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A decade of mammalian retinal stem cell research

M. LOCKER, W. EL YAKOUBI, N. MAZURIER, J.-P. DULLIN, M. PERRON

CNRS UPR 3294 Neurobiology and Development, Institut de Neurobiologie "A. Fessard", Université Paris Sud, ORSAY Cedex, France

ABSTRACT

Ten years have now passed since the discovery of quiescent neural stem cells within the mammalian retina. Beside the fascinating aspect of stem cell biology in basic science, these cells have also offered hope for the treatment of incurable retinal diseases. The field has thus rapidly evolved, fluctuating between major advances and recurring doubts. In this review, we will retrace the efforts of scientists during this last decade to characterize these cells and to use them in regenerative medicine. We will also highlight advances made in animal models capable of stem cell-mediated retinal regeneration.

Key words

Retina • Adult retinal stem cells • Ciliary body • Ciliary marginal zone

Discovery of quiescent retinal stem cells in mammals

In mammals, the two functional components of the retina, the inner neural retina and the outer retinal-pigmented epithelium (RPE) achieve their development by the early postnatal period and no additional retinal cells are produced thereafter. Based on this feature and the abscence of tissular repair following damage, the adult mammalian eye was thus considered to be devoid of retinal stem cells (RSCs). In contrast, the retina of many fish and amphibians continues to grow throughout the animal life and is able to regenerate (Reh and Constantine-Paton, 1983). New cells are indeed produced from a pool of multipotent and self-renewing stem cells, located in a circumferential region, known as the ciliary marginal zone (CMZ; Wetts and Fraser, 1988; Wetts et al., 1989). Comparative studies in different vertebrates have led to the idea that the CMZ became progressively reduced as species evolved from fish to mammals (Perron and Harris, 2000). Hatched chicken retinas still contain a zone of proliferating cells at the peripheral margin but its activity is only transient and its neurogenic potential more limited compared to that of fish and amphibians (Fischer and Reh, 2000). A few mitotically active cells resembling the CMZ were also identified in postnatal marsupials but never to date in other mammals.

In 2000, however, a hypothesis was raised that the mammalian ciliary body (CB) might nevertheless harbor retinal stem cells. The CB is located between the neural retina and the iris (Fig. 1). It contains the ciliary muscles, which are involved in the eye accommodation process by changing the shape of the lens. Additionally, it is composed of a ciliary epithelium (CE) responsible for the production of the aqueous humor and consisting of two cell layers, an inner non-pigmented epithelium, and an outer-pigmented epithelium (Fig. 1). Although this CE does not contain any neurons, it shares a common embryonic origin with the optic cup. Indeed, the non-pigmented

Corresponding author: Muriel Perron, CNRS UPR 3294 Neurobiology and Development, Bat. 445, Université Paris Sud, 91405 ORSAY Cedex, France - Email: muriel.perron@u-psud.fr

layer of the CE derives, like the neural retina, from the inner layer of the optic cup, and the pigmented epithelium is ontologically related to the RPE. In lower vertebrates, the CE is adjacent to the CMZ, which separates it from the neural retina (Fig. 1). Two independent teams demonstrated in 2000 that single pigmented cells from the CE of mouse retina could clonally proliferate *in vitro* to form sphere colonies (Tropepe et al., 2000; Ahmad et al., 2000). A small number of pigmented cells from these primary colonies repeatedly generated new secondary spheres indicating that the initial colony-forming cell had the capacity to self-renew. In addition, exposed to differentiation conditions, the colony forming cells were shown to express genes found in rod photoreceptors, bipolar neurons, and Müller glia, suggesting their multipotentiality. The idea was thus born that the CB of the adult mammalian eye harbors a population of retinal stem cells, yet in a mitotically quiescent state *in vivo*. A few years later, these cells were identified in the CB of other mammalian species, including human (Coles et al., 2004; Mayer et al., 2005; MacNeil et al., 2007; Xu et al., 2007a; Gu et al., 2007).

A second unexpected source of potential retinal stem cells was subsequently discovered within the iris. Similarly to the CE, the iris epithelium does not normally generate neurons but derives from the optic cup. Haruta and colleagues in 2001 revealed its ability to express neuronal markers *in vitro* uncovering a remarkable plasticity (Haruta et al., 2001). Additionally, a recent work proposed that the iris epithelium might harbor discrete heterogeneous populations of cells endowed with innate neural stem/progenitor properties, including the ability to differentiate into retinal specific neurons (Asami et al., 2007; MacNeil et al., 2007). Müller glial cells might also represent a source of potential stem cells within the neural retina of the mammalian eye, although their case will not be reviewed here (see for reviews Lamba et al., 2008; Locker et al., 2009; Karl and Reh, 2010).

The discovery of CB-derived stem cells generated much excitement and paved the way towards several research directions aimed at characterizing their in vitro properties, molecular signature, in vivo ability for tissue repair and potential for cell-transplantation medicine. We thus provide below an overview of the main advances made in the field during these past ten years.

In vitro proliferative properties of adult retinal stem cells

In contrast to diverse other neural stem cells (NSCs), iris- and CB-derived cells display the unusual property to proliferate as neurospheres in serum free media without exogenous growth factor addition (Tropepe et al., 2000). However, supplementation with EGF and/or FGF2 or bFGF (the most widely used growth factors in current RSC culture methods) significantly increases the number of neurospheres formed from dissociated cells (Tropepe et al., 2000; Coles et al., 2004; Asami et al., 2007; Gu et al., 2007). Although significant, the proliferation potential of CB-derived cells appears somehow limited, especially when compared with brain-derived stem cells (Yanagi et al., 2006; Moe et al., 2009) or retinal progenitor cells from neonatal retinas (Klassen et al., 2004b; Merhi-Soussi et al., 2006). First, no more than 0.2% to 2% of CB-derived cells seem to be endowed with proliferative capacities (Tropepe et al., 2000; Das et al., 2004; Xu et al., 2007a). Second, their self-renewal and proliferation rates appear to gradually decrease with subsequent passages (Coles et al., 2004; Xu et al., 2007a). Whether this corresponds to an intrinsic limitation or yet undetermined experimental causes remains to be established. Notably, the proliferative capacity of porcine, rodent and human RSCs maintained in monolayer was significantly increased compared to suspension sphere culture conditions (Klassen et al., 2004a; Coles et al., 2004; Gu et al., 2007; MacNeil et al., 2007). Besides, self renewal, monitored through the extent of secondary sphere formation, could be significantly enhanced following stimulation with exogenous molecules such as Wnt3a (Inoue et al., 2006), SCF (Stem Cell Factor; Das et al., 2004) and more recently, PEDF (Pigment Epithelium Derived Factor; De Marzo et al., 2010). An important issue in stem cell expansion is to ensure that cells exposed to mitogens for several generations do not undergo genetic changes. In a recent study, Djojosubroto et al. pointed out that CB-derived cell lines rapidly accumulated severe chromosomal aberrations upon prolonged cultivation and demonstrated tumorigenicity (Djojosubroto et al., 2009). Similar analyses are now needed to define whether such a genetic instability is also inherent in adult human cells, which would limit their clinical use.

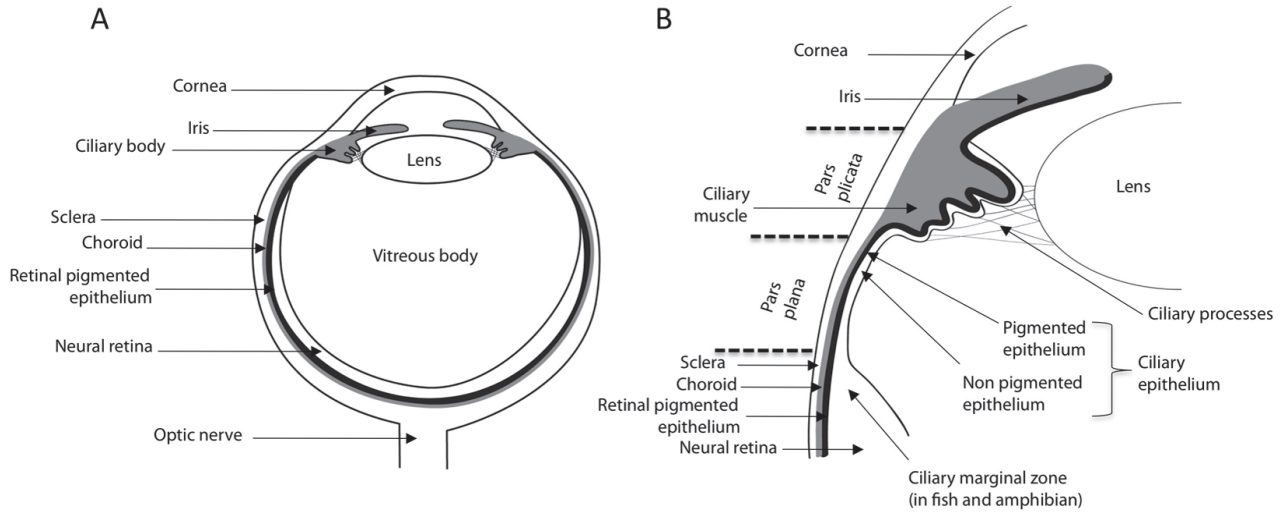

Fig. 1. - A. Schematic diagram of an adult vertebrate eye transverse section. B. Magnification of the region containing the ciliary body (CB). The CB can be subdivided in two zones: a flat region called *pars plana* and a folded one called *pars plicata*. The ciliary epithelium (CE) includes an outer layer which is pigmented and continuous with the retinal pigmented epithelium, and an inner layer, which is non pigmented and continuous with the neural retina. Quiescent retinal stem cells are located in the pigmented layer of the ciliary epithelium. In fish and amphibians continuous neurogenesis in the adulhood occurs in the ciliary marginal zone (CMZ).

Molecular signature of adult retinal stem cells

Numerous studies supported the presence of cells with retinal progenitor identity within CB spheres, based on the expression of several eye field transcription factors that have been shown to be critical for retinal development (Zuber et al., 2003; Viczian et al., 2009). Among them are the paired-class transcription factors, *Rx, Chx10* and *Pax6*, the homeodomain-containing transcription factors, *Six6* and *Six3* or the LIM homeodomain factor, *Lhx2* (Tropepe et al., 2000; Ahmad et al., 2004; Das et al., 2005; Lord-Grignon et al., 2006; Xu et al., 2007a; Gu et al., 2007). Moreover, comparative transcriptional profiling revealed that CB-derived cells and embryonic retinal progenitors share 80% identity of their expressed genes (Ahmad et al., 2004; Das et al., 2005). In addition, *Pax6, Rx, Chx10* and *Six3* were found to be expressed within the CE of adult rodents, monkeys and humans consistent with the presence of retinal stem/progenitor cells *in vivo* (Das et al., 2005; Lord-Grignon et al., 2006; Xu et al., 2007b; Martinez-Navarrete et al., 2008). It was recently shown that *Pax6* expression level in the murine CE was significantly higher in the pigmented outer layer, where putative retinal stem cells might reside, than in the non pigmented inner layer (Xu et al., 2007b). Additionally, Xu et al. demonstrated that only cells with the highest expression level of this gene could generate neurospheres, suggesting that *Pax6* represents a hallmark for RSCs within the CB. Besides, *Pax6* might be functionally required for the maintenance of these cells *in vivo*, since its conditional knockout results in impaired neurosphere formation from the adult CB (Xu et al., 2007b).

In vitro photoreceptor differentiation from retinal stem cells

A challenge in RSC biology is to direct undifferentiated cells towards the desired neuronal fate. Most efforts during the last decade have been made in setting up protocols driving *in vitro* photoreceptor differentiation. Spontaneous and consistent differentiation of CB- or iris-derived cells towards the photoreceptor lineage might be limited or at least infrequent. Indeed, although some groups reported rhodopsin expression (Asami et al., 2007; Tropepe et al., 2000; Coles et al., 2004), other failed to find photoreceptor markers (MacNeil et al., 2007; Cicero et al., 2009;

Gualdoni et al., 2010). Noticeably, Gualdoni et al. recently re-assessed the potential of RSCs for generating photoreceptors. By using the *Nrl.gfp* transgenic line that expresses GFP in developing and mature rod photoreceptors, they demonstrated that the rod differentiation program was not activated in CB-derived cells upon culture conditions previously reported to promote a photoreceptor fate Gualdoni et al., 2010). The authors thus concluded that these cells would require reprogramming to be useful as a source of new photoreceptors. In line with this, genetic manipulation of factors involved in photoreceptor fate and survival proved to be an efficient strategy to enhance *in vitro* photoreceptor differentiation. Overexpression of *Crx* or *Otx2* by retroviral infection of CB- or iris-derived cells, yielded high percentages of cells expressing rhodopsin as well as additional components of the phototransduction cascade (Haruta et al., 2001; Akagi et al., 2004; Akagi et al., 2005). In a recent study, Inoue et al. further improved photoreceptor differentiation from human RSCs by coexpressing OTX2 and CRX while blocking the function of CHX10 (Inoue et al., 2010). Promisingly, both murine and primate photoreceptor-like cells obtained from iris tissue were shown to respond to light stimulation and to integrate into explanted developing retinas in co-culture experiments (Akagi et al., 2005). The *in vitro* functionality of photoreceptors induced from mouse and human RSCs was recently demonstrated as well (Jomary and Jones, 2008; Jomary et al., 2010). Interestingly, attempts to induce photoreceptor differentiation through similar strategies of gene delivery in either mesencephalic or hippocampal NSCs (Haruta et al., 2001; Akagi et al., 2004), or corneal cells (Jomary et al., 2010) failed or resulted in incomplete acquisition of photoreceptor characteristics. These results strongly suggest the permissive action of intrinsic factors expressed in eye-derived stem cells.

Controversial stemness of retinal stem cells

The fact that pigmented cells from the CE can clonally generate spheres and express retinal neuron markers was considered as a strong argument for the existence of *in vivo* latent retinal stem cells in the CB (Tropepe et al., 2000). However, it was recently proposed that pigmented CE cells have the unexpected ability to form spheres made up of proliferating differentiated pigmented cells. Moe et al. found that neurospheres formed clonally from dissociated CE cells retain epithelial features (Moe et al., 2009). This was further demonstrated by the group of Dyer who reported that all cells in the CB-derived spheres, including those proliferating or expressing neural markers, were pigmented and displayed morphological characteristics of normal pigmented CE cells (Cicero et al., 2009). These data led the authors to claim that none of these cells are, nor become stem cells. The mammalian CE would thus not contain actual RSCs, but would instead harbor a population of differentiated cells that can proliferate, clonally expand, self-renew and express some neuronal markers, while retaining features of pigmented epithelial cells.

Do these experiments definitely rule out the existence of rare latent stem cells within the CB? It might be premature to state on that point because some data, such as the fact that sphere-initiating cells can be prospectively enriched from a cellular subpopulation expressing high *Pax6* levels, rather favor the stem cell hypothesis (Xu et al., 2007b; Inoue et al., 2010).

In addition, Kohno et al. proposed that sphere formation and neural differentiation of CB-derived cells can arise through distinct pathways according to the culture method (Kohno et al., 2006). In clonal assays, spheres would be formed by the expansion of rare neural stem-like cells. In contrast, when CB-derived cells are propagated in high-density culture, they also form spheres but in a proliferation-independent manner, by aggregating to each other and/or incorporating adjacent adherent cells. In these conditions, cells within spheres progressively lose their epithelial markers and gain the expression of neural ones. The authors thus proposed that such CB sphere formation constitute a reprogramming system allowing neural differentiation from epithelia-like cells. This might not be surprising as several studies highlighted the ability of CE, neural retina and RPE to transdifferentiate into each other (Fischer and Reh, 2001; Lee et al., 2001; Del Rio-Tsonis and Tsonis, 2003; Rowan et al., 2004; Liu et al., 2007). In addition, it has been known for more than half a century that the eye pigmented cells are capable of acting as a source of retinal regeneration in embryonic chicks and

adult amphibians (Moshiri et al., 2004; Araki, 2007; Yoshii et al., 2007; Vergara and Del Rio-Tsonis, 2009). Thus, although the adult mammalian RPE has lost the property to transdifferentiate, some cells within the ciliary body may have retained this plasticity and the consecutive ability to generate neuronal cells in response to growth factors.

In any case, whether these cells should be called "stem cells" might only be a semantic issue, or even not, if we consider that the stemness concept has to evolve along with recent discoveries of the plasticity and unexpected potential of more and more cell populations (reviewed in Seaberg and van der Kooy, 2003). Some investigators indeed suggest that the term "stem cell" should be used to refer to a biological function that can be inherent to or induced in many distinct cell types, even differentiated cells. For now, the existence of a cell population able to display some retinal stem cell properties *in vitro* may be nonetheless valuable in the context of cellular transplantation trials or for alternative therapeutic strategies that would consist in mobilizing them *in vivo* for retinal repair.

Transplantation trials using retinal stem cells

Retinal degenerative diseases, including age-related macular degeneration and retinitis pigmentosa, are the major causes of human blindness in the world. In these neurodegenerative diseases, photoreceptor cells are damaged or lost, which leads to subsequent visual impairment. Given the social and economic impact of vision loss, tremendous efforts have been put in trying to replace photoreceptors by cellular therapy. Researchers have put great expectations in stem cells as a source for cell transplantation, since they may have the migratory capacity and cellular plasticity needed to integrate and differentiate in the diseased retina. Attempts to replace photoreceptor loss have been made using embryonic, fetal or post-natal retinal stem/progenitor cells. These studies have generated variable data but as a whole demonstrated rather limited levels of integration and/or photoreceptor differentiation (reviewed in MacLaren and Pearson, 2007; Pellegrini et al., 2007; Djojosubroto and Arsenijevic, 2008; Lamba et al., 2008). An alternative strategy has been to examine the potential of adult neural stem cells. Transplanted rat hippocampal stem cells exhibited widespread incorporation into the host retina, but failed to properly differentiate into retinal cell types (Takahashi et al., 1998; Young et al., 2000).

The discovery in 2000 of adult retinal stem cells in the mammalian CB has thus prompted many researchers to investigate their therapeutic relevance, given their potential higher ability to differentiate into retinal neurons. Such cells could also offer the possibility of autologous transplantation, thereby avoiding immune rejection issues. Murine adult RSCs were shown to adopt retinal phenotypes when transplanted into diseased retinas, but not in healthy ones (Chacko et al., 2003). This work thus highlighted the benefice of injury-induced factors for the differentiation of stem cells into recipient eyes (Chacko et al., 2003). Regarding human adult RSCs, the group of Van der Kooy reported that their vitreous injection into neonatal animal eyes (which provides a more appropriate host environment compared to adult retinas) resulted in cell migration, integration, and differentiation towards retinal cell fates (Coles et al., 2004).

It has previously been shown that cells already engaged towards a photoreceptor fate have significantly better integration rates than immature stem cells (MacLaren et al., 2006). Along this line, a recent report from Inoue and colleagues indicated that human RSCs, genetically modified to promote their photoreceptor differentiation, exhibited higher level of retinal integration compared with unmodified RSC progeny following their transplantation into murine eyes. Importantly, these transplanted cells significantly improved visual function in transducin mutant mice, which lack functional rod photoreceptors (Inoue et al., 2010).

Obviously, such genetically modified cells are not suitable for clinical trials and the next challenge will be to generate photoreceptor precursors with procedures that do not alter the genomic integrity of donor cells. Another major difficulty in translating such strategies into clinical applications relies on the limited availability of adult RSCs and their reduced proliferative potential. This problem could be alleviated with the use of human embryonic stem (ES) cells. Indeed, because these pluripotent cells can proliferate indefinitely, they represent a potential inexhaustible source for human photoreceptor production (reviewed in Haruta, 2005 and in

Lamba et al., 2009b). Recent data demonstrated that human ES cells can be directed towards a retinal fate and that transplanted ES derived photoreceptors can restore some light response in visually deficient mice (Lamba et al., 2009a). The discovery of induced pluripotent stem (iPS) cells in 2006 in mouse (Takahashi and Yamanaka, 2006), and in 2007 in humans (Takahashi et al., 2007; Yu et al., 2007), also offers new opportunities for autologous cell based therapies. Tremendous advancements in the field have been made in just a few years, widening the prospects of regenerative medicine (reviewed in Jin et al., 2009 and in Comyn et al., 2010). However, several issues such as their oncogenic potential, have yet to be addressed before ES or iPS cells could be considered as an option in human trials for the treatment of retinal neurodegenerative diseases.

Awakening dormant retinal stem cells *in vivo*

Another angle of research, that could lead in the future to alternative therapeutical strategies to treat retinal dystrophies, relates to endogenous retinal stem cell mobilization (reviewed in Karl and Reh, 2010). Indeed, accumulating evidences suggest that, although quiescent or barely active, these cells may be amenable to drive regeneration following appropriate stimulation.

It has been proposed that *in vivo* activity of CB stem/progenitor cells is hampered by intrinsic limitations and/or lack of permissive factors in their microenvironment. Nonetheless, these cells retain the ability to respond to growth factor stimulation *in vivo*. Intraocular injection of FGF2 and Insulin was indeed reported to induce proliferation of quiescent ciliary body epithelial cells *in vivo* (Das et al., 2004; Zhao et al., 2005; Abdouh and Bernier, 2006). Abdouh and Bernier showed that these reactivated cells exhibited neuroepithelial characteristics (expression of *Chx10*, *Pax6*, *nestin*) but did not differentiate into neurons (Abdouh and Bernier, 2006). In contrast, Zhao et al. described the expression of several differentiated retinal cell markers in chased BrdU labeled cells. In addition, they found that these progenitors were able to migrate centrally towards the neural retina (Zhao et al., 2005), highlighting both their neurogenic potential and their ability to move away from their production site. Whether mitogenic, differentiation and migration signals are physiologically present in limiting quantities or counteracted by local inhibitory cues within the CB remains to be determined. Close et al. investigated the nature of extrinsic factors that might account for the postnatal decline in proliferation within the postnatal retina. They found that exogenous transforming growth factor β1 or β2 (TGFβ1/2) inhibited proliferation in rat retinal explants. Conversely, postnatal inhibition of TGFβ signaling *in vivo* extended the period of proliferation (Close et al., 2005). Based on these data, they proposed that a cytostatic TGFβ signal produced by retinal neurons is involved in the maintenance of mitotic quiescence in the postnatal retina. Postembryonic downregulation of Hedgehog (Hh) signalling might also contribute to silence the proliferative and neurogenic potential of adult retinal stem cells. Indeed, mice with a single functional allele of the Hh receptor Patched, in which Hh signaling is constitutively activated, exhibit persistent progenitor cells in the retinal margin, reminiscent of the CMZ of fish and amphibians. Importantly, those *Patched*$^{+/-}$ proliferative cells were shown to trigger limited but observable neuronal regeneration in mice with a retinal degeneration background (Moshiri and Reh, 2004). In line with this, upregulation of Hh signalling components have recently been correlated with increased proliferation in the CB of Royal College of Surgeons rats, an animal model for retinitis pigmentosa (Jian et al., 2009). Such data raise the possibility that a pathological context may favor reactivation of key embryonic signalling pathways and the consecutive initiation of repair processes. Accordingly, two distinct lesional paradigms, intraocular injection of *N*-methyl-*N*-nitrosourea and retinal ganglion cell injury induced by optic nerve axotomy, were shown to trigger proliferation of progenitors and upregulate the expression of the photoreceptor and bipolar marker recoverin in the adult mouse ciliary body (Nickerson et al., 2007; Nishiguchi et al., 2008; Wohl et al., 2009).

Finally, recent findings unexpectedly suggested that in physiological conditions, CB stem cells might contribute to retinal cell turnover of adult primates, by producing retinal neurons (Martinez-Navarrete et al., 2008). The authors indeed observed in the non laminated retinal margin of human and simian

retinas, a variety of cell types progressively adopting a mature neuronal morphology as moving away from the CB (Martinez-Navarrete et al., 2008). These cells express markers for virtually all retinal cell types, suggesting the possibility of spontaneous neurogenesis occurring in the peripheral margin of adult primates. To support this hypothesis, single cell labeling experiments allowing to chase CB cells over long periods of time are now required.

Learning from the ciliary marginal zone of lower vertebrate species

Our fundamental knowledge on RSCs must be improved to set up rational cell based therapy procedures aiming at efficiently stimulating their neurogenic potential *in vivo*. Among the critical issues that undoubtedly need to be addressed are their precise molecular signature, the differences that may distinguish them from their embryonic counterparts, the nature of their niche and the molecular cues that regulate their maintenance and activity. Investigating these issues is an arduous task in mammals. Most data thus come from *in vitro* or *ex vivo* studies and consequently reflect situations where stem cells have been removed from their natural microenvironment. In contrast to the mammalian situation, retinal stem cells in the CMZ of fish and amphibians are active throughout life in physiological as well as regenerative conditions. This gives credit to the use of cold-blooded vertebrates as animal models to study retinal stem cells. Although caution should be taken before extrapolating to humans, such models already proved useful in understanding several aspects of post-embryonic retinogenesis. The CMZ of hatched chick, despite its transient and more limited activity, also revealed to be a prime model notably for dissecting signalling interactions at work during retinal regeneration.

Fish and amphibians as animal models to characterize specific stem cell markers

Retinal stem cell biology is presently limited by the lack of reliable markers that could allow formal identification and segregation of these immature cells within heterogeneous proliferating cell populations. The CMZ of fish and amphibians offers an exceptional model to screen for such markers, as stem cells are confined in an identified area (Fig. 2). The spatial organization of the amphibian CMZ have indeed been shown to mirror the temporal sequence of retinal development, with stem cells residing in the most peripheral margin, successively followed by actively dividing progenitors and their post-mitotic progeny more centrally (Perron et al., 1998; Casarosa et al., 2005; reviewed in Harris and Perron, 1998; Henningfeld et al., 2007; Locker et al., 2009). Few genes have been described as specifically expressed in the stem cell zone and absent in the surrounding progenitors. Among them are components of the Hh signaling pathway, *Gli2*, *Gli3* and *X-Smoothened* (Perron et al., 2003), as well as the collagen encoding gene *DrCol15a1b* (Gonzalez-Nunez et al., 2010). Additionally, we recently identified a dozen specific novel retinal stem cell markers through an ongoing large-scale hybridization screen (our unpublished data).

The zebrafish allows forward genetic screens that already proved useful in identifying mutants that likely affect stem cell maintenance (Wehman et al., 2005). In their study, Wehman et al. isolated 18 mutant strains, some of them exhibiting phenotypes consistent with an absence of stem cell activity. Strikingly, these mutations disrupt larval growth of the retina, while leaving embryonic development of the eye largely unaffected. Moreover, gene linkage analyses revealed no match with candidate genes known to regulate retinogenesis, stem cell biology or growth. Altogether, their results suggest that many genes controlling adult stem cell activity remain to be identified and might not necessarily be found within the pool of genes previously described in embryonic stem/progenitor cells.

Signalling pathways controlling CMZ retinal stem cell behavior in physiological and regenerative conditions

Several lines of evidence suggest that key signalling pathways such as Wnt, Hh, Notch and BMP, known to control NSCs in the adult brain, might be involved as well in the regulation of RSC ontogenesis, maintenance and/or activity (Zhao et al., 2002; Alexson et al., 2006; Inoue et al., 2006; Das et al., 2006b; Bhattacharya et al., 2007). However, whether they act on stem or progenitor cells, which cellular parameters they control *in vivo* (cell division modality, impact on cell cycle progression, regula-

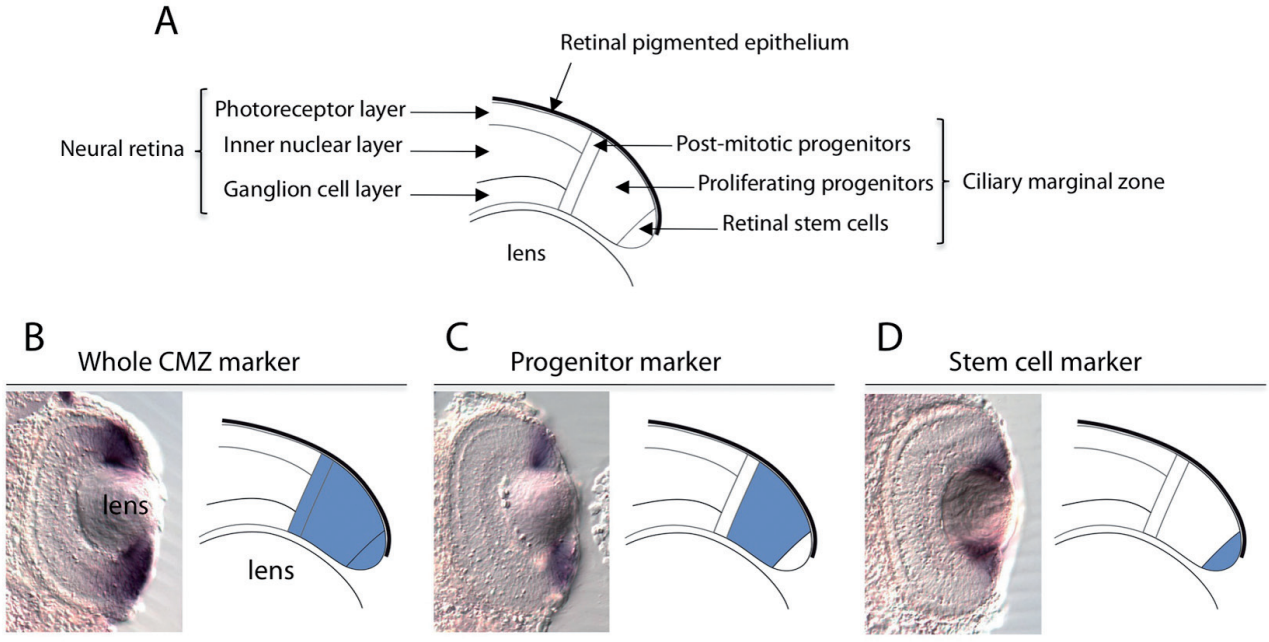

Fig. 2. - A. Schematic of a ciliary marginal zone (CMZ) in the periphery of a *Xenopus* tadpole retina at stage 41. The spatial organisation in this region mirrors the temporal sequence of retinal development, with stem cells residing in the most peripheral margin. Noticeably, the CB is not represented here since it will form at later tadpole stages. B-D. Retinal sections following *in situ* hybridization labelling and schematic diagrams illustrating three types of gene expression profiles within the CMZ of *Xenopus tropicalis* tadpoles. B. Illustration of an expression pattern in the whole CMZ. C. Illustration of an expression pattern, such as that of bHLH factors *Xath5* or *Hes2* (Kanekar et al., 1997; Solter et al., 2006), restricted to progenitor cells and excluded from the stem cell region. D. Illustration of an expression pattern, such as that of *Gli2, Gli3* or *Smo* (modified from Perron et al., 2003), restricted to the stem cell region.

tion of cell exit) and what is the nature of their target genes are still poorly documented.

As mentioned above, constitutive activation of Hh signalling results in persistent proliferative cells in the murine ciliary margin (Moshiri and Reh, 2004). In line with this, Sonic Hh protein has been found to promote proliferation within the CMZ of post-hatched chicken eyes (Moshiri et al., 2005). Our group demonstrated that Hh signalling exerts ambivalent effects on both cell cycle kinetics and cell cycle exit of retinal precursors, thereby simultaneously promoting proliferation and differentiation (Locker et al., 2006). We proposed that Hh signalling may regulate the transition from slow cycling retinal stem cells to fast cycling neuronal progenitors, bringing them closer to cell cycle exit (Locker et al., 2006; Agathocleous et al., 2007). Besides, in retinotectomized embryonic chicks, Hh signalling was found to act as a prominent regulator of stem cell dependent regeneration, in close interdependency with the FGF pathway (Spence et al., 2004; Spence et al., 2007). A third player in the process was found to be the BMP pathway, which also interacts with FGF signalling although in a stage dependent manner, first activating FGF-receptor expression and then downregulating it (Haynes et al., 2007).

Both Wnt and BMP signalings participate during embryogenesis to the determination and patterning of anterior eye structures including the iris and CB (Zhao et al., 2002; Cho and Cepko, 2006; Liu et al., 2007; Kubo and Nakagawa, 2009). The Wnt pathway might also be critical for the formation of the CMZ by keeping cells of this region in a proliferative and undifferentiated state (Kubo et al., 2003; Stephens et al., 2010). Recently, it was found that this function is mediated by *c-hairy1*, a member of the *Hairy and Enhancer of split* gene family (Kubo and Nakagawa, 2009). A role for the Wnt pathway in adult retinal stem cell maintenance has been suggested as well. Both Wnt3a treatment or overexpression of a stabilized form of β-catenin enhanced proliferation of murine CB-derived cells *in vitro* (Inoue et al., 2006; Das et al., 2006a). In addition, canonical Wnt activity has been observed in the CMZ of diverse vertebrates suggesting that Wnt responsiveness might be a fea-

ture of adult retinal stem/progenitor cells (Denayer et al., 2008; reviewed in Kubo and Nakagawa, 2008). We recently directly addressed this question *in vivo* by using hormone inducible constructs allowing perturbations of the canonical Wnt pathway in a temporally controlled manner. Activation of Wnt signaling in transgenic tadpole retinas increased the number of CMZ proliferative cells, while blocking the pathway almost completely abolished proliferation (Denayer et al., 2008). These results substantiate the hypothesis that, in lower vertebrates, the Wnt pathway plays a crucial role in adult retinal stem cell maintenance.
Altogether, it appears that a growing number of studies have addressed the influence of diverse growth factors and signaling pathways on retinal stem cells behaviour. The major challenge is now to unravel the interactions and cross-talks between these pathways to elucidate how they work together to orchestrate the maintenance and proliferation potential of retinal stem cells.

Final Summary

The remarkable discovery, in the 90s, of NSC dependent neurogenesis in the adult brain, ended a central dogma in neurobiology. Since then, surprises kept on occurring, with a variety of NSCs being identified in many places of the nervous system, including the retina. As summarized in the present review, in vitro and in vivo studies of RSCs already produced a rich understanding of their proliferation and differentiation potential, and thereby allowed to foresee the possible design of new therapies for retinal degeneration diseases. Endogenous stimulation of photoreceptor production in the mammalian eye was completely unanticipated only ten years ago and might now constitute a future therapeutical option. However, RSC applications in cell based therapy and regenerative medicine still rely on necessary progresses of basic research regarding the molecular basis of their self-renewal, fate determination, survival, integration potential and plasticity. Importantly, future work in the field may also provide knowledge relevant to target cancer stem cells in various brain tumors. Significant advances are expected from relatively recent aspects of RSC biology such as epigenetic or miRNA gene regulation. The study of the molecular and cellular composition of RSC niche is also of utmost importance. Although it has been initiated (Raymond et al., 2006), it clearly remains to be further explored to better evaluate how environmental cues are integrated by retinal stem and progenitor cells. Besides, novel insights in the field of RSCs should rapidly emerge from recent technical breakthroughs. Complex 3D-culture systems (Dutt and Cao, 2009) and biomaterials (Ballios et al., 2010), as carriers or substrate matrices, should for instance allow to improve conditions for in vitro RSC differentiation and implantation of cellular progeny into injured or degenerating retinas. Rapid advances in the identification of factors impacting on RSC identity and behavior can also be anticipated thanks to the development of high-throughput technologies. As a whole, we can hope that the next decade will provide as much excitements as the previous one, and that future work will unravel other unexpected aspects of RSC biology, that will be relevant to set up therapies for retinal dystrophies.

Abbreviation list

BMP: Bone Morphogenetic Protein; CB: Ciliary Body; CE: Ciliary Epithelium; CMZ: Ciliary Marginal Zone; ES cells: Embryonic Stem cells; iPS cells: Induced Pluripotent Stem cells; FGF: Fibroblast Growth Factor; Hh: Hedgehog; NSC: Neural Stem Cell; RPE: Retinal Pigmented Epithelium; RSC: Retinal Stem Cell; TGFβ: Transforming Growth Factor β.

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