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The peripheral eye: A neurogenic area with potential to treat retinal pathologies?

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ABSTRACT

Numerous degenerative diseases affecting visual function, including glaucoma and retinitis pigmentosa, are produced by the loss of different types of retinal cells. Cell replacement therapy has emerged as a promising strategy for treating these and other retinal diseases. The retinal margin or ciliary body (CB) of mammals has been proposed as a potential source of cells to be used in degenerative conditions affecting the retina because it has been reported it might hold neurogenic potential beyond embryonic development. However, many aspects of the origin and biology of the CB are unknown and more recent experiments have challenged the capacity of CB cells to generate different types of retinal neurons. Here we review the most recent findings about the development of the marginal zone of the retina in different vertebrates and some of the mechanisms underlying the proliferative and neurogenic capacity of this fascinating region of the vertebrates eye. In addition, we performed experiments to isolate CB cells from the mouse retina, generated neurospheres and observed that they can be expanded with a proliferative ratio similar to neural stem cells. When induced to differentiate, cells derived from the CB neurospheres start to express early neural markers but, unlike embryonic stem cells, they are not able to fully differentiate *in vitro* or generate retinal organoids. Together with previous reports on the neurogenic capacity of CB cells, also reviewed here, our results contribute to the current knowledge about the potentiality of this peripheral region of the eye as a therapeutic source of functional retinal neurons in degenerative diseases.

1. Introduction

The ciliary marginal zone (CMZ) is a neurogenic region located at the peripheral retina of lower vertebrates that is specialized for retinal neuron generation. During embryonic development, different types of retinal neurons, including retinal ganglion cells (RGCs) and photoreceptors, are generated from this area, and this neurogenic capacity is maintained throughout the adult life of the animal. In mammals, a region that spontaneously contributes to the generation of new neurons has not been found in the adult peripheral retina, initially supporting the hypothesis that this area does not have the capacity to generate new neurons. Subsequent studies postulated that the anterior mammalian eye contains a niche of cells with neurogenic capacity (Ahmad et al., 2000; Martinez-Navarrete et al., 2008; Tropepe et al., 2000), and more recent works have shown that cells located in the periphery of the retina in adult homeothermic vertebrates can proliferate *in vivo* (Kokkinopoulos et al., 2011; Reh and Levine, 1998; Wang et al., 2010). These observations opened the possibility that cells located in the periphery of the eye hold potential for retinal degenerative disease treatments. However, other studies have distrusted the neurogenic capacity of these peripheral eye cells questioning the idea that they are actual stem cells (Cicero et al., 2009; Froen et al., 2013; Gualdoni et al., 2010). To better understand and interpret the therapeutic possibilities of these cells, we herein review the current knowledge regarding the origin of the retinal edge as well as the proliferative and differentiation capacity of cells derived from this area. We also describe experiments we performed to shed light on the current debate on the neurogenic capacities of cells peripherally located in the adult mouse eye and

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List of abbreviations

CB	ciliary body
CB-NS	ciliary body neurospheres
CE	ciliary epithelium
CMZ	ciliary marginal zone
CPE	ciliary pigmented epithelium
mESC	embryonic stem cells
iPSCs	induced pluripotent stem cells
NPE	non-pigmented epithelium
NR	neural retina
NSCs	neural stem cells
RPCs	retinal progenitor cells
RPE	retinal pigmented epithelium
RSCs	retinal stem cells
WCP	Wnt/β-catenin signaling pathway

comment on the possibilities of therapy strategies directed to treat pathological retinal conditions.

2. The embryonic peripheral retina

To properly evaluate the potential for using cells located at the periphery of the mammalian eye in therapeutic approaches it is essential to have basic notions about the embryonic origin of these cells as well as some evolutionary aspects regarding their biology and evolution. Therefore, in this section, we describe the formation of the mouse eye rim providing pertinent references to chicks, frogs and zebrafish when necessary to highlight the evolutive aspects of this region.

2.1. Early eye morphogenesis

By the end of gastrulation, two major events lead to the formation of the nervous system in vertebrates: the anterior-posterior subdivision of the neural plate and the induction of neural tissue in the presumptive ectoderm. At this time, the prospective eye territory arises from the anterior neural plate located in the prosencephalon driven by a series of inductive interactions involving the neuroectoderm, mesoderm and neural crest cells (Chow and Lang, 2001; Fuhrmann, 2010). The specification of the eye field in diencephalic territories gives rise to the emergence of the optic vesicle, an epithelial ball-like structure that eventually transforms into a multilayered neural retina (NR) surrounded by the retinal pigmented epithelium (RPE) (Wilson and Houart, 2004; Zaghloul et al., 2005).

In mice, the morphogenesis of the optic vesicle begins on embryonic gestational day (E) 8.5 and is mainly controlled by the action of the transcription factors Rx, Pax6, Six3 and Lhx2 (Adler and Canto-Soler, 2007; Canto-Soler and Adler, 2006; Lagutin et al., 2001; Mathers et al., 1997; Yun et al., 2009; Zuber et al., 2003) (Fig. 1A). On E9.5, the distal portion of the vesicle contacts with the overlying surface ectoderm, which is then induced to form the lens placode (Cvekl and Zhang,

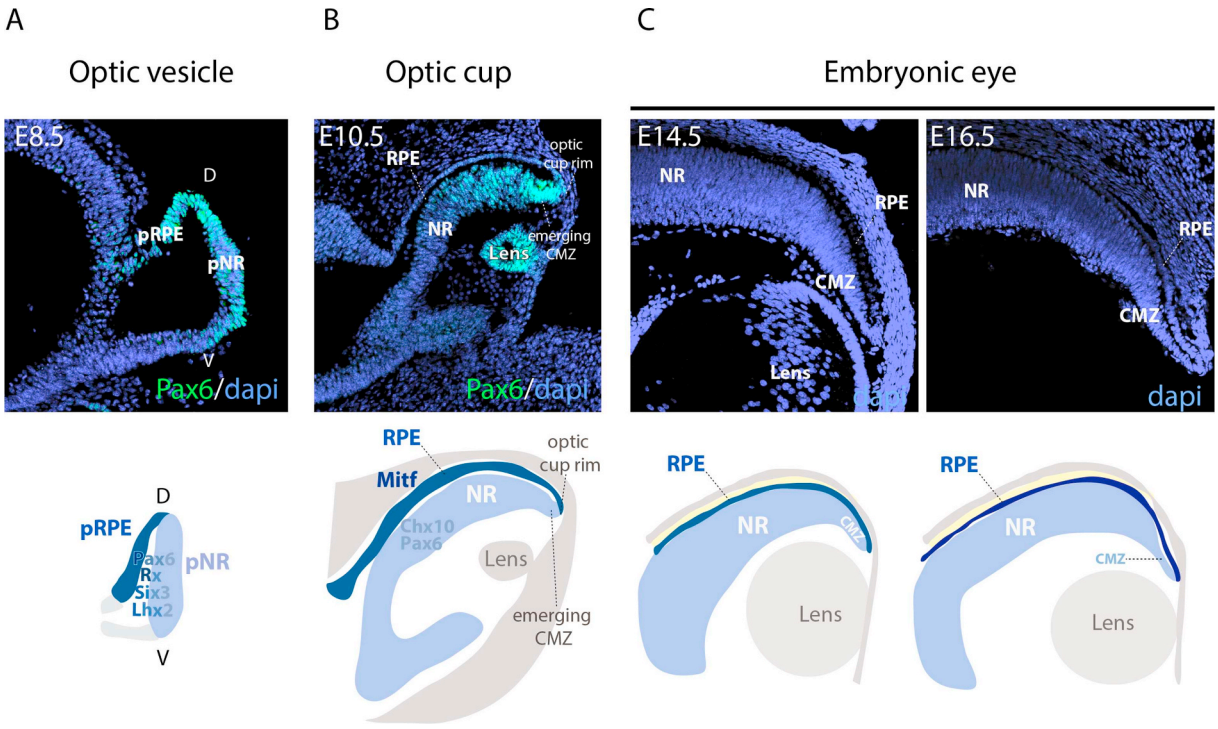

Fig. 1. Development of the mouse eye.
The top panels show coronal sections from mouse embryos at different stages, stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). The schemes at the bottom summarize the different steps of eye development.
(A) The optic vesicle derives from the diencephalon and invaginates into an optic cup. Pax6 (green), together with Rx, Six3 and Lhx2 define the eye field. At E8.5 the prospective neural retina (pNR) and the prospective pigmented epithelium (pRPE) start to specify.
(B) Between E9.5 and E10.5 the optic cup is formed. The neural retina (NR) develops from the non-pigmented inner layer of the optic cup and is surrounded by the retinal pigmented epithelium (RPE), which derives from the outer layer. The transition between the pigmented and the non-pigmented layers is known as the optic cup rim and by E10.5 the ciliary marginal zone (CMZ) emerges at the tip of the inner layer. At this stage, the NR and the RPE are specified by the expression of the transcription factors Chx10 and Mitf, respectively. Pax6 (green) is required for maintenance of the dorsal and nasotemporal patterns in the NR.
(C) The elongation of the distal tip of the retina is initiated at subsequent stages (E14.5-E16.5) forming the CMZ, which may be anatomically distinguished by RPE and NR flanking and will give rise to the ciliary body, iris and some retinal neurons at the peripheral retina.

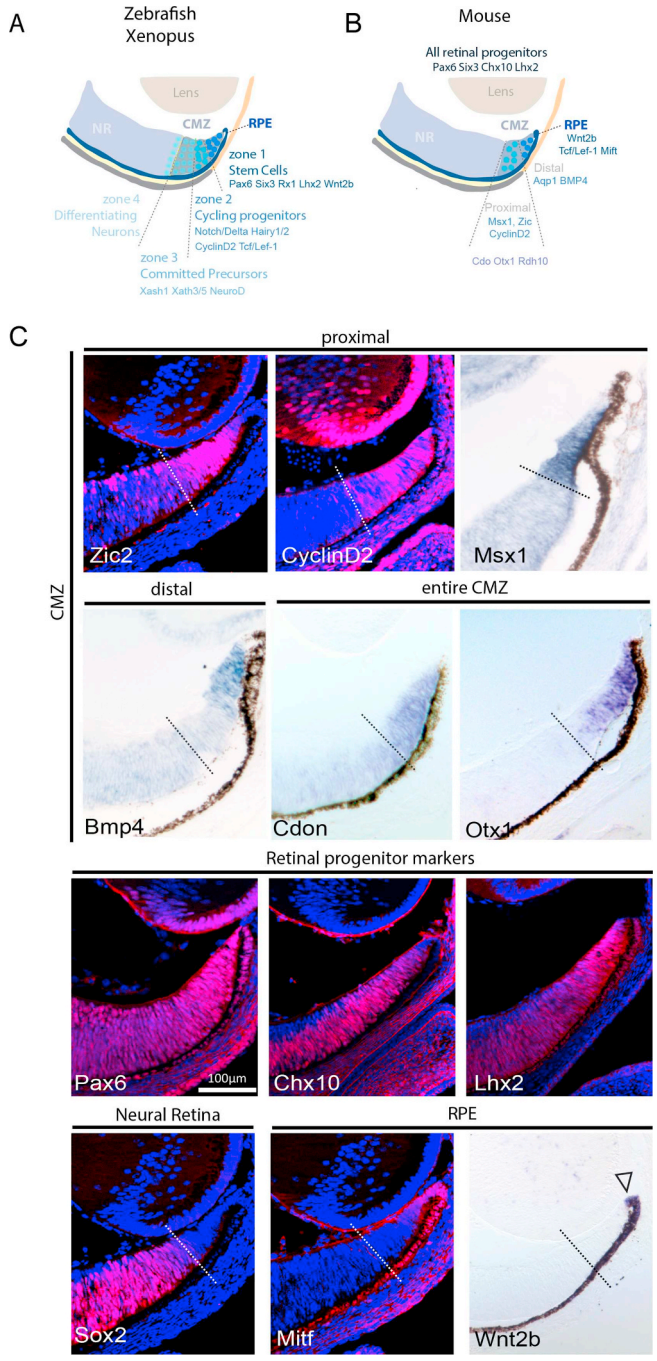

Fig. 2. CMZs in lower vertebrates and mammals.
(A) Depending on the maturity of cells, the ciliary marginal zone (CMZ) of lower vertebrates is organized into 4 regions that can be recognized by the expression of different markers.
(B) The mouse CMZ is not clearly organized into four regions. Only some markers have been shown to be differentially expressed in the distal (Bmp4) and the proximal (Msx1, CyclinD2) areas of the CMZ.
(C) In situ hybridization and immunofluorescence of an E14.5 mouse retina. Zic2, Msx1 and CyclinD2 (red) are expressed in the proximal CMZ, while Bmp4 labels the distal CMZ. The transmembrane glycoprotein Cdon and the transcription factor Otx1 are general CMZ markers. Pax6, Chx10 and Lhx2 are expressed throughout the entire CMZ and in retinal progenitors. Both Mitf and Wnt2b are markers of RPE, but the latter is also expressed in the junction between the RPE and CMZ. Sox2 is not expressed in the CMZ. Sections were stained with DAPI (blue). In all the panels, the dashed black lines indicate the boundery between the neural retina and CMZ delineated by Sox2 staining.

2017). Both the lens placode and the distal optic vesicle eventually invaginate, leading to the formation of a bilayered optic cup, and the initial lens in a process controlled by physical forces (Hosseini et al., 2014; Martinez-Morales et al., 2017; Oltean et al., 2016; Oltean and Taber, 2018) and genetic and molecular factors. The cellular and molecular mechanisms that regulate the positioning and differentiation of the lens placode and optic vesicle are not fully understood in mammals, but the signaling mediated by diffusible factors, such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Sonic hedgehog (Shh) and Wingless-like proteins (Wnts) are known to play critical roles in this process (Gunhaga, 2011). Inactivation of BMP signaling disrupts lens induction (Furuta and Hogan, 1998; Huang et al., 2015; Rajagopal et al., 2009), and inhibition of FGF signaling affects the formation of the lens placode (Faber et al., 2001). Shh suppresses the lens fate, and Wnt signaling restricts caudal expansion of the lens (Kreslova et al., 2007; Smith et al., 2005; Zhang et al., 2013).

Between E9.5 and E10.5, concomitant with the induction of the lens, the NR and the RPE domains are specified in the optic cup by the expression of the transcription factors Chx10 (also called visual system homeobox 2 (Vsx2)) and Microphthalmia-associated transcription factor (Mitf), respectively. Mitf is initially expressed in the entire vesicle (Nguyen and Arnheiter, 2000), but once NR specification occurs in the distal region, the RPE emerges in the dorsal area, and Mitf expression becomes restricted to dorsal regions. At this moment, the distal/ventral portion of the optic vesicle begins to express Chx10 (Nakayama et al., 1998; Nguyen and Arnheiter, 2000), while Pax6 maintains the dorsal and nasotemporal pattern of the optic cup (Baumer et al., 2002) (Fig. 1B). BMPs and Wnts, which are both released from the surface ectoderm (Carpenter et al., 2015; Cho and Cepko, 2006; Grocott et al., 2011; Liu et al., 2003b, 2006; Steinfeld et al., 2013), are not only involved in the induction of the lens but also influence the specification of the NR and RPE. BMP induces the RPE fate and inhibits the formation of the NR (Zhang et al., 2013; Zhao et al., 2002), while the absence of Wnt/β-catenin signaling in the distal retina abolishes the expression of Mitf and induces NR formation (Fujimura et al., 2009; Westenskow et al., 2009).

2.2. The ciliary margin zones in different species

In the embryonic eye, the transition area between the NR and the RPE is called CMZ from the moment that these two structures are specified until birth (Bharti et al., 2006; Fotaki et al., 2013; Martinez-Morales et al., 2001; Monaghan et al., 1991) (Fig. 1C).

In lower vertebrates, the area of transition between the NR and RPE is also known as the CMZ, which contains a pool of progenitor cells that maintain ability to generate new retinal neurons throughout life. In these species, the retina grows via the addition and integration of concentric rings of newly generated cells from the CMZ (Hitchcock and Raymond, 1992; Raymond and Hitchcock, 1997) and also from a central-to-peripheral wave of neural differentiation that initiates in the dorso-central region of the optic cup (Cepko et al., 1996; Hollyfield, 1971; Livesey and Cepko, 2001; Straznicky and Gaze, 1971). Both the embryonic and the adult CMZs in zebrafish and *Xenopus* contain spatially ordered retinal stem cells (RSCs) and retinal progenitor cells (RPCs) that are gradually differentiated and finally added to the NR (Fischer et al., 2013; Ohnuma et al., 2002). The expression of different markers distinguishes the CMZ of lower vertebrates into 4 differentiated zones. Zone 1 is located at the most distal tip of the CMZ and is populated by RSCs, which are proliferative and multipotent. Some of the RSCs are dormant, while others are proliferative, and their daughter cells remain within this zone (Tang et al., 2017). Cells in this area express the early eye field transcription factors Pax6, Six3 and Rx1 (Raymond et al., 2006). Zone 2 located next to this area, undergoes intensive cell proliferation by asymmetric cell division. One daughter

cell remains multipotent in this peripheral niche, and the other one becomes an RPC that migrates into the next zone. Cells in zone 2 express components of the Shh (Gli1, Gli3, X-smoothened), Wnt (Tcf/Lef-1) and Notch-Delta (Hairy1 and Hairy2, the respective orthologues of human Hes1 and Hes4) signaling pathways as well as the enhancer of split-related genes (ESR1 and ESR3) (Perron and Harris, 2000a; b) and CyclinD2, a member of the CyclinD family that regulates cell-cycle progression during the G1/S transition (Kozar and Sicinski, 2005; Ohnuma et al., 2002). Zone 3 is a region compressing committed precursors that facilitate the final round of division and progress into postmitotic cells. Xash1, atonal genes, such as Xath3 and Xath5, and proneural genes that stimulate the generation of retinal precursors (e.g., NeuroD) (Harris and Perron, 1998), are expressed in this zone. These cells successively develop into postmitotic zone 4 wherein cells further differentiate into retinal neurons and eventually integrate into the different layers of the NR (Perron et al., 1998) (Fig. 2A).

The mouse CMZ also expresses different components of the Shh (Gli2, Boc, Cdo, LRP2), Wnt (Tcf/Lef-1), and Notch-Delta signaling (Hes1) pathways (Bao and Cepko, 1997; Christ et al., 2015; Jo et al., 2012; Liu et al., 2006, 2007; Maurer et al., 2014; Sanchez-Arrones et al., 2013), supporting the idea that many of the mechanisms that control proliferation and differentiation in the CMZs of all vertebrates are conserved. The four-zone organization of the CMZ observed in *Xenopus* and zebrafish is not apparent in mammals, but at least two areas, the distal and proximal regions, are differentiable according to the different markers expressed in this area between E14.5 and birth. The distal area is positive for BMP4, while the proximal area is positive for CyclinD2 and the transcription factors Msx1 and Zic1/2 (Belanger et al., 2017; Kuwahara et al., 2015; Marcucci et al., 2016). CMZ cells also express Pax6, Chx10 and Lhx2, as well as specific markers, such as the transcription factor Otx1 and the retinol dehydrogenase Rdh10 (Horsford et al., 2005; Kuwahara et al., 2015; Martinez-Morales et al., 2001). The retinal progenitor marker Sox2 expressed in the NR, sharply delimitates the border between the NR and the CMZ (Fig. 2B and C) (Heavner et al., 2014).

Genetic experiments using a tyrosinase-related protein-2 (Tyrp2)-Cre mouse line to remove Pax6 from the distal CMZ showed that the ablation of Pax6 from this area abolishes the formation of the ciliary epithelium (CE) but does not affect the development of the NR (Davis et al., 2009). Therefore, the mammalian CMZ was thought to give rise to non-neuronal structures, the ciliary body (CB) —the structure responsible for the production of the aqueous humour implicated in ocular accommodation (Coulombre and Coulombre, 1957; McDougal and Gamlin, 2015; Napier and Kidson, 2007) — and the iris, which controls the diameter and size of the pupil. However, recent observations demonstrated that different types of neurons in the peripheral adult mouse retina derive from the proximal CMZ (Belanger et al., 2017; Marcucci et al., 2016). Time-lapse experiments using a transgenic reporter mouse line in which CMZ cells positive for the transcription factor Zic2 are labelled (Herrera et al., 2003; Nagai et al., 1997), revealed that a number of proximal CMZ cells move laterally toward central positions, to eventually becoming ganglion neurons (Marcucci et al., 2016), and that CyclinD2 is crucial for the generation of those retinal neurons located at the borders of the NR, including the subpopulation of ipsilaterally projecting ganglion cells (Marcucci et al., 2016). A second lineage tracing approach designed to label CMZ cells positive for the transcription factor Msx1, also expressed in the proximal CMZ, confirmed that most Msx1-positive CMZ cells populate the internal layer of the CB, also called the non-pigmented epithelium (NPE) and the iris of the adult eye (Belanger et al., 2017). However, consistent with the work of Marcucci and colleagues, this study also revealed that cells from the proximal CMZ yield multipotent intermediate RPCs that can proliferate and generate all seven major retinal cell types at the periphery of the adult retina (Belanger et al., 2017) (Fig. 3). Thus, the CMZ is a gradual transition area, with the proximal region giving rise to retinal and NPE cells, while the distal region contributes only to the CE.

2.3. Molecular mechanisms controlling CMZ differentiation

The structurally well-defined four areas of the CMZ in lower vertebrates have facilitated functional analyses of some factors and signaling cascades involved in the generation and differentiation of retinal neurons originating from this area. Different components of the Wnt, Notch, BMP, and Shh signaling pathways as well as the Hippo pathway and posttranscriptional regulators of gene expression, such as microRNAs (miRNAs), have been implicated in the formation and differentiation of the CMZ in both lower and higher vertebrates (de Iongh et al., 2006; Maiorano and Hindges, 2012; Maurer et al., 2014). The role of these regulators in the development of the peripheral retina is becoming elucidated, and their precise functions in cell proliferation and differentiation as well as their complex interactions, particularly in mammals, are still under intense investigation. In this section, we summarize the most relevant findings on the functions of these pathways in the differentiation of the CMZ throughout its maturation process.

2.3.1. The Wnt/Notch/BMP pathways

Together with Wnt2b, downstream targets of the Wnt pathway, such as Lef1, Axin2 and Hairy, are highly expressed in the CMZ at the junction with the RPE (Fuhrmann et al., 2009; Kubo and Nakagawa, 2009; Kubo et al., 2003; Liu et al., 2006). Activation of the Wnt canonical pathway (WCP) in the chick NR induces the ectopic expression of markers typically expressed in the CMZ, such as BMPs, or that of the transcription factors Otx1 and Zic2 (Trimarchi et al., 2009) and inhibits NR maturation (Kubo et al., 2003, 2005). Similarly, in the mouse retina,

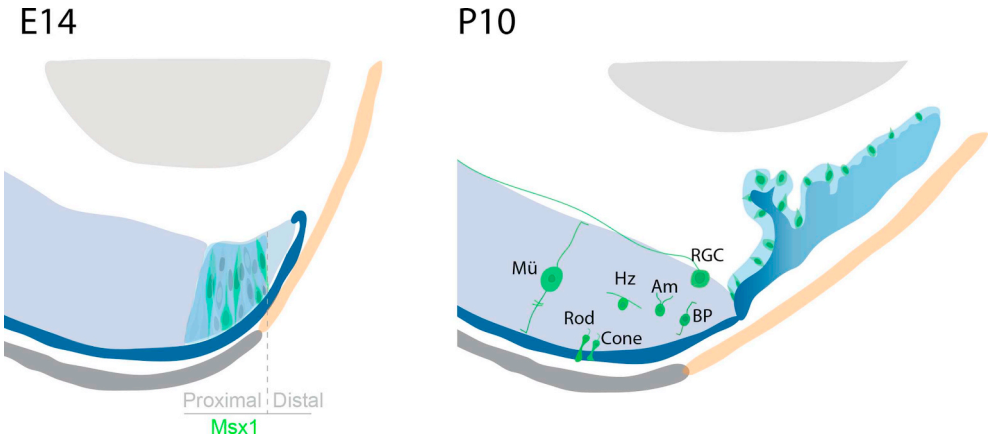

Fig. 3. Artistic illustration of the lineage tracing of Msx1-CMZ cells.
(A) On E14.5, mouse retinal progenitors in the proximal ciliary marginal zone (CMZ) express Msx1 (green cells).
(B) After birth, Msx1-lineage cells mostly populate the non-pigmented epithelium (NPE) in the ciliary body (CB) and iris. In addition, some Mxs1-derived cells generate different types of retinal cells including retinal ganglion cells (RGCs horizontal cells (Hz), amacrine cells (Am), photoreceptors (rods and cones), bipolar cells (BP) and Muller cells (Mü) (green cells).

activation of the WCP has been associated with an increased expression of CMZ markers, such as Msx1, Otx1 and BMP4 (Liu et al., 2007). Total ablation of BMP signaling by ectopically expressing the BMP antagonist Noggin leads to the complete suppression of Otx1 and Msx1 expression in the CE, which consequently alters the fate of CE cells to express Brn3b, a marker specific for RGCs (Thut et al., 2001; Zhao et al., 2002). A recent study has shown, however, that the conditional removal of BMP4 from the peripheral retina after optic vesicle invagination and lens induction has no impact on ocular development (Rausch et al., 2018), suggesting that the expression of BMP4 in early stages what determines the CE fate. In the dorsal part of the retina, the border between neurogenic and non-neurogenic regions is also influenced by the forkhead transcription factor Foxg1, which is expressed in the NR and acts as a repressor of the WCP (Fotaki et al., 2013).

The Notch signaling pathway is active in undifferentiated precursor cells throughout the entire optic cup. Continuous activation of the Notch signaling pathway inhibits cell differentiation and maintains the cells in an undifferentiated state in *Xenopus* (Dorsky et al., 1995, 1997). In mice, Notch2 is strongly expressed in the RPE and expressed at low levels or not expressed at all in the retina at perinatal stages. Notch2 maintains active BMP signaling in the CB, and its inactivation leads to a reduction in RPE cell proliferation (Zhou et al., 2013; Zhu et al., 2013). In addition, the endocytic adaptor protein Numb, which mediates the interaction between the Notch and the Wnt signaling pathways (Cheng et al., 2008), promotes the CE fate at the expense of the retinal fate (Belanger et al., 2017; Kechad et al., 2012), suggesting that Numb is a key differentiating factor between neural and non-neural fate.

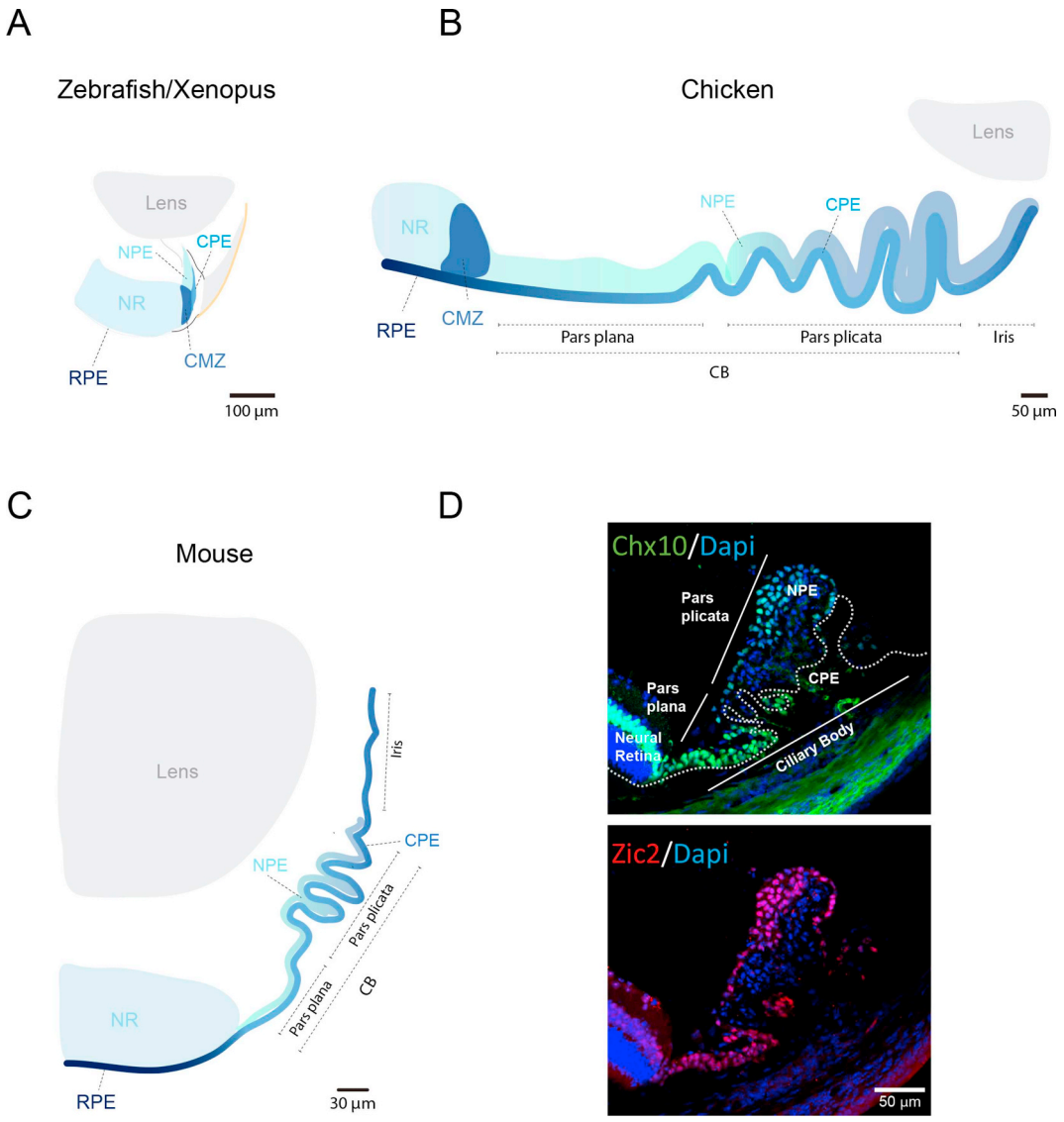

Fig. 4. Anatomical structure of the adult CMZ and CB.
Comparative schema representing the anatomical structures of the adult the ciliary marginal zone (CMZs) and the ciliary bodies (CBs) of zebrafish/*Xenopus*, chicken and mice.
(A) The CMZs of fish and frog contains progenitor cells that contribute to retinal regeneration of the retina throughout the lifetime of the animal.
(B) Unlike the CMZs of fish and frogs, birds have a very small CMZ and a large ciliary epithelium (CE).
(C) In mammals, the CB can be subdivided into the *pars plicata*, the distal region close to the iris, and the *pars plana*, proximal to the neural retina (NR). The CB and iris are two-layered structures containing the ciliary pigmented epithelium (CPE) and the non-pigmented epithelium (NPE).
(D) Immunofluorescence analysis of Chx10 and Zic2 in the CB of a retinal section from a P60 mouse. These two markers are expressed in the NPE. Nuclei were counterstained with DAPI (blue).

2.3.2. The Shh signaling pathway

Different Shh signal transduction molecules, such as the Shh receptors, Cdon and Boc, are also expressed in the mouse CMZ (Perron et al., 2003; Sanchez-Arrones et al., 2012, 2013; Tenzen et al., 2006). Although the precise function of this pathway in the CMZ remains under investigation, preliminary data suggest a role for Shh signaling in promoting proliferation and inhibiting differentiation in the CMZ. For instance, a mouse line engineered to overactivate the Shh pathway contains a zone in which CMZ cells persistently proliferate (Moshiri et al., 2004). In agreement with these observations, LRP2, a member of the low-density protein receptor family and modulator of the Shh pathway, is expressed at the apical surface of the NPE, and its absence results in the ectopic induction of Shh signaling and aberrant expansion of the progenitor pool at the CMZ, provoking peripheral retina hyperplasia (Christ et al., 2015).

2.3.3. The Hippo signaling pathway

The Hippo signaling pathway regulates the cell cycle exit and terminal differentiation in multiple types of proliferating cells, including RPCs. In zebrafish, the Hippo pathway is involved in regulating the balance between RPCs self-renewal and differentiation (Asaoka et al., 2014; Miesfeld et al., 2015). During mouse retinogenesis, Yap (Yes-associated protein), a major effector of the Hippo pathway together with Taz (transcriptional coactivator with a PDZ-binding domain), is expressed in all optic neuroepithelial compartments, and the loss or knockdown of Yap decreases the proliferation of RPCs and promotes retinal cell differentiation, leading to an early reduction of RPCs (Lee et al., 2008; Zhang et al., 2012). The loss of Yap in the optic vesicle results in transdifferentiation of the RPE into the NR (Kim et al., 2016).

Neurofibromin 2 (Nf2), a protein strongly expressed in the RPE and the CMZ, activates the Hippo signaling pathway at multiple levels (Moon et al., 2018). The Nf2-Hippo pathway restricts the growth capacity of the RPE and CMZ in the developing eye (Moon et al., 2018) because ablation of Nf2 from the optic neuroepithelium induces hyperplasia of this area. Mouse CMZ cells express higher levels of Yap/Taz than early RPCs, suggesting that high levels of Yap/Taz are required to maintain the neurogenic potential of CMZ progenitors. In line with this data, new RPCs derived from Nf2-deficient CMZ progenitors do not differentiate into neurons in the embryonic mouse retina but rather proliferate, increasing the accumulation of Yap/Taz proteins (Moon et al., 2018). This result is also supported by experiments performed in frog, in which Yap artificially expressed in active neural stem cells (NSCs) in the retina maintain cell cycle progression (Cabochette et al., 2015).

2.3.4. Small noncoding RNAs

Micro RNAs (miRNAs) or small non-coding RNAs, are key regulators of developmental events in vertebrates that control gene expression posttranscriptionally by regulating either mRNA stability, translation efficiency or both (Lee and Ambros, 2001; Pasquinelli et al., 2000; Reinhart et al., 2000). The RNaseIII ribonuclease Dicer1 cleaves pre-microRNA into a short double-stranded RNA fragment called miRNA. In *Xenopus* embryos, Dicer1 plays an important role in retinal morphogenesis, controlling the cell cycle exit, survival and time of cell differentiation (Decembrini et al., 2008). Dicer1 is detected in a mosaic pattern in the inner layer of the optic cup, playing a role in the generation and survival of all retinal cells types (Damiani et al., 2008). Dicer1 is not expressed in the distal optic cup and it has been suggested to be necessary for the compartmentation of neuronal versus non-neuronal progenitors (Davis et al., 2011). In fact, ablation of Dicer1 in the mouse retina results in the formation of an ectopic progenitor population that creates a CMZ-like region and provokes changes associated with alterations in Notch and Hh signaling-dependent genes (Davis et al., 2011).

Despite all these advances, substantial work is needed to fully understand the details regarding the interactions and synergies among the Wnt/Notch/BMP, Shh and Hippo pathways and how post-transcriptional and posttranslational regulation precisely control the balance between proliferation and differentiation in the CMZ, ultimately resulting in non-pigmented ciliary epithelia as well as some retinal neurons that populate the peripheral retina.

3. The peripheral retina in the adult eye

In the adult eye, the retina is a layered structure formed by different types of cells that collect and then transmit visual information to visual processing centers. In the innermost layer, RGCs extend their axons outside the retina, forming the optic nerve, to reach the visual targets in the brain (Coombs et al., 2007; Erskine and Herrera, 2014; Herrera et al., 2017). Bipolar, amacrine and horizontal cells form the inner nuclear layer and rod and cone photoreceptors define the outer nuclear layer. The outer segments of photoreceptors are embedded in the RPE, which supports the structure and function of photoreceptors. At the periphery of the retina, the RPE extends to form the external part of the CE, which is defined as the ciliary pigmented epithelium (CPE). In lower vertebrates, the area between the iris and the NR is occupied by the proliferative CMZ and CB. However, the adult mammalian retina does not have a proliferative CMZ, and the nexus structure between the NR and the iris is the non-pigmented layer of the CB. Because their similar locations in the retina, the CB of the mammalian retina and the adult CMZ of lower vertebrates have been considered as topologically analogous (Perron and Harris, 2000b). In the next sections, we describe the anatomical and functional features of the adult peripheral eye in lower vertebrates, birds and mammals and then discuss the current knowledge regarding the potential regenerative features of CB cells.

3.1. Anatomical features of the CB in different species

In zebrafish and amphibians, the CB and iris are small regions located at the extreme tip of the retina and have no clear anatomical distinction (Fig. 4A). The CMZ of lower vertebrates contributes to the generation of new neurons throughout the life of the animal (Johns, 1977), but the CMZ is not the only region capable of regenerating retinal neurons in these species, as cells in the RPE can also transdifferentiate and regenerate retinal neurons (Yoshii et al., 2007) as well as the Müller glia (Wan and Goldman, 2016).

In birds, the CB and the iris are large and anatomically distinguishable while the CMZ is proportionally smaller (Fischer and Reh, 2000; Morris et al., 1976) (Fig. 4A). In the chick, CMZ cells are spontaneously able to generate bipolar and amacrine cells, although their proliferative capacity decreases with age (Fischer and Reh, 2000) and, the migration of CMZ cells is limited to a relative small peripheral region of the retina (Reh, 1987). Avian cells located in the adult CMZ can not only spontaneously differentiate into amacrine and bipolar neurons but also gain additional capacities of proliferation and differentiation in response to treatment with external factors. For instance, the injection of insulin or Shh promotes the proliferation and/or differentiation of the CMZ in posthatched chickens *in vivo* (Fischer et al., 2005; Moshiri et al., 2005). The delivery of insulin, fibroblast growth factor 2 (FGF2) or a combination of both also stimulates cell proliferation in the NPE of the CB, and a few of these cells even express RGC markers, such as the neurofilament-associated antigen (NAA) and RA4, although they do not integrate into the NR (Fischer and Reh, 2003). These results suggested that both the CMZ and NPE have neurogenic capacity in chicks. CMZ cells can differentiate into specific cell types and integrate into the NR spontaneously, while NPE cells, although capable of proliferating and differentiating, do not integrate into the visual circuit.

In mammals the CB and the iris are large structures like those in chicks but the CMZ is not noticeable. Consequently, the CB is adjacent to the NR in the mammalian eye (Coulombre and Coulombre, 1957; McDougal and Gamlin, 2015; Napier and Kidson, 2007). The

mammalian CB is subdivided into the *pars plana*, a smooth surface zone proximal to the NR, and the *pars plicata*, which has a folded surface and is located in the more distal region of the CB, close to the iris (Fig. 4A). The CB and iris of mammals are also two-layered structures containing the CPE and NPE. The CPE and NPE may be distinguishable because they express different markers, P-Cad (Cdh3) is expressed by the CPE, while N-Cad (Cdh2), Zic2 and Chx10 are expressed in the NPE (Coles and van der Kooy, 2017)(Fig. 4D).

Based on the anatomical similarity between the CBs and the CMZs of lower vertebrates, the CB was initially predicted to retain regenerative potentiality. This idea was later reinforced with the observation that in primates cells in the *pars plana* express retinal progenitor molecular markers (Fischer et al., 2001; Martinez-Navarrete et al., 2008). In recent years, whether or not the peripheral mammalian adult retina retains a certain neuroregenerative capacity has generated an intense debate. Lineage tracing experiments have demonstrated that in mice, Msx1-positive cells of the CB cannot spontaneously generate new neurons (Belanger et al., 2017). Even though mammalian CB cells do not proliferate spontaneously, as in chickens, external stimulation of proliferation, induced by the loss of ganglion and amacrine cells through elevation of the intraocular pressure, ischemia or optic nerve lesions, promoted cell proliferation in the NPE of the CB (Karl et al., 2008; Nickerson et al., 2007; Ooto et al., 2004; Wohl et al., 2009, 2012).

3.2. Proliferative features of mammalian CB cells

The proliferative potentiality of mammalian CB cells was evaluated *in vitro* by Tropepe and colleagues and they found that under specific culture conditions, cells isolated from the CB were capable of generating neurospheres. These results suggested that CB cells harbor stem cell-like properties, such as self-renewal and sphere formation abilities (Ahmad et al., 2000; Tropepe et al., 2000), an observation that was later reproduced by other groups testing cells from different species including humans (Bhatia et al., 2010; Liu et al., 2007; Moe et al., 2009; Wohl et al., 2012; Xu et al., 2007). Neurospheres derived from the CB (CB-NS) are formed by pigmented and non-pigmented cells and comprise a mixture of cells positive for different markers, including the neural progenitor marker Nestin, and Claudin-1, a marker of differentiated epithelial cells, which suggests that these spheres contain both epithelial cells and cells with a more neural progenitor-like phenotype (Ahmad et al., 2000; Engelhardt et al., 2005; Froen et al., 2011, 2013; Jasty et al., 2012; Tropepe et al., 2000).

In principle, cells in the NPE were the best candidate to retain proliferative properties and give rise to neurospheres because in chick and mice cells acquiring proliferative capacity in response to external factors are located in this structure (Fischer and Reh, 2003; Karl et al., 2008; Nickerson et al., 2007; Ooto et al., 2004; Wohl et al., 2009, 2012). However, experiments comparing their ability to form clones in mouse cells from the pigmented layer versus those on the non-pigmented side revealed that cells with the capacity to form neurospheres mostly reside in the CPE, a finding that agrees with the observation that these cells express P-cadherin, which is essential for the formation of adherent sphere colonies *in vitro* (Coles and van der Kooy, 2017). In addition, CB-NS obtained from albino mice yield results similar to those from pigmented mice (Ahmad et al., 2000; Tropepe et al., 2000), supporting the idea that the pigment does not interfere with proliferation. Although unexpected, this result was not completely surprising because CPE cells share a common neuroectodermal origin with those of the RPE (Bertolotti et al., 2014), a structure that has been demonstrated to contain “quasiprogenitor state” cells that can functionally transdifferentiate into NSCs or progenitor cells in chicks and *Xenopus* (Fuhrmann et al., 2014; Yoshii et al., 2007).

Neurospheres expressing different NSC markers such as Nestin and Pax6, have also been generated from the human CB and are more frequently formed from the *pars plicata* area than from the iris or the *pars plana* (Coles et al., 2004; Mayer et al., 2005). Isolation of the human pigmented and non-pigmented epithelium from the CB and the iris, demonstrated that, like in mice, pigmented cells from the CB were more efficient than non-pigmented cells at generating neurospheres (Jasty et al., 2012).

3.3. Signaling pathways that influence CB cell proliferation

CB-NS cells initially express a set of molecules involved in the Notch pathway, including Notch1, Delta1, HES-1 and HES-5, but they partially lose their expression after passaging (Yanagi et al., 2006), and the perturbation of Notch signaling affects the generation of CB-NS (Ahmad et al., 2004; Das et al., 2004). Perturbation of the Wnt signaling pathway also influences the proliferation of CB-NS cells *in vitro* (Ahmad et al., 2004; Liu et al., 2003b). Another pathway that has an important role in the maintenance of CB-NS is the signaling mediated by the receptor tyrosine kinase c-Kit and its ligand stem cell factor (SCF). They are both expressed in CB cells and regulate their self-renewal and differentiation abilities (Ahmad et al., 2004). Impairment of the c-Kit signaling by blocking the interaction with SCF decreases the generation and proliferation of neurospheres (Das et al., 2004). Members of the Shh signaling pathway, such as Ptc1 and Smo, are also expressed in the CB. However, disruption of the Shh pathway does not significantly alter the neurospheres formation capacity (Ahmad et al., 2004).

4. Use of CB cells for therapeutic purposes

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can both be reprogramed and generate photoreceptors *in vitro* (Lamba et al., 2010; Osakada et al., 2008; Tucker et al., 2011, 2013; Zhou et al., 2015). This protocol could potentially be used for therapeutic purposes in retinal pathologies involving photoreceptor degeneration or damage. However, transplantation of these cells presents certain limitations. ESCs must be differentiated before transplantation to avoid the formation of tumors (Binder, 2011; Cui et al., 2013; Mead et al., 2015; Shirai et al., 2016; West et al., 2012) and iPSCs from diverse somatic cells may acquire genetic and epigenetic aberrations during the reprograming process, including changes in the number of gene copies, point mutations, aberrant patterns of DNA methylation and impaired functioning of imprinted genes (Ben-David et al., 2010; Hussein et al., 2011; Sun et al., 2012; Vaskova et al., 2013).

Therefore, the observation that adult CB cells have the capacity to proliferate and be expanded, has been perceived as an opportunity for regenerative therapy, as the CB is a region that is surgically accessible, originates from a retinal environment, and is potentially free of the epigenetic marks that reprogrammed stem cells may present. CB cells could thus be used for autologous transplantation because they can be harvested from adult patients, expanded or modified *in vitro* and retransplanted into the original patient (Liu et al., 2003a).

To test this possibility, spheres derived from the mouse CB have been transplanted into the subretinal space, but they seem to be unable to activate the photoreceptor differentiation program and accumulate into a group failing to integrate into the tissue (Cicero et al., 2009; Gualdoni et al., 2010). In contrast, human retinal CB-NS cells transplanted into developing mouse and chick retinas were able to migrate and express the photoreceptor marker Rom1 (Coles et al., 2004), suggesting that human cells may be able to differentiate into specific retinal cell types *in vivo* when they are in an adequate environment. In addition, cells derived from the *pars plicata* of the human retina, that were genetically modified to express a constitutively active form of Chx10 and transplanted into the vitreous cavity of the mouse eye lacking functional rod photoreceptors, integrate into the photoreceptor layer, show electrophysiological properties and even seem to improve visual function (Inoue et al., 2010).

The observation that proliferating CB cells exhibit pigment and membrane interdigitations, features that are more typical of specialized cells than undifferentiated cells (Cicero et al., 2009; Moe et al., 2009),

together with the disparate results *in vivo* injecting mouse and human CB, have questioned the neurogenic capacity of cells derived from CB-NS. In fact, Cicero and colleagues suggest that CB cells represent a population of CE cells that can transdifferentiate into neural-like cells because they acquire some neural markers but do not lose ciliary epithelial identity. Intense investigations are currently ongoing in an attempt to clarify the multipotent capacity of CB cells, which have been cultured under different conditions to characterize their ability to produce retinal cells. In this regard, mouse CB-NS induced to differentiate in the presence of fetal calf serum (FCS) and absence of growth factors, stop proliferating, begin to attach to the dish and express early neural progenitor markers, such as Pax6, Chx10, Nestin (Ahmad et al., 2000; Engelhardt et al., 2004, 2005; Jasty et al., 2012; Tropepe et al., 2000), doblecortin, ßIII-Tubulin, mAP2, Enolase, O4, GFAP, A2B5, NG2 and GalC (Engelhardt et al., 2005), but they do not express specific markers for differentiated retinal cell types.

CB-NS generated from the human CB also express Nestin, Pax6 and other markers of neural fate after 3 weeks of cultured on laminin-coated plates in the presence of fetal bovine serum (FBS) and epidermal growth factor (EGF) (Coles et al., 2004; Mayer et al., 2005). The efficiency of these differentiation assays is very low in all the cases, but if the medium is supplemented with retinoic acid and brain-derived neurotrophic factor (BDNF), these human CB-NS derived cells may even express markers of different retinal cells types, including Brn3b (RGCs), Thy1 (RGCs), rhodopsin (rod photoreceptor), recoverin (rod and cone cells), s-Opsin (cone cells), Nrl (rod cells), syntaxin1 (synapses) and calretinin (amacrine cells) (Jasty et al., 2012; Mayer et al., 2005), and some of these cells have ionotropic glutamate receptors activity (Jasty et al., 2012). Therefore, the differentiation of cells derived from the CPE seems to be highly conditioned by the culture medium (Reynolds and Weiss, 1992) and/or may be species-dependent.

To characterize CB-NS cells in more detail and gain further insight into their potential capacity to differentiate into retinal cell types, we isolated CB cells from the adult mouse retina, generated neurospheres according to established protocols (Coles and van der Kooy, 2010), determined their ratio of proliferation and stained for different markers. Upon addition of BrdU to the culture medium (at a concentration of 28 μM) and subsequent staining with an anti-BrdU antibody, we confirmed that neurospheres did not appear as a result of cell aggregation and were growing at a proliferative rate of 19% per day, a rate similar to that of other NSCs (Fig. 5A). As previously reported immunofluorescence analysis showed that neurospheres cells express Nanog and other neural progenitor markers such as Nestin or Sox2 (data not shown). Then, we plated these cells on polylysine-coated coverslips in medium supplemented with 4% FBS in the absence of growth factors (EGF and FGF2) and observed that the cells began to emerge from the neurosphere on day 1. While still in the neurosphere, the cells exhibited a large pigment content, but the pigment was lost as time progressed and the cells moved away from the neurosphere (Fig. 5C). After one week of growth in conditioned media, cells derived from CB-NS were positive for the early neuronal differentiation marker Map2 showing different levels of expression. Some cells also expressed the early neuronal marker Tuj1 and in most of them Sox2—associated to neural progenitors— was downregulated. Lhx2, a marker for neural progenitors, is expressed by both cells inside and outside the neurosphere. Cells derived from CB-NS were all positive for Calbindin and Calretinin. These markers label subsets of amacrine and ganglion cells in the adult retina and given the large diversity that we observe in the expression of other makers, we tend to believe that the homogenous Calbindin and Calretinin labeling observed in these cultures is artefactual. In addition, none of the cells derived from CB-NS were positive for Brn3a or rhodopsin, which are markers for RGCs or photoreceptors respectively (Fig. 6). Thus, in agreement with previous reports, our results indicate that neurospheres isolated from the mouse CB contain pigment, have the ability to proliferate and can be induced to stop proliferation while start to express some neural differentiation markers and lose the pigment during this process. However, although a number of CB-NS derived cells express certain neuronal markers they do not seem to fully differentiate to retinal cells under these culture conditions. These restuls raise the question of whether specific media would be needed for

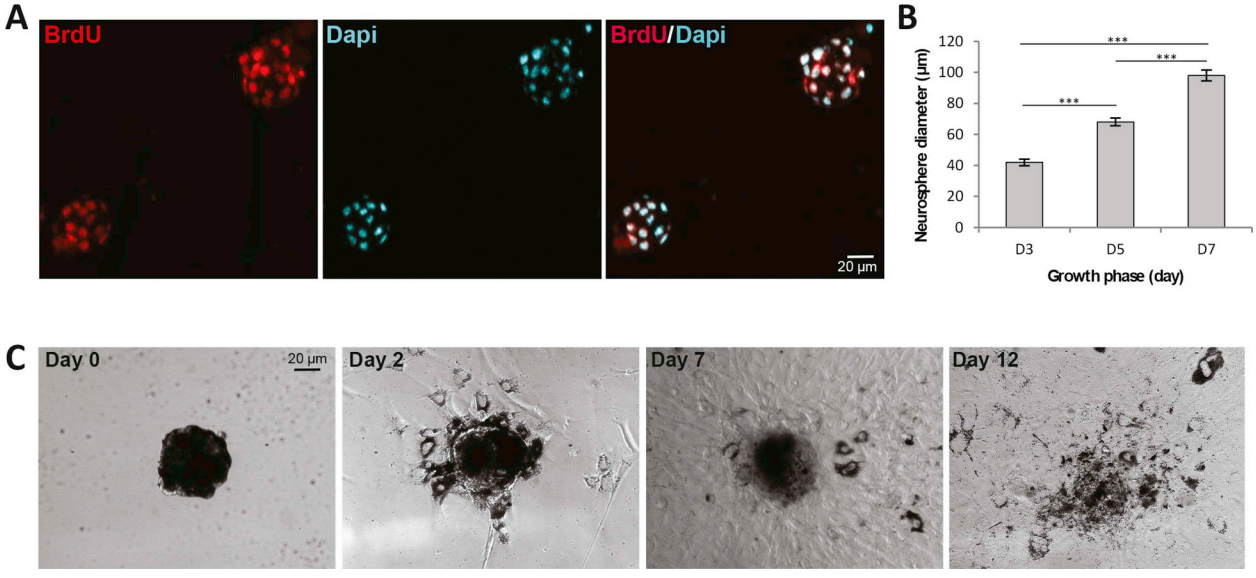

Fig. 5. Generation and characterization of neurospheres from mouse CB cells.
(A) Immunofluorescence analysis of BrdU (red) in 7-days neurospheres generated from cells isolated from the ciliary bodies (CB-NS) of 2 months-old WT mice. Nuclei were counterstained with DAPI (cyan).
(B) Quantification of their proliferation rate, indicating their increase in size over time (growth Rate = current diameter/past diameter$^{(1/\text{days in culture})-1)}$X100) (****pvalue* < *0.001*).
(C) Progression of cells from the CB-NS after the induction of differentiation in a medium supplemented with 4% fetal bovine serum at in the absence of growth factors (EGF and FGF2) and plating on a polylysine-coated coverslip. Initially, cells from the neurosphere are pigmented. When incubated under differentiation conditions, the cells begin to concentrically migrate from the neurosphere, losing the pigment in the process. By day 12 the neurosphere has disappeared, and all the cells are attached to the dish.

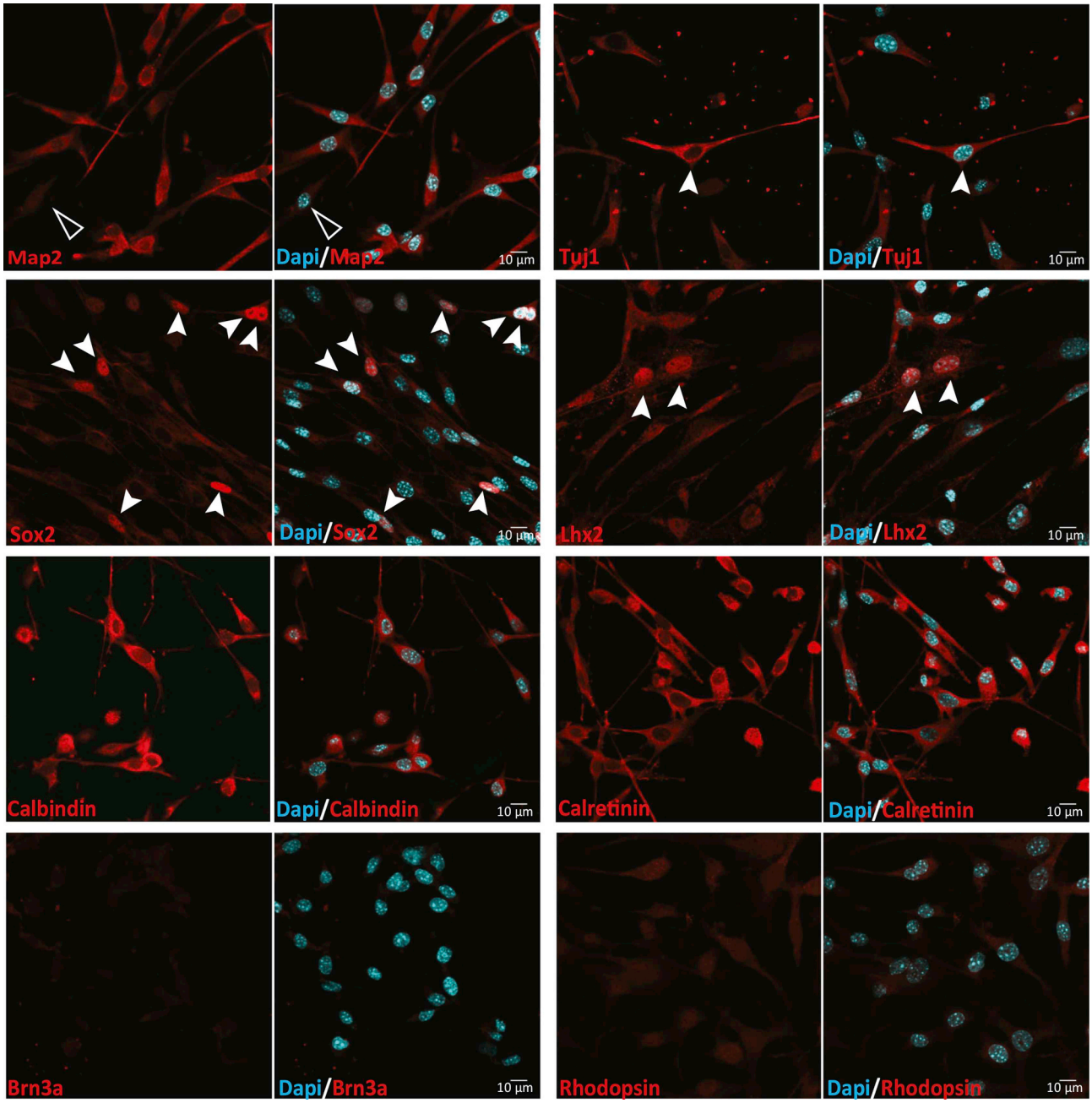

Fig. 6. Characterization of cells derived from CB-NS.
Immunofluorescence analysis of different markers in CB-NS plated on polylysine-coated coverslips and incubated in a medium supplemented with fetal bovine serum in the absence of growth factors. Empty arrows indicate lack of staining while arrowheads highlight cells that are positive for that particular marker.

the differentiation of each retinal cell type.

To further explore the multipotent capacities of CB-NS cells overcoming the limitation of each retinal cell type requiring a specific medium, we decided to use a protocol that allows the generation of optic cups *in vitro* creating a three dimensional (3D) retinal organoid (Eiraku and Sasai, 2011; Eiraku et al., 2011). These organoids contain self-patterning retinal neurons forming a layered structure and their generation recapitulates retinal development *in vitro*, serving as an ideal platform to explore the endogenous potentiality of cells isolated from the CB to produce different retinal cell types. We attempted to generate 3D retinal tissues from CB-derived cells and compare their potential with that of a mouse embryonic stem cell line (mESCs) (Eiraku et al., 2011). We first generated CB-NS as described above. After one week they were disaggregated and plated in parallel with the mESCs and with cells directly isolated from the adult mouse CB under the same conditions and in the same quantity. The differentiation protocol generally used to produce retinal organoids (Chen et al., 2016; Eiraku and Sasai, 2011) was then applied to the three type of cells. Ten days after differentiation, optic cups similar to those previously reported expressing Nanog, were visible in the cultures coming from mESC (Fig. 7B and C). In contrast, cells isolated from CB-NS, although formed secondary spheres, did not generate organoids. Cells isolated from the CB were not even able to attach to each other and form spheres under these conditions (Fig. 7B). Therefore, CB cells do not generate 3D optic cups *in vitro*, revealing that unlike mESCs, CB-NS do not have the capacity to generate a self-forming retina with different retinal cell types, at least in these culture conditions.

Here, we have used 3D-retinoids to specifically test the multi

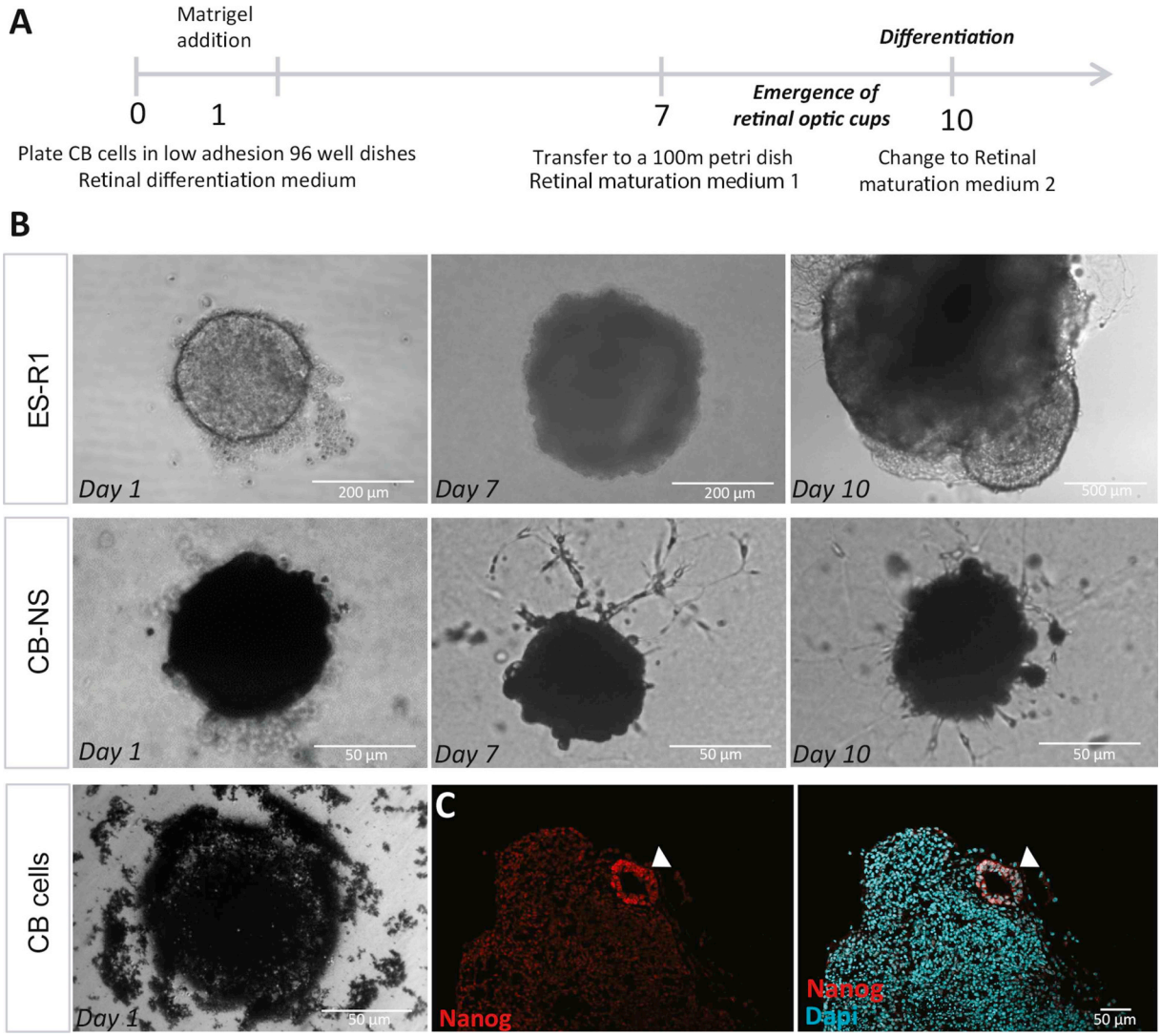

Fig. 7. Generation of retinal organoids from mouse CB-NS.
(A) Schematic of the protocol followed to generate retinal organoids. Retinal differentiation medium: GMEM, nonessential amino acids, pyruvate; 2-ME, KSR. Retinal maturation medium 1: DMEM/F12 with GlutaMax, N2 supplement, penicillin/streptomycin. Retinal maturation medium 2: DMEM/F12 with GlutaMax, N2 supplement, penicillin/streptomycin, FCS, taurine, retinoic acid.
(B) Comparison of the generation of retinal organoids using three different cells types: ES-R1, an established mouse embryonic stem cell line; CE CECB-NS, cells derived from mouse neurospheres and ciliary body (CB) cells; and CB cells directly isolated from the ciliary epithelium (CE) from a two-month-old mouse.
(C) Immunostaining analysis of a section of organoids derived from ES-R1 cells with a Nanog antibody (red) after 10 days in culture.

potency of CB cells, but this approach has recently emerged as an additional line of research to obtain different types of retinal cells. Recent modifications to the protocol consisting on the addition of the glycogen synthase kinase 3 (GSK-3) and FGF receptor (FGFR) inhibitors, have demonstrated that these artificial structures may even form CMZ-like zones at the NR-RPE junctions (Kuwahara et al., 2015) and shown that under hypoxic conditions (5% oxygen) the efficiency on the generation of 3D optic cups increased (Chen et al., 2016). Even more importantly, retinal organoids can be produced not only from mice but also from human ESCs (Nakano et al., 2012) and rod photoreceptors produced in the organoids showed transcription profiles similar to those of rods purified from the mouse retina (Chen et al., 2016). All these data pinpoint the generation of retinal organoids as a promising path to explore the possibility of obtaining retinal progenitors for therapeutic purposes.

5. Conclusions and future directions

Unlike in lower vertebrates, new neurons differentiating from the peripheral retina are not spontaneously added to the adult mammalian retina but CB cells are able to generate proliferative neurospheres. This observation raised the promising idea that the CB could serve as a source of plastic cells to be delivered into the eye and incorporated into a damaged visual circuit. However, although some studies suggest that human CB cells have the potential to generate different types of retinal neurons, most current data indicate that mouse CB cells are neural-progenitor like cells unable to fully differentiate to retinal neurons. Further experiments comparing human and mouse CB cells in parallel as well as the evaluation of the long-lasting potential of these cells *in vivo* are still required to definitively close this debate. Meanwhile, other strategies, such as the generation of 3D retinal organoids, are emerging as promising approaches with potential for applications in regenerative medicine (Fig. 8).

Authors' contributions

MFN has performed the experiments involving neurospheres/organoids generation. VBM has performed in situs and immunostainings of different markers in retinal sections. MFN, VBM and EH prepared the

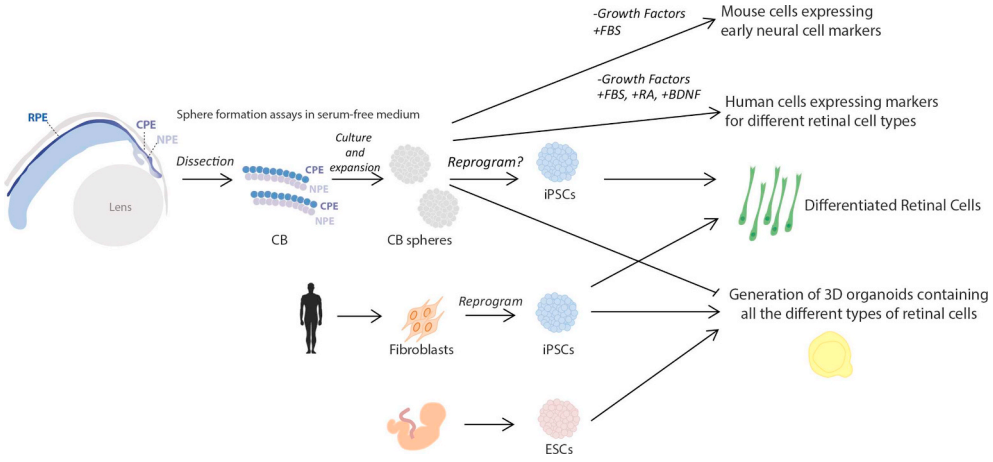

Fig. 8. Different strategies to obtain cells for a potential use in cell replacement therapies.
Neurospheres obtained after the dissection and disaggregation of cells from the adult ciliary body (CB) can be cultured and expanded *in vitro*. The withdrawal of growth factors and the addition of fetal bovine serum (FBS) favors the expression of different early neuronal markers in cells that emerge from the neurospheres. The addition of specific media containing brain derived neurotrophic factor (BDNF) and retinoic acid (RA) seems to be favorable for the expression of different retinal markers. However, up to date the potential of CB cells to fully differentiate to retinal neurons is controversial. Neurospheres derived from CB cells could be potentially reprogrammed to generate induced pluripotent cells (iPSCs) via the ectopic expression of transcription factors. Then retinal cell types could be obtained as photoreceptors have been shown to be generated from iPS cells. On the other hand, ciliary body neurospheres (CB-NS) do not generate 3D retinal organoids *in vitro,* revealing that CB-NS do not have the capacity to generate a self-forming retina with different retinal cell types like embryonic stem cells (ESCs) and iPSCs.

figures and wrote the manuscript. HYC provided the protocols and advice for the generation of organoids.

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