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Identification of genes expressed in retinal progenitor/stem cell colonies isolated from the ocular ciliary body of adult mice

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Abstract

Rare pigmented cells showing retinal stem cell characteristics have been identified in the ocular ciliary body (CB) of adult mammals. In vitro, these cells were reported to clonally proliferate and generate pigmented sphere colonies (PSC) containing multipotent retinal progenitor-like cells. Because these cells may have important clinical applications and because their embryonic origin is unclear, we have analyzed their local environment and gene expression profile. We found that transcription factors Pax6, Six3, and Rx, all involved in early eye morphogenesis, were expressed in the CB of adult mice. By sequencing a PSC cDNA library, we found that PSC expressed at high levels transcripts involved in the control of redox metabolism and cellular proliferation. PSC also expressed the retinal transcription factor Six6, which expression was not detected in the CB epithelium. By in situ hybridization screen, we found that *Palmdelphin* (*Palm*), *Hmga2*, and a novel transcript were expressed in the central nervous system of early embryos. *Palm* expression delineated the pigmented epithelium of the future CB and the developing myotome. *Hmga2* was expressed in the ventricular zone of the telencephalon, the developing retinal ciliary margin and lens. Several genes expressed in PSC were also expressed in the nasal anlagen. Taken together, our study reveals that PSC isolated from the ocular CB express genes involved in the control of embryonic development, retinal identity, redox metabolism, and cellular proliferation.

Keywords: Ciliary body; Stem cell; Retinal progenitor; Pax6; Rx; Six3; Palmdelphin; Hmga2

1. Results and discussion

In the anterior neural plate of vertebrate embryos, overlapping expression patterns of the homeobox genes Pax6, Six3, Six6, Rx, Otx2, and Lhx2 demarcate the presumptive eye field. Gain- and loss of function experiments have revealed that this limited set of transcription factors is necessary and sufficient to control early vertebrate eye development (Hill et al., 1991; Walther and Gruss, 1991; Oliver et al., 1995; Furukawa et al., 1997; Mathers et al., 1997; Porter et al., 1997; Chow et al., 1999; Loosli et al., 1999; Zuber et al., 1999; Bernier et al., 2000; Lagutin et al., 2001; Carl et al., 2002; Zuber et al., 2003). In *Xenopus*, retinal progenitor/stem cells located at the tip of the neural retina, the ciliary marginal zone (CMZ), can perpetually self-renew and regenerate the mature retina upon physical damage. Cells of the CMZ are spatially ordered with respect to their "developmental stage", primitive retinal progenitor/stem cells being the most distal and commited progenitor cells being more proximal to the central retina (Wetts et al., 1989; Dorsky et al., 1995). Notably, the genetic hierarchy of retinal development is apparently maintained where *Pax6*, *Six3*, and *Rx* are expressed the most distally while downstream basic helix-loop-helix (bHLH) transcription factors are expressed at a relatively more proximal position in the CMZ (Perron et al., 1998). In contrast to amphibians, retinal progenitor cells of mammals loose their proliferative capacity and become terminally differentiated once development is completed.

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Therefore, they cannot contribute to the regenerative process when needed (Pacione et al., 2003). Notably, a rare population of pigmented cells displaying stem cell properties has been isolated from the eye of adult mice (Ahmad et al., 2000; Tropepe et al., 2000). Intriguingly, these cells were only found in the ocular ciliary body (CB), a structure that is anatomically located, together with the iris, at the most distal tip of the neural retina.

The CB is composed of two distinct epithelium apposed by their basal surface. This double epithelium forms a complex tri-dimensional network with capillaries (Morrison and Freddo, 1996). To investigate the potential expression of transcription factors involved in eye development in the ocular CB of adult mice, RNA in situ hybridization experiments were performed with *Mitf*, *Rx*, *Six3*, and *Six6* cDNA. *Mitf* encodes a bHLH-zipper transcription factor that is expressed in the developing retinal-pigmented epithelium (RPE) and which mutations are linked to the *microphthalmia* phenotype in mice (Fig. 1A) (Hodgkinson et al., 1993). In adult mice, *Mitf* expression was detected in the iris but not in the CB (Fig. 1B). Expression of *Rx* was observed in most epithelial cells of the CB and this result was confirmed using non-radioactive RNA in situ hybridization (Fig. 1C–E). At embryonic stage 13.5 (e13.5), robust expression of *Six3* and *Six6* was detected in the neural retina (Fig. 1F and H). *Six3* expression was also present in the developing RPE (Fig. 1F) (Oliver et al., 1995; Jean et al., 1999). In adult, *Six3* was predominantly expressed in the non-pigmented cells of the CB epithelium, while *Six6* expression was not detected (Fig. 1G and I). Expression of Six3 in the CB epithelium at the protein level was also confirmed using a specific antibody (provided by Guillermo Oliver, St. Jude Children's Research Hospital Memphis, Tennessee) (data not shown). We previously reported on *Pax6* expression in the ciliary margin of the neonatal mouse retina (Bernier et al., 2001). Because the CB is a derivative of the developing ciliary margin and RPE, it seemed plausible that *Pax6* is also expressed in the CB. Using X-gal staining, *Pax6* expression was analyzed in whole eye preparations from adult transgenic mice ($Pax6^{LacZ/+}$) carrying the reporter gene β-galactosidase in frame with the endogenous *Pax6* coding sequences (St-Onge et al., 1997). In these preparations, intense X-gal staining was observed in the region corresponding to the CB (Fig. 2A and B). No X-gal staining was observed in eyes of wild type littermates (data not shown). To localize Pax6 expression in the CB, indirect immunofluorescence (IF) on eye sections from wild type Albinos adult mice was performed (Fig. 2C–F). At low magnifications, robust Pax6 expression was observed in the CB and iris as well as in the neural retina, where Pax6 is present in ganglion cells and amacrine neurons (Fig. 2C). At higher magnifications (viewed on a distinct plan of section), Pax6 was present at high level in most cells composing the CB epithelium, with the exception of blood and vascular endothelial cells (Fig. 2E and F, and data not shown). We also investigated for the expression of the neural stem/progenitor cells marker nestin by IF (Temple, 2001). No evidence of nestin expression was found in the CB, while robust staining was

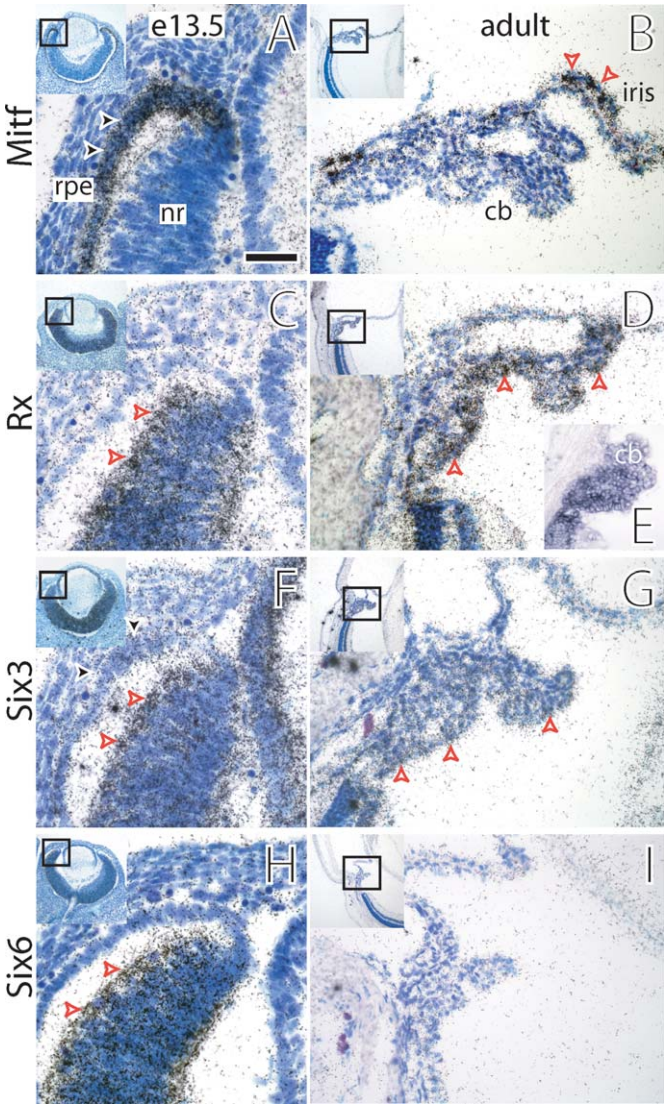

Fig. 1. Transcription factors expressed in the ocular CB. In situ hybridizations on sections are showed (A–I). (A) *Mitf* is expressed in the retinal-pigmented epithelium (rpe) of developing embryos and (B) iris of adult mice (arrowheads). *Rx*, *Six3* and *Six6* (C, F and H) are expressed in the embryonic neural retina (nr). (F) *Six3* expression is also detected in the rpe (black arrowheads). (D and G) *Rx* and *Six3* are expressed in the CB of adult mice. (I) *Six6* expression was not detected in the CB of adult mice. (E) Non-radioactive in situ hybridization showing expression of *Rx* in the CB. Scale bar: 50 μm.

observed at e14.5 in the neocortex (data not shown). The summary of these observations is depicted in Fig. 2G.

We cultured pigmented sphere colonies (PSC) from the ocular CB of adult mice as described previously (Tropepe et al., 2000). Rare pigmented colonies (30–60 clones/eye), which were initially adhesive, developed from single cells in culture conditions that promoted neural stem cells proliferation (Fig. 3A). These colonies were already visible after 48 h in culture (data not shown). Growing colonies detached from the uncoated plastic dishes after 4 days in culture, and formed large pigmented floating spheres (neurospheres) containing about 10000–15000 cells after 7–9 days (data not shown) (Tropepe et al., 2000). In clonal