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Isolation and characterisation of neural progenitor cells from the adult *Chx10*$^{orJ/orJ}$ central neural retina

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ABSTRACT

Retinal stem cells have been isolated from the ciliary epithelium (CE) of the mammalian retina. However, the central neural retina (CNR) lacks the capability to regenerate, a phenomenon retained by lower vertebrates. Mutations in the *Chx10* homeobox gene cause reduced proliferation of retinal progenitor cells during development, leading to microphthalmia. Recently, we showed that in *Chx10*$^{orJ/orJ}$ mice, dividing cells persist in the adult CNR, suggesting the existence of a dormant progenitor population. Here, we show that these cells are proliferative and give rise to neurospheres *in vitro*, a characteristic of neural stem cells. However, these adult-derived CNR progenitors differ from those of the wildtype CE, leading to de-pigmented, larger and more numerous neurospheres expressing Müller glial cell markers. Our results suggest that lack of Chx10 leads to maintenance of a dormant neural progenitor population in the adult CNR. Furthermore, Chx10 is not required for *in vitro* proliferation of these progenitors.

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Introduction

Lower vertebrates display the capacity to generate new retinal neurons in response to injury (Hollyfield, 1971; Reh and Levine, 1998). New neurons arise mainly from a region called the ciliary marginal zone (CMZ) located around the anterior margin of the retina, which maintains a population of retinal stem cells throughout life. Regenerative potential in the central retina is more limited. A population of rod precursor cells exists in teleost fish that can regenerate all retinal neuronal types after damage (for review, see Raymond, 1991). Similarly, recent work showed that in zebrafish, the Müller glial cells function as multipotent retinal stem cells that respond to loss of photoreceptors by specifically regenerating the missing neurons (Bernardos et al., 2007). Conversely, acute damage of the post-natal chick retina induced Müller glial cells to undergo limited cell division and rarely to express markers characteristic of retinal neurons (Reh and Levine, 1998; Dyer and Cepko, 2000; Fischer and Reh, 2001). Although the adult mammalian eye lacks these regenerative capabilities, recent evidence has demonstrated that the ciliary epithelium (CE; Fig. 1), part of the ciliary body, a structure analogous to the lower vertebrate CMZ, contains a population of retinal stem cells (RSC) (Ahmad et al., 2000; Tropepe et al., 2000), but their function *in vivo* is unclear (Das et al., 2005b). While quiescent during adult life, *in vitro* these cells demonstrate characteristics typical of neural stem cells, including multipotentiality and self-renewal (Ahmad et al., 2000; Tropepe et al., 2000). Whether the mammalian central neural retina (CNR) contains a dormant stem cell population, similar to the Müller-like progenitor cell observed in lower vertebrates, remains an important question. Characterisation of adult stem cells offers insight into the regenerative potential in the mammalian nervous system and may have application in the development of novel therapies for the treatment of retinal disease.

Recent literature describes two different cell populations; retinal stem cells (RSCs) and retinal progenitor cells (RPCs) (Cepko et al., 1996; Reh and Levine, 1998; Ahmad et al., 2000; Tropepe et al., 2000; James et al., 2004; Zaghloul et al., 2005). The latter is purported to derive from asymmetric proliferation of the former (Zhong, 2003). During retinal histogenesis, RPCs undergo a finite number of divisions to generate the full complement of retinal neurons and Müller glia, which is complete by around post-natal day 11 in the mouse (Young, 1985a,b). By contrast, RSCs appear to persist during adulthood in the CE and when stimulated by growth factors *in vitro*, proliferate and show properties of multipotentiality and self-renewal. However, there is limited evidence of either specific cell markers or assays that permit the distinction between these two cell types (Bhattacharya et al., 2003; Das et al., 2005a). For this reason, here we use the term progenitor cell to describe stem-like cells in

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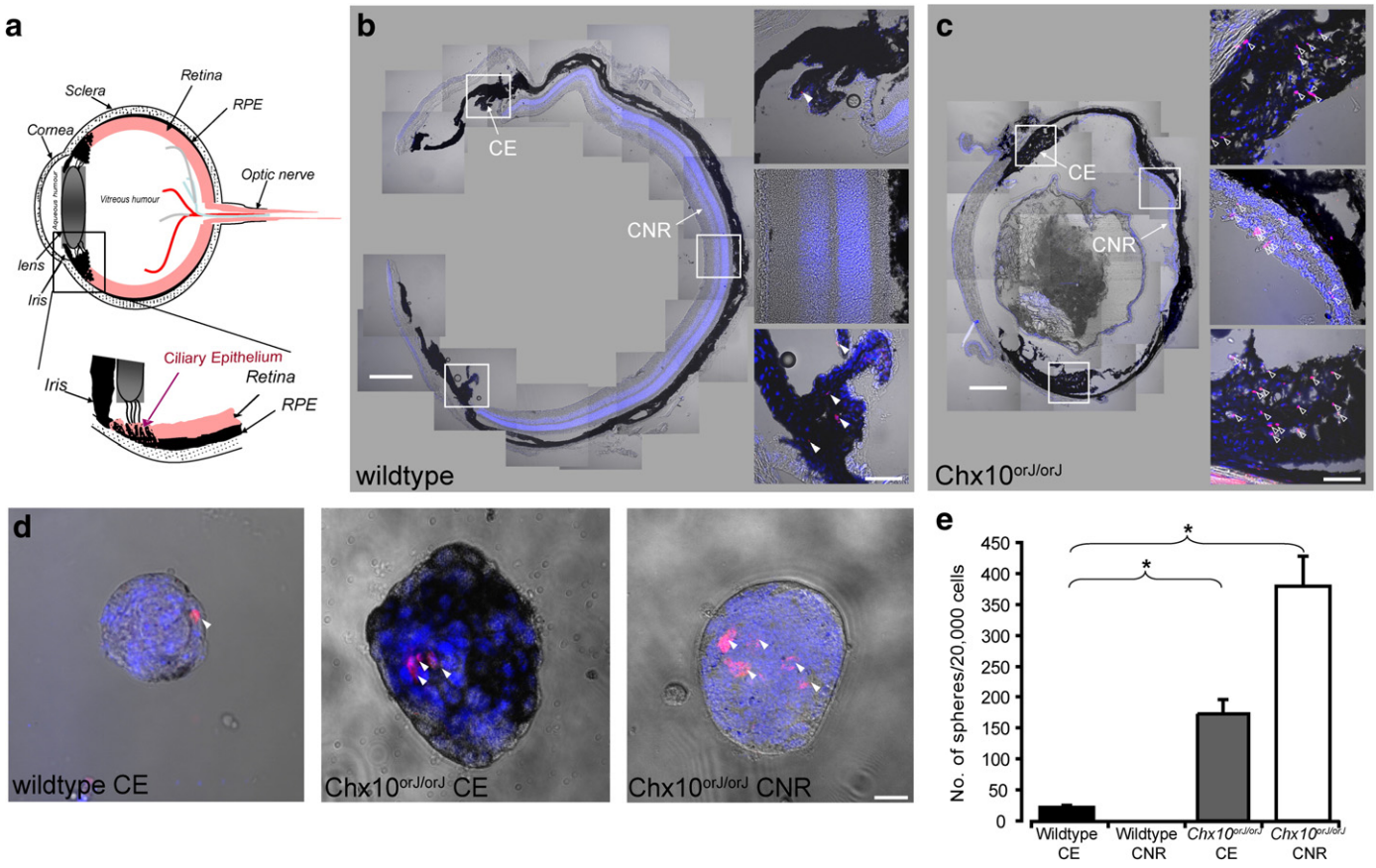

Fig. 1. Proliferating cells remain in the Chx10-deficient central neural retina and give rise to neurospheres *in vitro*. a, Schematic of the eye, showing the anatomical location of the ciliary epithelium (CE) and associated structures. b, confocal montage of a sagittal section through a wildtype eye (lens removed). Sections show Normarski images in which nuclei are counter-stained with Hoechst 33342. Inserts, BrdU-positive (red) dividing cells are occasionally found in the ciliary epithelium (top and bottom). No BrdU-positive cells were found in the central neural retina (CNR) (middle). c, Confocal montage of a section through a *Chx10*$^{orJ/orJ}$ eye. Inserts, numerous BrdU-positive (red) dividing cells are found in the ciliary epithelium (top and bottom) and the central neural retina (CNR) (middle). Scale bars 200 μm (main images b, c) and 50 μm (inserts). d, Neurospheres are derived from the population of slowly dividing cells present *in vivo*. In wildtype animals, neurospheres could only be derived from the CE and not the CNR. In contrast, neurospheres could be derived from both the *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CE and CNR. Neurospheres from the CNR of *Chx10*$^{orJ/orJ}$ animals and the CE of wildtype and *Chx10*$^{orJ/orJ}$ animals which had been pre-labelled with BrdU, prior to sacrifice, were found to contain small numbers of BrdU-positive (red) cells (arrow heads) together with many cells that were BrdU-negative ($N = 3$; $n = 20$ for each region, counter-stained with Hoechst 33342). Scale bar 20 μm. e, Histogram showing the average number of primary neurospheres derived per 20,000 cells after 7 days *in vitro* from wildtype CE, *Chx10*$^{orJ/orJ}$ CNR and *Chx10*$^{orJ/orJ}$ CE. $N = 13$ eyes for each region; one-way ANOVA $^*P<0.01$.

the adult neural retina with the ability to proliferate and form neurospheres *in vitro* and to differentiate to express retinal cell markers.

Mutations in several transcription factors, as well as cell cycle proteins, result in major alterations in eye size, often due to effects on the expansion of the progenitor pool during early development (Fantl et al., 1995; Kobayashi et al., 2001). *Chx10*, a paired-like homeobox transcription factor gene, is one of the earliest markers of the presumptive neural retina expressed in the invaginating optic vesicle (Liu et al., 1994). RPCs express *Chx10* throughout retinal development (Liu et al., 1994; Burmeister et al., 1996; Ferda Percin et al., 2000). After terminal division, expression is restricted to bipolar cells (Liu et al., 1994; Burmeister et al., 1996; Ferda Percin et al., 2000) and a recently described subpopulation of Müller glia (Rowan and Cepko, 2004; Rowan et al., 2004). Mutations in both the human and mouse *Chx10* genes lead to microphthalmia (Burmeister et al., 1996; Ferda Percin et al., 2000; Bar-Yosef et al., 2004). The naturally-occurring *Chx10* mouse mutant, *ocular retardation* (*Chx10*$^{orJ/orJ}$) (Burmeister et al., 1996), is characterised by a severely underdeveloped neural retina and expanded CE (compare Figs. 1b and c), absence of bipolar cells and disrupted photoreceptor differentiation (Robb et al., 1978; Silver and Robb, 1979; Burmeister et al., 1996; Bone-Larson et al., 2000; Rowan and Cepko, 2004; Rowan et al., 2004; Rutherford et al., 2004). In cultures of cells derived from the adult CE, notably more neurospheres arise from the CE of the *Chx10*$^{orJ/orJ}$ mice than from wildtype mice (Tropepe et al., 2000) and it has been proposed that loss of RPC proliferation causes an expansion in the number of RSCs in the mutant CE (Coles et al., 2006). Similarly, we have previously shown that there are increased numbers of CE cells proliferating *in vivo* in the mutant compared to the wildtype (Dhomen et al., 2006).

Although retinal stem or progenitor cells have not been isolated from the adult mouse CNR (Tropepe et al., 2000; Coles et al., 2006), we recently showed that a population of dividing cells persist *in vivo* in

Fig. 2. Neurospheres derived from the Chx10-deficient retina are larger, proliferate more and are more numerous than those derived from the wildtype retina. a, A limiting dilution assay revealed a linear relation between the number of cells plated and the formation of neurospheres (mean ± SEM; $N = 3$). b, Neurospheres do not result from cell aggregation. Mixed cultures of cyan fluorescent protein (CFP) and green fluorescent protein (GFP) cells result in neurospheres that comprise either of only GFP-positive cells or of CFP-positive cells. Nuclei are counter-stained with propidium iodide (PI). Scale bar 10 μm. c, Graph showing diameter of neurosphere derived from wildtype CE (black squares; $N = 10$ eyes, $n = 132$ neurospheres), *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CNR (white triangles; $N = 10$, $n = 154$) and *Chx10*$^{orJ/orJ}$ CE (grey triangles; $N = 10$, $n = 153$). d, Graph showing the number of cells per neurosphere from wildtype CE ($n = 22$), *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CNR ($n = 30$) and *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CE ($n = 33$). e, Graph showing the percentage of cells per neurosphere that were BrdU-positive following a 4h pulse *in vitro* from wildtype CE ($n = 22$), *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CNR ($n = 30$) and *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CE ($n = 33$). One-way ANOVA tests used throughout, $^*P<0.005$. f, Examples of confocal sections through neurospheres from wildtype CE (top), *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CNR (middle) and *Chx10*$^{orJ/orJ}$Chx10$^{orJ/orJ}$ CE (bottom) following a 4h pulse of BrdU (red). Nuclei are labelled with Hoechst (blue).

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