optic vesicle and optic cup cell phenotypes. By day 40 of neurosphere suspension, Mitf and Chx10 are co-expressed. Importantly, nearly 26% of total cells are positive for Chx10 and more than 99% of them express also Pax6. Taken together, this information indicate that the culture contains early retinal progenitors cells.

By day 80 of differentiation in suspension, nearly 12% of total cells expresses Crx and among them 46.4% of cells is positive for more mature photoreceptor markers (Recoverin, cone Opsin). These results are shared with the ones obtained by differentiating human iPS cell line IMR90-4, even if changing cell line drastically affects Pax6 expression levels during the procedure.

Recently, a new protocol has been described specifically for mouse induced pluripotent stem cells (Parameswaran et al., 2010) (Fig.4).
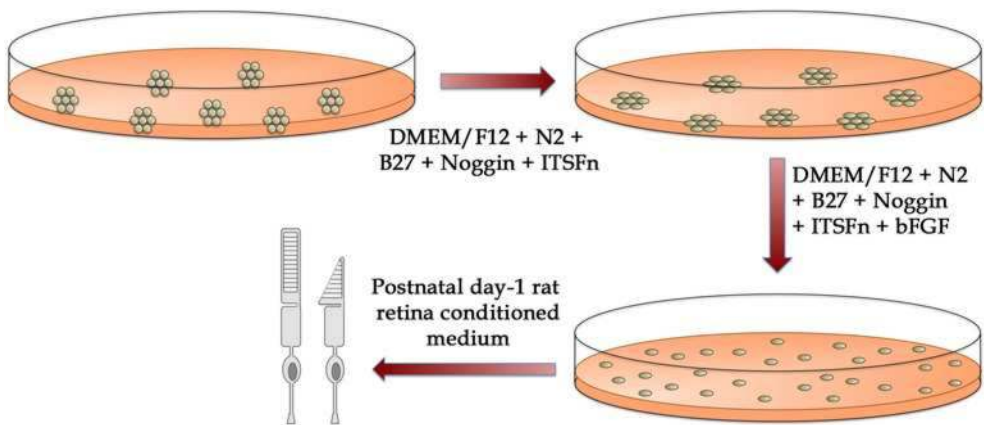

Fig. 4. Representation of the protocol of differentiation of mouse induced pluripotent stem cells into photoreceptors, described by Parameswaran et al., 2010.

A neural phenotype is obtained promoting embryoid bodies formation and culturing them in the presence of N2 supplement, B27 supplement, ITSFn and Noggin, following a previously described protocol (Okabe et al., 1996). Subsequently, the resulting colonies are trypsinized and expanded in the same medium added with bFGF for 25 days (day 40 of

differentiation). At this point, nearly 22.2% of total cells co-expresses Rx and Pax6. The differentiation is induced with N2 supplement and a medium conditioned by postnatal day-1 rat retinal cells. In 10 days, nearly 23% of total cells starts expressing Crx, while 15% expresses Nrl, 9% expresses Rhodopsin and 11.3% expresses S-Opsin.

Regarding the differentiation of stem cells into retinal pigmented epithelium cells, some of the protocols are shared with the ones described for neural retina cells induction.

Kawasaki and colleagues describe the production of neural cells from mouse embryonic stem cells using stromal cell – derived inducing activity (Kawasaki et al., 2000). In the same way, the co-culture of primate embryonic stem cells with the mouse stromal cell line PA6 allows for the induction of large patches of pigmented cells in nearly 8% of the colonies (Kawasaki et al., 2002). Further analyses confirm the presence of pigment granules and apical microvilli, both typical of RPE cells, and their capability to digest latex-beads as proof of the phagocytic function (Haruta et al., 2004). Pigmented cells may be obtained also by letting human embryonic stem cells spontaneously propagate and differentiate for 6-8 weeks as monolayer, in the absence of exogenous factors (Klimanskaya et al., 2004). Once they have reached confluence, patches of retinal pigmented epithelium appear and express typical RPE markers, such as Cellular Retinaldehyde-Binding Protein (CRALBP), Bestrophin and Pigmented Epithelium – Derived Factor (PEDF).

Culturing mouse embryonic stem cells on the stromal cell line PA6 in the presence of basic Fibroblast Growth Factor, Dexamethasone and Cholera Toxin, as previously mentioned (Hirano et al., 2003), leads to formation of eye – like structures. After 10 days, nearly 70% of colonies in culture comprises masses of cells displaying the first pigment granules (Aoki et al., 2006): depending on when these cells are collected and replated onto PA6 layer, it is possible to obtain other eye – like structures, RPE patches or single pigmented cells.

Retinal pigmented epithelium cells derived from embryonic stem cells can be used to induce the formation of neural retina cells. Primate embryonic stem cells can be differentiated into Pax6+ RPE cells by means of co-culture with Sertoli cells for 2 weeks (Yue et al., 2006). When primate embryonic stem cells are cultured for 3 days on these patches and Retinoic Acid is added to the medium, 25% of primate ES – derived cells are found expressing Rhodopsin in rosette – like structures (Yue et al., 2010). However Recoverin+ cells are only a few and they do not seem to be consistently affected by RA treatment (0.6% of total cells).

Following the protocol described by Osakada and colleagues, retinal pigmented epithelium cells can be obtained from monkey (Osakada et al., 2008) and human (Osakada et al., 2008; Osakada et al., 2009a) embryonic stem cells, positive for ZO-1 (40.1% and 34.7% of total cells respectively).

Meyer and colleagues describe using their protocol to obtain also RPE cells. If neural rosettes are kept in adhesion, by day 40 of differentiation patches of polygonal, pigmented cells positive for Mitf (25% of total cells) and ZO-1 are observed (Meyer et al., 2009).

Interesting experiments of retinal differentiation have been recently performed on *Xenopus laevis* using the animal cap essay (Yamada & Takata, 1961; reviewed in Ariizumi et al., 2009). Our group has demonstrated that Noggin overexpression is sufficient to induce the formation of a complete eye in this model, but only at high doses (Lan et al., 2009). Embryos at 2 – cells stage are microinjected with Noggin and GFP synthetic mRNAs: the animal caps are subsequently explanted at blastula stage and cultivated until stage 39 – when, during embryogenesis, the eye is almost completely formed. At this point, the cells within the aggregates express typical retinal markers (such as Opsin for photoreceptors, Vsx1 for

bipolar cells and Hermes for ganglion cells) and display a structure reminiscent of a wild type retina (Fig.5).
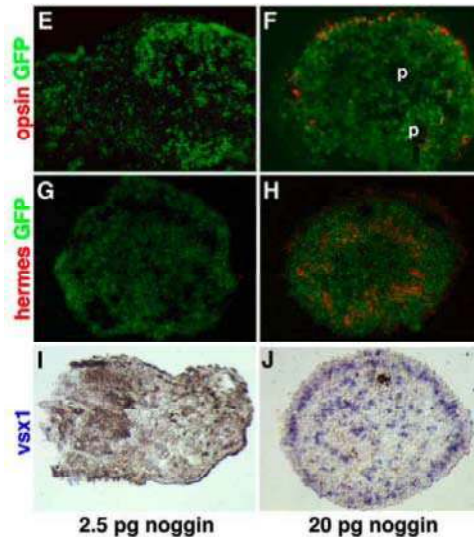

Fig. 5. Immunohistochemistry (E-H) and *in situ* hybridization (I-J) of animal caps treated with Noggin at low doses (2.5 pg) and high doses (20 pg). GFP fluorescence prove the correct procedure of microinjection. p: pigment (*from Lan et al., 2009*).

Moreover, an embryo precociously deprived of a single eye field can develop a complete eye when a treated animal cap is transplanted in the same position. Electrophysiological recordings demonstrate that the new eye elicits a correct response to light stimuli. Even more posterior transplants lead to the formation of an almost complete eye, but its cytoarchitecture seems to be more compromised (Fig.6).
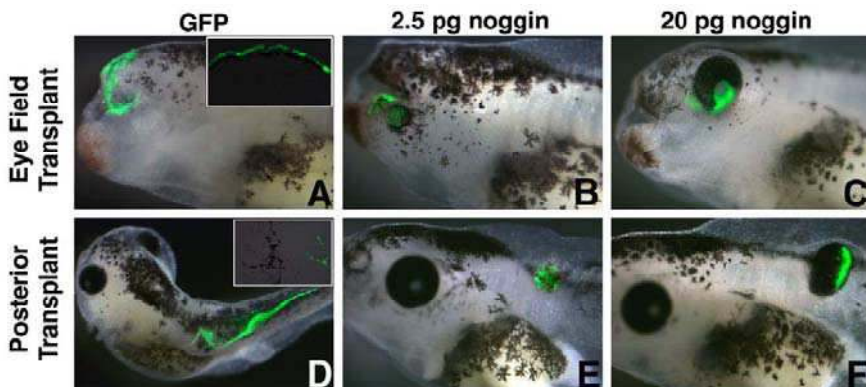

Fig. 6. Transplants of animal caps treated with GFP (A, D), GFP + Noggin at low doses (B, E) and GFP + Noggin at high doses (C, F). Anterior (A-C) and posterior (D-F) transplants are shown (*from Lan et al., 2009*).

Similar experiments have been carried out culturing *Xenopus* animal caps in the presence of mouse recombinant Noggin protein (Viczian et al., 2009). The results are equivalent to the ones we have described: Noggin enhances the formation of a complete eye, similarly to what the overexpression of the eye field transcription factors exert in the same model (Zuber et al., 2003).

4.2 Future strategies

The future of cell - based substitutive therapy seems to rely on direct reprogramming: instead of reprogramming somatic cells to a pluripotent state – and then using previously established protocols to induce differentiation toward specific fates – somatic cells can be directly reprogrammed into a neural phenotype (Vierbuchen et al., 2010). Mouse embryonic fibroblasts and tail – tip fibroblasts can be converted into mature neurons (19.5% of total cells) by a lentiviral transfection of Ascl1, Brn2 and Myt1l. Few experiments had been previously described in this direction, all focused on chick RPE cells reprogramming into retinal neurons (Ma et al., 2009; Li et al., 2010). However, at the moment there is no evidence of direct reprogramming of mouse or human fibroblasts into retinal progenitors or mature photoreceptors. Thus, a feasible strategy could aim to induce this type of differentiation, in order to avoid using embryonic stem cells and long manipulations. In a second moment, it will be necessary to perform the same procedure without using lentiviral delivery. This would bring research one step closer to actual therapy.

5. In vitro differentiated retinal cells as a tool for regenerative medicine and drug discovery

Retinal diseases are characterized by a permanent loss of retinal neurons that is accompanied by a gradual irreversible loss of visual capacity.

With the progression of the technologies and the adventure of stem cells era, many efforts are focused on the possibility to differentiate specific cell types *in vitro* and to transplant them in the injured organs including retina. During the initial phases of retinal degeneration, photoreceptors or RPE cells die, but the inner microarchitetture of the retina is not yet altered, suggesting the possibility to introduce *in vitro* differentiated retinal cells to replace the lost cells.

In parallel with regenerative medicine, another important application of *in vitro* differentiated retinal cells is represented by drug discovery. In fact, a large portion of current therapies for retinal diseases takes in consideration treatments with bioactive molecules (endobiotics or xenobiotics), that prevent or retard the pathology insurgence. The possibility to have an inexhaustible source of retinal cells *in vitro* could be a way to develop a strategy to screen a large numbers of new molecules and possible drugs in short periods of time.

5.1 Cell replacement strategies for retinal repair

Cell replacement therapies are innovative therapeutic strategies that allow the restoration of visual responses to the degenerate adult neural retina and represent an exciting area of regenerative medicine. So far, it has been shown that transplanted postmitotic photoreceptor precursors are able to functionally integrate into the adult mouse neural retina. Recent reviews discuss the differentiation of photoreceptor cells from both adult and embryonic-derived stem cells, their potential for retinal cell transplantation and possible strategies used

to overcome barriers present in the degenerate neural retina and improve retinal cell integration (Lamba et al., 2009b; West et al., 2009).

5.2 Future possibilities for drug discovery

The availability of libraries of chemical compounds is today a critical tool in drug discovery. There is now the possibility to perform High – Throughput - Screenings (HTS) using small molecule libraries, to search for new molecules able to reduce cell death and/or to enhance survival of degenerating photoreceptors. Photoreceptor cell death is the major hallmark of most inherited retinal dystrophies. Although the genetic causes are not always identified, and the mechanisms leading to photoreceptor degeneration poorly defined, some common features of the cell death have been identified (Marigo, 2007; Wright et al., 2010). It is thus possible to use as a readout of the HTS a TUNEL assay, that allows to efficiently isolate those molecules able to reduce cell death. The selected (and subsequently validated) molecules could be new interesting pharmaceutical leads, that could then be further analyzed and exploited. In this way, it is possible to identify new uses of drugs that are already pharmacologically and toxicologically well characterized. This can accelerate and speed up animal and clinical trials, permitting to obtain a new pharmacologically active drugs in a relatively short time (Osakada & Takahashi, 2009).

6. Conclusions

In the past years the molecular mechanisms underlying retinal development and differentiation have been at least partially elucidated, probably enabling the retina to become the best characterized region of the CNS and at the same time to be an attractive system for regenerative medicine. Moreover, the functional contribution of transplanted and integrated cells can be assessed by the analysis of visually guided behavior, thus making it possible to study in this system neuronal regeneration and replacement in the CNS. Over the next few years some of the hurdles to overcome to make cell replacement a clinical reality concern the efficiency of both in vitro retinal differentiation protocols, and functional integration of the transplanted cells. Non mammalian vertebrates will remain to be interesting systems to study retinal differentiation and regeneration mechanisms, that will allow to draw important conclusions, to be used also in mammalian retinal biology.

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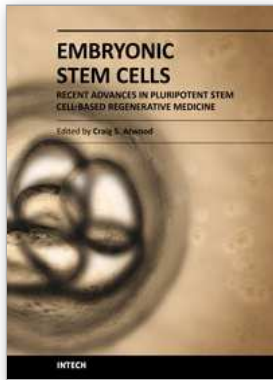
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