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Loss of retinal progenitor cells leads to an increase in the retinal stem cell population *in vivo*

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Abstract

Retinal stem cells [with the potential to produce either neural retinal progenitors or retinal pigment epithelial (RPE) progenitors] exist in the mammalian eye throughout life, and indeed the greatest absolute increase in the stem population occurs postnatally. The stem cells proliferate embryonically and thus may help to build the retina initially, but in postnatal mammals they clearly do not proliferate to regenerate the retina in response to injury. Using *Chx10*$^{orJ/orJ}$ and *Mitf*$^{mi/mi}$ mice, with small eye phenotypes due to the reduction of the neural retinal progenitor population and the retinal pigmented epithelial progenitor population, respectively, we now report that the retinal stem cell population, when assayed from the ciliary margin, increases 3–8-fold in both mutants. These findings suggest that the mammalian retinal stem cell population may be capable of responding to genetically induced signals from the progenitor populations.

Introduction

Although new neurons are added to the retinas of fish and amphibian eyes in response to injury throughout life (Hollyfield, 1971; Johns, 1977; Wetts *et al*., 1989), no such additions occur in adult mammals. However, a source of retinal stem cells (RSCs) was identified in the adult mammalian eye ciliary margin (Ahmad *et al*., 2000; Tropepe *et al*., 2000). Recent evidence shows that in various species, some cells in the ciliary margin are able to proliferate (Kubota *et al*., 2002) and are capable of responding to a genetic injury *in vivo* (Moshiri & Reh, 2004). We now find that RSCs within the pigmented ciliary epithelium proliferate in mice with inherited defects that impair the normal development of retinal pigmented epithelium (RPE) progenitor or neural retina progenitor populations.

The homeobox gene *Chx10* is expressed in the developing optic cup in neural retina progenitor cells (Liu *et al*., 1994). *Mitf* is expressed throughout the optic vesicle in mouse prior to lens induction (Bora *et al*., 1999; Nguyen & Arnheiter, 2000). Upon lens induction, *Mitf* is extinguished in the presumptive neural retina, and concurrently, *Chx10* expression is activated in this region (Liu *et al*., 1994; Bora *et al*., 1999). Thus, *Chx10* and *Mitf* become expressed in complementary domains that identify neural retina progenitors and RPE progenitors, respectively. The *Chx10*$^{orJ/orJ}$mouse has a small eye phenotype (Truslove, 1962), characterized by a reduction in proliferation of the neural retina progenitors, a loss of mature bipolar cells, and a severely hypocellular neural retina in adulthood (Burmeister *et al*., 1996; Bone-Larson *et al*., 2000; Rowan *et al*., 2004; Horsford *et al*., 2005) (Fig. 1A, B, E and F). *Chx10* has also been implicated in G1-phase cell cycle regulation, and *Chx10* mutations may cause cells to lengthen their cell cycle time (Green *et al*., 2003; Horsford *et al*., 2005). The *Mitf*$^{mi/mi}$ mouse is characterized by a small eye phenotype (Fig. 1C and D), due to abnormalities and partial loss of the RPE progenitor population. *Mitf* is required for the normal proliferation and differentiation of the RPE layer (Yasumoto *et al*., 1998; Nguyen & Arnheiter, 2000). The *Mitf*$^{mi/mi}$ mutation causes complete loss of the differentiated RPE population, with the dorsal RPE transdifferentiating to form a double neural retina that expresses *Chx10* (Bumsted & Barnstable, 2000; Nguyen *et al*., 2000). Given the expanded ciliary margin in adult animals (Fig. 1E and F; Tropepe *et al*., 2000) and recent evidence that *Chx10* and *Mitf* may function together to regulate retinal cell identity (Rowan *et al*., 2004; Horsford *et al*., 2005), we examined whether *Chx10* and *Mitf* also regulate the stem cell population in the ciliary margin. We hypothesized that these enlarged ciliary margins may result from a noncell autonomous expansion of the stem cell population in each mutant, in response to the loss of the stem cell progeny (neural retina progenitors or RPE progenitors where the mutations act cell autonomously). A clonal, colony forming assay showed that the stem cell population of the pigmented ciliary epithelium expands in each of these two small eye progenitor mutant phenotypes.

Materials and methods

Animals

The University of Toronto and Hospital for Sick Children Research Institute, Animal Care Committees approved these experiments. All mice were purchased from the Jackson Laboratories (Bar Harbor, ME). *Chx10*$^{orJ/+}$ 129/SvJ background, or *Mitf*$^{mi/+}$ (B6C3Fe background) heterozygous mutants were bred to obtain wild-type and homozygous individuals for each mutant. Females with a vaginal plug from an overnight breeding pair were estimated to have embryos at E0.5. Animals were killed using cervical dislocation. *Chx10*$^{orJ/orJ}$

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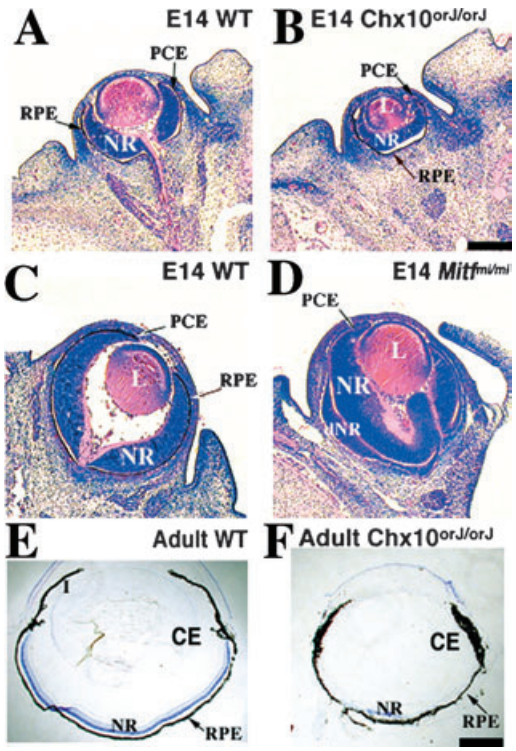

FIG. 1. Eye morphology. H & E staining of embryonic eyes (A–D) and Nissl staining of adult eyes (E and F). (A) E14 wild-type (*129/SvJ*); (B) E14 *Chx10*$^{orJ/orJ}$; (C) E14 wild-type (*B6C3Fe*); (D) E14 *Mitf*$^{mi/mi}$; (E) adult wild-type (*129/SvJ*) and (f) adult *Chx10*$^{orJ/orJ}$. NR, neural retinal layer; dNR, double neural retina; L, lens; PCE, presumptive ciliary epithelium; I, iris; CE, ciliary epithelium overlying the ciliary body. Scale bar, 0.1 mm (A, B, C and D); 0.5 mm (E and F).

mice were genotyped as previously described (Burmeister *et al.*, 1996). Heterozygous *Mitf*$^{mi/+}$ mice were genotyped by heteroduplex analysis from a PCR (forward: GGTGTGCCTCAGTCACTAATG, reverse: CTGGATCATTTGACTTGGGG). The forward intronic primer and was identified from a library screen of a 129 mouse genomic library (gift from J. Rommens, Hospital for Sick Children, Toronto, ON) using the mouse Mitf cDNA probe (gift from H. Arnheiter, National Institute of Health, Bethesda, MD).

Clonal sphere assay

RSCs were derived from the peripheral retinal pigmented epithelium (RPE) and presumptive ciliary margin from the eyes of mice at embryonic day 14 and postnatal day 10, and from the ciliary epithelium of adult mice. Postnatal mice were killed by cervical dislocation, and their eyes were harvested in oxygenated artificial cerebral spinal fluid (containing 124 nM NaCl, 5 nM KCl, 1.3 nM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose). The neural retina was first dissected free of the RPE. The ciliary margin area was then dissected from the rest of the eye. The ciliary margin area was treated with dispase (Collaborative Research, VWR, Mississauga ON) for 10 min at 37 °C and then moved to an enzyme solution at 37 °C for another 10 min [Trypsin 1.33 mg/mL, Hyaluronidase 0.67 mg/mL, and Kynurenic acid 0.2 mg/mL (Sigma, Burlington, ON)] to facilitate the removal of the ciliary epithelium from the underlying basement membrane. Cells were then centrifuged at 150 × *g* for 5 min, the enzyme solution removed and replaced with serum free media containing 1 mg/mL trypsin inhibitor (Roche, Laval, QC). Cells were triturated until a single cell suspension was achieved and then centrifuged again and the supernatant removed and replaced with the appropriate culturing media (see below). The clonal spheres that formed were counted after 7 days in culture. All of the cells were cultured at 10 cells per μL, as previous experiments have shown that all the spheres that form at 20 cells per μL or less are clonally derived from single cells (Tropepe *et al.*, 2000).

E14 embryonic mice (determined by plug date = 0.5 days) were extracted from the dam after cervical dislocation and placed in Dulbecco's phosphate buffered saline. The eyes were removed from the embryos and placed in dispase for 1 min and then rinsed in Dulbecco's phosphate buffered saline. Under a dissecting microscope (Carl Zeiss, Germany), the extra-ocular tissue was first removed using fine forceps and then the retinal epithelial layer was dissected away from the neural retina. A glass pipette was then used to transfer the peripheral RPE tissue to a tube containing serum-free media and 0.1% Trypsin-EDTA (Sigma), and the neural retina was placed in another tube containing serum-free media. All of the tissue was mechanically dissociated into a single cell suspension. The cells were then spun down in a centrifuge for 5 min, the supernatant removed and resuspended in serum-free media containing FGF2 and heparin (Sigma), and cultured. Dissociated embryonic and postnatal cells were plated in serum-free media with exogenous growth factors used at a final concentration of 10 ng/mL FGF2 (human recombinant; Sigma) and 2 μg/mL heparin (Sigma). Embryonic peripheral RPE and ciliary margin were plated at a density of one eye/well, given the very small number of cells isolated from these dissections. The postnatal ciliary margin was plated 10 cells/μL. All the tissue from each retina was plated, to allow an estimate of the number of sphere forming cells per eye.

Incorporation of BrdU in vitro

Cells from the adult *Chx10*$^{orJ/orJ}$and wild-type mouse ciliary epithelium were plated as above. On day three *in vitro*, the cells were pulse labelled with BrdU (0.6 nM) for 2 h, and the cells then rinsed and replated in serum-free media + FGF2 + heparin and allowed to grow for an additional 5 days. The clonal spheres that arose were fixed in 4% paraformaldehyde for 15 min, and then transferred to a cryoprotectant 30% sucrose solution overnight. The spheres were embedded in Tissue Tek and sectioned at 14 μm using a Bright Cryostat. The sphere sections were processed for BrdU immunohistochemistry as previously described (Craig *et al.*, 1996). The sections were exposed to 1 M HCl for 30 min at 65 °C to denature cellular DNA. Rat anti-BrdU (1 : 500; AbCam, Cambridge, UK) and anti-pan histone (1 : 500; Chemicon USA), followed by FITC donkey anti-rat (1 : 400; Jackson Immunoresearch, Westgrove PA), and TRITC goat anti-mouse IgG; Jackson Immunoresearch) were used for BrdU staining and pan-histone nuclear staining. The percentage of cells that were BrdU-positive was determined for each genotype by counting the number of BrdU-labelled and histone-labelled cells in each sphere section.

Self-renewal assay

Single retinal stem cell sphere colonies were dissociated by exposing for 90 min in an artificial CSF enzymatic solution (see dissection procedure). After 90 min in the enzyme solution sphere colonies were transferred to microcentrifuge tubes and centrifuged at 15 000 ***g***. for 10 s. Neural retina sphere colonies were exposed to 1% Trypsin-EDTA in Dulbecco's phosphate buffered saline (without $MgCl_2$ or

$CaCl_2$) for 10 min at 37 °C, and spun down at 10 000 r.p.m. for 10 s. Subsequently, the enzyme solution was replaced in both preparations with serum-free media containing trypsin inhibitor (1 mg/mL). Sphere colonies were mechanically triturated with fire-polished Pasteur pipettes and centrifuged once more at 15 000 ***g***. for 10 s. The solution was replaced with culture media and plated in 96-well plates. The number of stem cells is probably underestimated by this method because only 30% of the cells generated from this dissociation survive the procedure. The spheres were passaged every 7 days to test self-renewal capacity of the retinal stem and progenitor cells.

Differentiation

To assay the differentiation potential of the RSCs, individual clonally derived spheres were plated in 24-well plates on a substrate of 50 ng/mL laminin (Sigma) in the presence of 1% FBS (Invitrogen Canada), 10 ng/mL FGF2 and 2 μg/mL heparin. The media was changed every 3–4 days and the cells were allowed to migrate and differentiate over the course of 3 weeks at which time the plates were fixed in 4% PFA. Immunohistochemical analysis of the cells was tested using antibodies to retinal ganglion cells (Brn3b, Santa Cruz, CA), photoreceptors (Rho4D2, R. Molday, University of British Columbia, Vancouver, BC, and Rho1D4, AbCam, Cambridge, UK), bipolar cells (PKCα, Amersham, and Chx10, R McInnes, Hospital for Sick Children, Toronto, ON), amacrine and retinal progenitor cells (Pax6, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and Muller glia (10E4, C. Barnstable, Oxford University, New Haven, CT). The secondary antibodies all were purchased from Jackson Immunologicals (TRITC goat anti-mouse IgG and FITC goat anti-rabbit) and Invitrogen (Alexa488 goat anti-rabbit and Alexa568 goat anti-mouse IgG. The staining was visualized using either a Nikon inverted fluorescent microscope or an Olympus IX80 inverted fluorescent microscope and imaged using Olympus Micro-suite Imaging Software.

Results

Expansion of retinal stem cells

To assay the *in vivo* retinal stem cell population, the *in vitro* clonal sphere assay was utilized, whereby a single stem cell proliferates *in vitro* to form a clonal, floating sphere of cells. Thus the number of spheres reflects the endogenous stem cell number (Tropepe *et al*., 2000; Morshead & van der Kooy, 2001). We tested the $Chx10^{orJ/orJ}$ mutant at embryonic day 14, postnatal day 10, and as an adult, and the $Mitf^{mi/mi}$ mutant at embryonic day 14 and postnatal day 10 as these mice die at weaning due to malformations of bone structure (Packer, 1967).

To determine whether RSCs were present in increased numbers in the embryos of $Chx10^{orJ/orJ}$ and the $Mitf^{mi/mi}$ mice, we quantified the numbers of stem cells at E14 using the sphere-forming assay. The embryonic eye appears morphologically similar in wild-type and $Chx10^{orJ/orJ}$ animals, except the mutant eye was notably smaller than wild-type controls at E14 (Burmeister *et al*., 1996) (Fig. 1C and D). However, the cultured E14 $Chx10^{orJ/orJ}$ presumptive pigmented ciliary epithelial cells still gave rise to a two-fold increase in the number of primary clonal spheres compared to wild-type controls ($t_{29} = 4.7$; $P < 0.05$; Fig. 2A). Similarly, by E14, the $Mitf^{mi/mi}$ mutation produced a three-fold increase in the total number of pigmented ciliary epithelial spheres derived *in vitro* in comparison with their wild-type littermate controls ($t_{16} = 3.2$; $P < 0.05$; Fig. 2B).

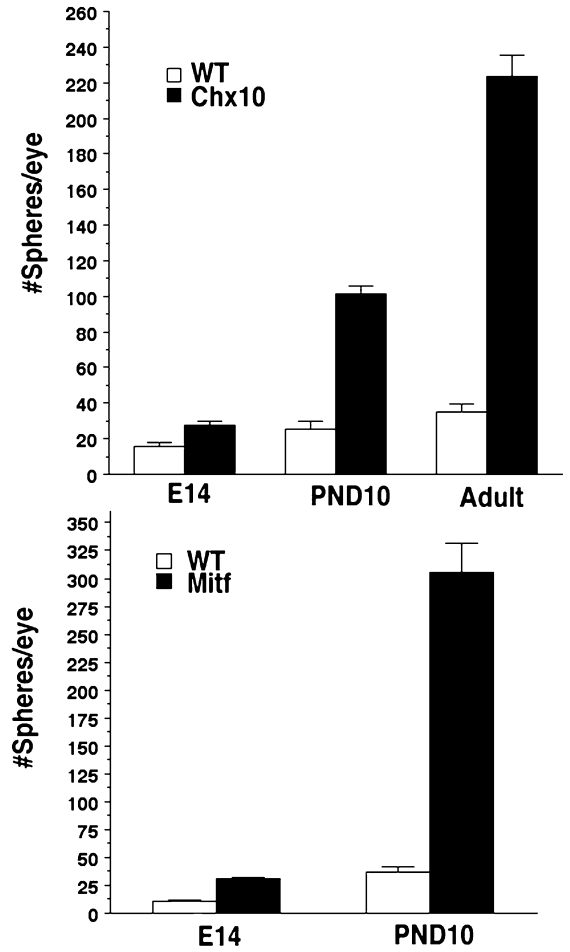

FIG. 2. Primary retinal stem cell sphere formation. (A) The E14 $Chx10^{orJ/orJ}$ mutant mouse ($n = 18$) has a two-fold increase in the number of stem cell spheres isolated compared to its wild-type control eye ($n = 14$). There is a four-fold increase in the number of sphere forming cells isolated from the ciliary margin of the postnatal day 10 $Chx10^{orJ/orj}$ compared to wild-type (wild-type $n = 8$, $Chx10^{orJ/orj}$ $n = 15$). There is a six-fold increase in the number of stem cell spheres in the mutant compared to its wild-type adult control ($n = 10$). Error bars = standard error mean. (B) Sphere forming cells can be isolated from the E14 presumptive ciliary margin of the $Mitf^{mi/mi}$ mouse. There is a three-fold increase in the number of retinal stem cell spheres isolated from the $Mitf^{mi/mi}$ mutant mouse eye ($n = 12$) compared to its wild-type control ($n = 7$). There is an eight-fold increase in the number of sphere forming cells isolated from the ciliary margin of the postnatal day 10 $Mitf^{mi/mi}$ mouse eye compared to E14.

These findings demonstrate that in both mutants, the RSCs have undergone more self-renewing divisions *in vivo*, to generate larger retinal stem cell populations.

To ask whether the clonal pigmented ciliary epithelial spheres retained the capacity for self-renewal, single pigmented ciliary epithelial spheres from both mutants were dissociated into single cells and replated every seven days. The single spheres, from both the wild-type and $Mitf^{mi/mi}$ mice, were able to demonstrate self-renewal, such that a range of one to six new pigmented spheres arose from each single pigmented ciliary epithelial sphere that was dissociated [only a few cells within each sphere are RSCs with the ability to form new spheres, with the majority of the cells within the sphere being progenitor cells with less proliferation capacity (Tropepe *et al*., 2000)]. The clonal spheres contain cells that have pigment, but these cells are still either retinal pigment epithelial progenitor cells (due to the fact that they can still divide and are not immuno-stained with

differentiated RPE markers) or stem cells (because secondary spheres always arise clonally from pigmented cells; Tropepe *et al*., 2000). Single pigmented ciliary epithelial spheres from both wild-type and $Chx10^{orJ/orJ}$ mice could also be passaged, but never gave more than three new spheres per each wild-type or $Chx10^{orJ/orJ}$ sphere that was dissociated. The wild-type, $Chx10^{orJ/orJ}$, and $Mitf^{mi/mi}$ pigmented ciliary epithelial spheres could be passaged at least eight times, indicating that the stem cells were able to self-renew.

In view of the fact that retinal spheres are composed primarily of progenitor cells, any significant difference in the size of the sphere may indicate a proliferation deficit that may be attributed to an intrinsic factor within the progenitor or stem cell population. Consistent with the reduced ability of neural retina progenitors to proliferate in $Chx10^{orJ/orJ}$ mice (Burmeister *et al*., 1996), the majority of pigmented ciliary epithelial stem cell spheres from the $Chx10^{orJ/orJ}$ mutant were significantly smaller (Tropepe *et al*., 2000) in diameter (1.7 times smaller) compared to their wild-type controls (as illustrated in Fig. 3A and B). To ask if the stem and progenitors cells from the $Chx10^{orJ/orJ}$ mutant have a slower cell cycle time compared to wild-type, the spheres were supplemented and kept in culture for seven more days to determine whether the $Chx10^{orJ/orJ}$ spheres would grow. The diameter of the spheres did not increase significantly with the additional culture times. However, when we added BrdU *in vitro* at day 3, the percentage of BrdU labelled cells was significantly lower in the $Chx10^{orj/orj}$ mutant spheres compared to wild-type spheres (average wild-type $= 86.7 \pm 5.5$, $n = 10$, $Chx10^{orJ/orJ} = 54.0 \pm 4.4$, $n = 19$; $t_{(27)} = 4.5$; $P < 0.001$). These data indicate that slower cell cycle may prevent the $Chx10^{orJ/orJ}$ spheres from proliferating as much as the wild-type sphere cells, but we can not exclude the possibility that cell death also may contribute to the small $Chx10^{orJ/orJ}$ sphere diameters. The cell diameter is similar in both genotypes (average wild-type $= 10.2$ μm ± 0.4; $Chx10^{orJ/orJ} = 9.9 \pm 0.6$), and therefore the difference in sphere diameters translates into approximately three times fewer cells in the *Chx10* mutant clonal spheres when the three-dimensional structure is taken into account. However, $Mitf^{mi/mi}$ pigmented ciliary epithelial spheres are not significantly different in diameter (average size $= 260.0$ μm ± 14.2, $n = 20$) from their wild-type controls (276.8 μm ± 28.4, $n = 20$). The lack of effect of the $Mitf^{mi/mi}$ mutation on sphere size does not exclude the possibility that the $Mitf^{mi/mi}$ mutation may be working on the RPE progenitor cells, as the loss of this smaller number of RPE progenitors cells (compared to the large majority of neural retina progenitors) may not be reflected in the overall sphere size.

To determine when the retinal stem cell population increases in number during the development of the eye, mice were analysed at postnatal day 10, using the clonal spheres to assay the stem cell population *in vivo* at this time. At postnatal day 10, the primary number of pigmented ciliary epithelial spheres isolated *in vitro* from the presumptive pigmented ciliary epithelium of $Mitf^{mi/mi}$ mice rose to eight times greater than that of the wild-type controls (average: wild-type $= 37.0 \pm 0.5.5$, $n = 8$; $Mitf^{mi/mi} = 305 \pm 26.5$, $n = 24$; $t_{30} = 12.6$; $P < 0.05$) (Fig. 2B), suggesting that most of the expansion of pigmented ciliary epithelial stem cells in the $Mitf^{mi/mi}$ mutant occurred in the late embryonic or early postnatal period. Similarly, the $Chx10^{orJ/orJ}$ adult pigmented ciliary epithelium produced six times more RSCs than wild-type adult controls (wild-type $= 34.9 \pm 4.6$, $n = 10$; $Chx10^{orJ/orJ} = 223.8 \pm 11.4$, $n = 15$; $t_{23} = 12.9$; $P < 0.05$; Fig. 2A). This result also suggests that even after E14, the $Chx10^{orJ/orJ}$ stem cells are still undergoing symmetric divisions. To test whether the delay in the development of the $Chx10^{orJ/orJ}$ eye delays the onset of retinal stem cell expansion, we analysed the postnatal day 10 $Chx10^{orJ/orJ}$ mutant animals as well, and found a four-fold increase in the number of pigmented ciliary epithelial spheres (wild-type $= 25.6 \pm 3.8$, $n = 12$; $Chx10^{orJ/orJ} = 101.3 \pm 4.1$, $n = 12$; $t_{22} = 12.6$, $P < 0.05$) (Fig. 2A). The fold difference in the number of retinal stem cell spheres increased from a two-fold increase at E14 to a four-fold increase at P10 and a six-fold increase in the adult. Thus, $Chx10^{orJ/orJ}$ RSCs appear to continue to respond during postnatal development, to the reduction of their neural retinal progenitor population by undergoing an increasing number of symmetrical divisions compared to controls (Fig. 2A).

When differentiated on an adhesive substrate, both the wild-type (Fig. 4A, D, G and J) and $Mitf^{mi/mi}$ (Fig. 4C, F, I and L) pigmented ciliary epithelial spheres give rise to neural (including photoreceptor) and glial lineages, demonstrating the other cardinal principle of a stem cell: multipotentiality. The $Mitf^{mi/mi}$ is able to form all of the cell types of the neural retina *in vivo* so it is not unexpected that all the same cell types can be differentiated from clonal $Mitf^{mi/mi}$ spheres *in vitro*. The spheres derived from $Chx10^{orJ/orJ}$ pigmented ciliary epithelial stem cell spheres were able to differentiate on an adhesive substrate. Both the wild-type and $Chx10^{orJ/orJ}$ pigmented ciliary epithelial spheres gave rise to Muller glia, neural cell types (retinal ganglion cells, amacrine and photoreceptors), but only the wild-type pigmented ciliary epithelial spheres were able to produce bipolar cells (Fig. 4B, E, H and K; PKCα: wild-type $= 2.1\% \pm 0.8$, $n = 5$; $Chx10^{orJ/orJ} = 0\% \pm 0$, $n = 7$; $t_{(9)} = 2.9$; $P < 0.05$; Table 1). The absence of retinal bipolar neurons differentiation from $Chx10^{orJ/orJ}$ stem cell progeny *in vitro* corresponds to the total loss of the mature bipolar neuron population *in vivo* in the $Chx10^{orJ/orJ}$ mouse retina (Burmeister *et al*., 1996).

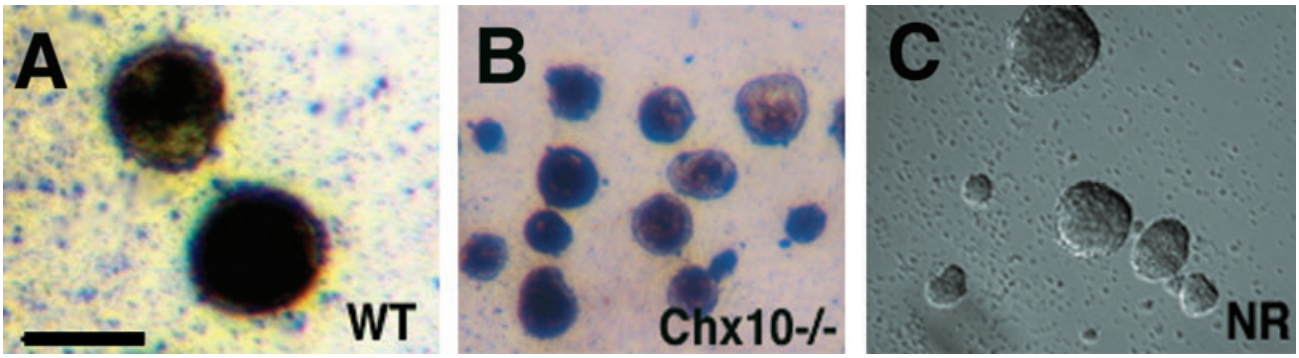

FIG. 3. Spheres. (A) Wild-type retinal stem cell spheres plated at clonal density of 10 cells/μL. The spheres in A, B and C have been moved to the centres of the fields for photographic purposes. Scale bar, 200 μm (A, B and C). (B) $Chx10^{orJ/orJ}$ spheres plated at clonal density of 10 cells/μL. (C) Neural retinal spheres from a wild-type mouse.

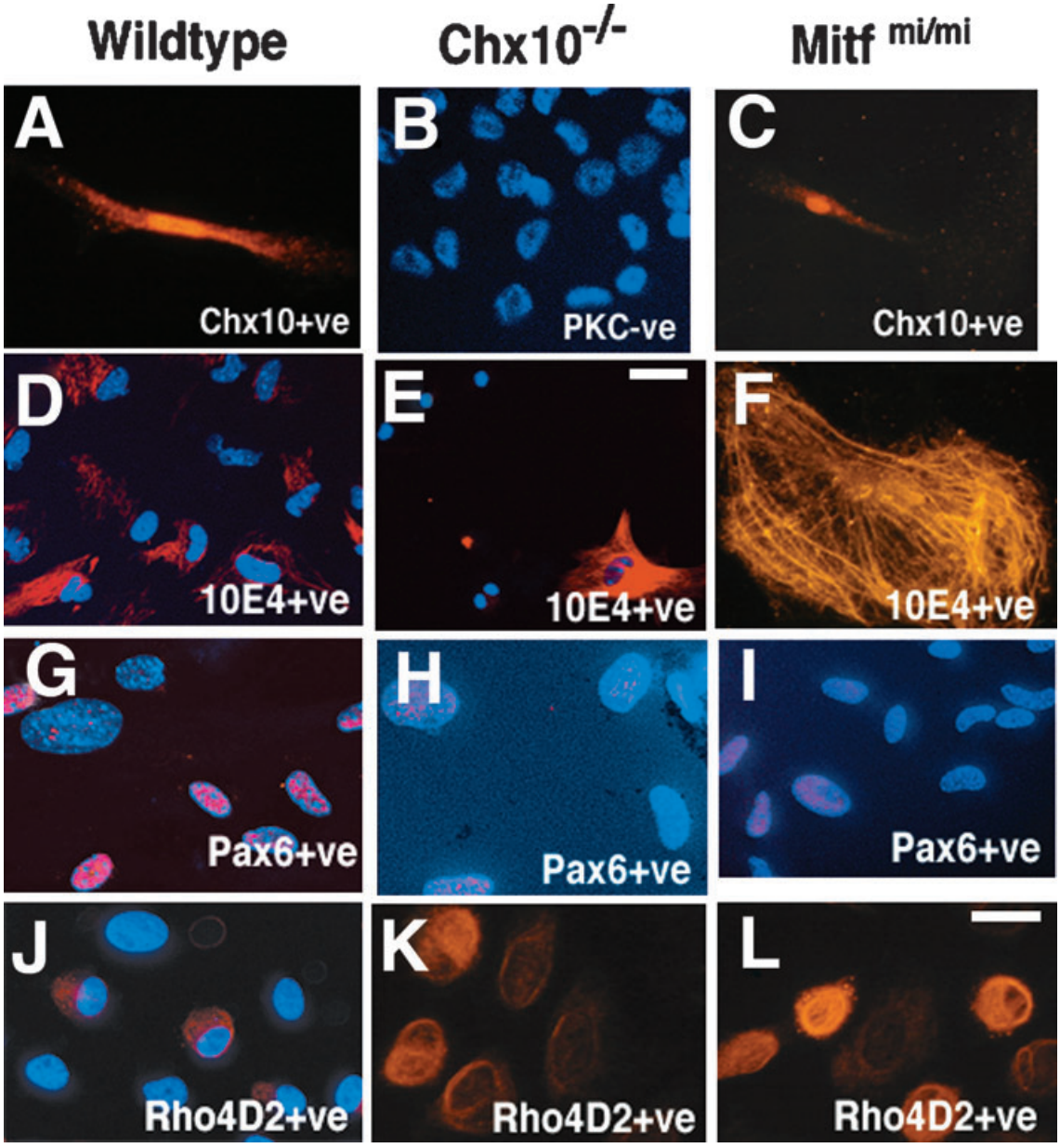

FIG. 4. Differentiation. Differentiation of wild-type (A, D G and J), *Chx10*$^{orJ/orJ}$ (B, E, H and K) and *Mitf*$^{mi/m}$ (C, F, I and L) retinal stem cell spheres. (A and C) Bipolar cells. Wild-type and *Mitf*$^{mi/m}$ stained with Chx10 antibody. (B) *Chx10*$^{orJ/orJ}$ stained with PKC antibody, no staining observed. (D–F) Muller glial cells. Wild-type and the mutants stained with 10E4. (G–I) Undifferentiated or Amacrine cells in the wild-type and mutants stained with Pax6. (J–L) Photoreceptor cells in the wild-type and mutants stained with Rho4D2 antibody. Hoechst staining of the cell nucleus is represented by the blue staining in panels B, D, E and G–J). The antibody staining are all represented in red. Scale bar 50 μm (C and E); 20 μm (A, B, D and F–J).

Limited self-renewal of progenitor cells in the mutants

To establish whether the proliferating spheres that arose from the embryonic neural retina of the *Chx10*$^{orJ/orJ}$ and the *Mitf*$^{mi/mi}$ mutants were progenitor cell derived, or originated from stem cells that had migrated or mislocalized in the neural retina, the spheres from each mutant were tested for their self-renewal ability. We previously demonstrated that progenitor cells in the embryonic neural retina can proliferate to form nonpigmented clonal spheres, but these neural retina derived spheres have limited self-renewal ability (Tropepe *et al*., 2000), never passage *in vitro,* and are not found in the adult neural retina *in vivo* (Tropepe *et al*., 2000). We dissociated the neural retina from the E14 wild-type, *Mitf*$^{mi/mi}$ and *Chx10*$^{orJ/orJ}$ mice to examine sphere formation and continuous long-term passagability of neural retinal precursors. Unexpectedly, we found that the numbers of E14 neural retinal progenitors spheres were greatly reduced in the *129/SvJ* wild-type background strain of the *Chx10*$^{orJ/orJ}$ mutation compared to the *B6C3Fe* wild-type background of the *Mitf*$^{mi/mi}$ mutation (Fig. 5A). Importantly, this difference contrasts with the similarity in the numbers of ciliary margin derived retinal stem cell spheres at the same age (E14) in the same two background strains (see wild-type numbers in Fig. 2A). These background strain differences in the ability to form neural retina progenitor cell spheres vs. pigmented ciliary epithelial stem cell spheres (derived from the presumptive ciliary margin) identifies an important dissociation of background genetic influence on the two different types of retinal precursor cells. These

TABLE 1. Differentiation of $Chx10^{orJ/orJ}$ retinal stem cell spheres

Cell type	Antibody used	Control positive (%)	$Chx10^{orJ/orJ}$ positive (%)
Bipolar cell	PKCα	2.0 ± 0.8	0 ± 0*
Retinal ganglion cell	Brn3b	4.5 ± 0.9	3.3 ± 1.4
Muller glia	10E4	14.6 ± 2.2	13.8 ± 6.4
Photoreceptor	Rho4D2/Rho1D4	11.8 ± 7.1	4.7 ± 1.7
RPC and amacrine	Pax6	31.7 ± 14.6	36.8 ± 4.9

Data are presented as percentages ±SEM. *Chx10*$^{orJ/orJ}$ RSC spheres are multipotential for neural retinal differentiation, but are unable to differentiate into bipolar neurons. Plated spheres were immunostained with markers for cell types of the neural and undifferentiated cells. The percent positive columns indicate the average numbers of positive cells in each well compared to the number of nuclei stained with Hoechst. Rho4D2 and Rho1D4 both mark rhodopsin in photoreceptor cells so they were combined together in the table. Pax6 marks retinal progenitor cells and differentiated amacrine cells. * indicates that *Chx10*$^{orJ/orJ}$ is significantly different from the control ($P < 0.05$).

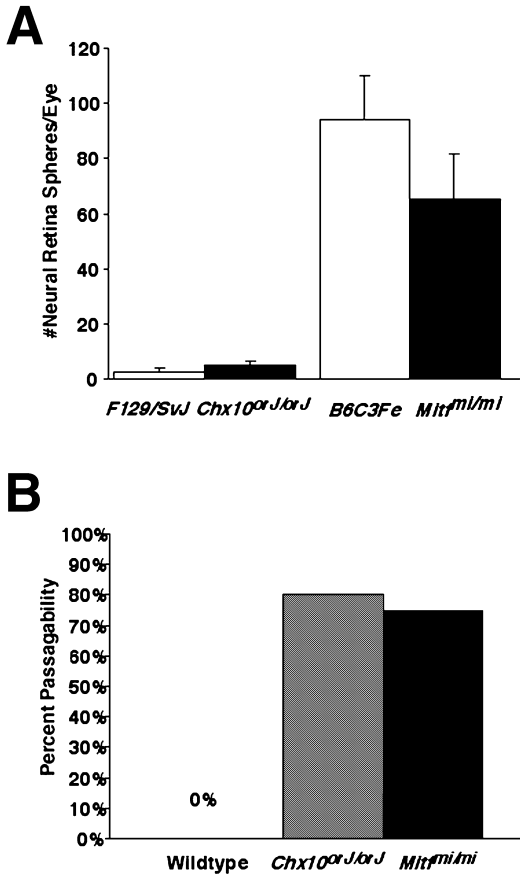

FIG. 5. Neural retinal progenitor spheres. (A) Sphere formation from primary E14 neural retina of both the *Chx10*$^{orJ/orJ}$ and *Mitf*$^{mi/mi}$ mutants demonstrate that the embryonic neural retinas are capable of forming clonal spheres in both of the mutant neural retinas compared to their appropriate wild-type controls, in spite of the much larger numbers of neural retina progenitors spheres in the *129SvJ* wild-type strain (*Chx10* background) than in the *B6C3Fe* wild-type (*Mitf* background). (B) Single primary E14 neural retina spheres never passage from wild-type control animals of either background genotype, but 75–80% of the *Chx10*$^{orJ/orJ}$ and *Mitf*$^{mi/mi}$ neural retinal spheres are capable of forming at least one secondary sphere.

findings highlight the power of the *in vitro* sphere assays to study the behaviour of retinal stem cells vs. progenitor cells. Regardless of the background strain differences in neural retinal progenitor cells, neither the *Chx10* nor *Mitf* mutations produced any significant changes in the numbers of E14 primary neural retinal progenitor spheres compared to their appropriate wild-type controls (Fig. 5A).

In contrast, both the *Chx10*$^{orJ/orJ}$ and *Mitf*$^{mi/mi}$ neural retinal primary progenitor spheres demonstrated an increased ability to passage, compared to their appropriate wild-type controls. Whereas wild-type E14 neural retina progenitor spheres, of either background strain, were incapable of self-renewal, 75–80% of the individually passaged spheres from each type of mutant animal formed secondary spheres (Fig. 5B). Some of the *Mitf*$^{mi/mi}$ and *Chx10*$^{orJ/orJ}$ E14 neural retinal progenitor spheres could be passaged as single spheres to a maximum of four times. However, with each subsequent passage between passages two and four, the percentages of clonal spheres that formed new spheres diminished until none of the spheres self-renewed at all after passage four. These results indicate that the proliferating progenitor cells from the E14 *Mitf*$^{mi/mi}$ and *Chx10*$^{orJ/orJ}$ neural retinas have a moderately expanded capacity for self-renewal. Given that stem cells last the lifetime of the animal, we also tested the remnant of the neural retina in the adult *Chx10*$^{orJ/orJ}$ mutant for its ability to form spheres, but no spheres could be isolated *in vitro*, further strengthening the hypothesis that the embryonic neural retina contains only progenitor cells with an increased, but limited, self-renewal capacity in the *Chx10*$^{orJ/orJ}$ mice.

Discussion

Our studies of *Chx10*$^{orJ/orJ}$ and *Mitf*$^{mi/mi}$ mutant mice demonstrate that loss of either the neural retina or RPE progenitor populations, respectively, results in an increase in the resident stem cell population *in vivo*. Strikingly, although the mutant retinas overall are small, one rare population (pigmented ciliary epithelial stem cells) is significantly increased. These data suggest that the number of RSCs has increased in these mutants during development, possibly in response to the loss of their progeny (neural retina or RPE progenitors). Ultimately, the increased numbers of stem cells leads only to an abortive proliferative progenitor response, possibly reflecting the mutant genotypes studied. This is the first report of an endogenous, proliferative regeneration response *in vivo* by pigmented ciliary epithelial stem cells, and offers encouragement for studies aimed at stimulating endogenous precursor cells to regenerate retinal tissue.

We propose that the increases in pigmented ciliary epithelial stem cell numbers in both mutants reflect a putative noncell autonomous feedback signal on the stem cell population from the neural retinal progenitor cells (which are the only cells in which *Chx10* is expressed) and the RPE progenitor cells (which are the only cells in which *Mitf* is expressed) (Fig. 6). The loss of either specific progenitor cell type may decrease a signal that negatively regulates stem cell proliferation, and consequently increases the RSC population. Our suggestion that the *Chx10*$^{orJ/orJ}$ and *Mitf*$^{mi/mi}$ effects are noncell autonomous effects on the stem cells (and are not due to a loss of expression of the genes in the RSCs themselves) is based on the primary expression of *Chx10* in neural retinal progenitors (Liu *et al.*, 1994; Rowan *et al.*, 2004; Horsford *et al.*, 2005) and of *Mitf* in RPE progenitors (Bora *et al.*, 1999; Nguyen *et al.*, 2000) and on the observation that the *Chx10* and the *Mitf* expressing cell populations in the E14 retina are nonoverlapping (Nguyen *et al.*, 2000). However, RSCs are a very rare population [1 in 500 cells within the pigmented ciliary epithelium of the adult retina (Tropepe *et al.*, 2000)], and it remains to be seen whether there are low levels of expression of either gene in the RSCs themselves and therefore, it remains possible that a cell-autonomous mechanism could contribute to the present results. In our model, the

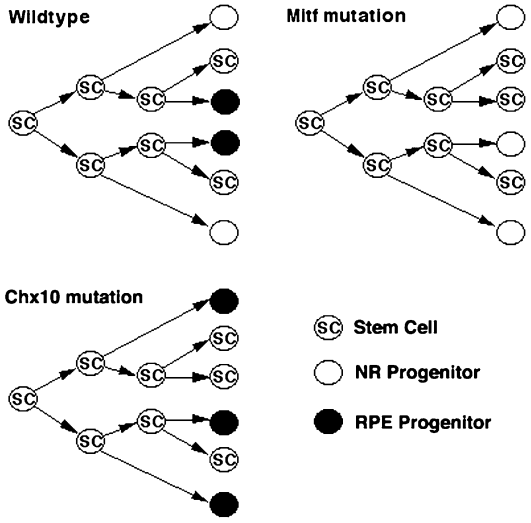

FIG. 6. Model of retinal stem cell expansion. Wild-type: stem-cell expansion during eye development demonstrating that the stem cell self-renews, both by symmetric division to produce two stem cells and by asymmetric division to form the neural retinal and the retinal pigmented epithelium (RPE) progenitor lineages. *Chx10*$^{orJ/orJ}$ mutation: The mutation results in a loss of cells of the neural retina progenitor population. In response, the stem cell population undergoes more symmetric divisions, resulting in an expansion of the stem cell population, which then produces more RPE progenitors by asymmetrical divisions. Although no neural retina progenitors are shown in the model, there are some neural retinal progenitors produced but many fail to proliferate. *Mitf*$^{mi/mi}$ mutation: the mutation prevents the RPE progenitors from expanding their lineage. In response, the stem cell again undergoes more symmetric divisions, resulting in an expansion in the stem cell population, which then produces more neural retina progenitors by asymmetrical divisions.

loss of *Chx10* or *Mitf* function directly affects neural retina or RPE progenitors, respectively, and leads through a putative feedback signalling mechanism (either a diminished negative regulator or increased expression of a positive regulator of retinal stem cells) to increase the number of stem cells. An alternative model of retinal development, that could also account for our findings, suggests that all embryonic retinal precursor cells are bipotential, and capable of giving rise to either an RPE progenitor cell (expressing *Mitf*) or a neural retinal progenitor cell (expressing *Chx10*) (Zhao *et al*., 1995; Nguyen *et al*., 2000; Shatos *et al*., 2001; Rowan *et al*., 2004; Horsford *et al*., 2005). Indeed it has been shown that in a *Chx10:CRE* transgenic; *Chx10*$^{orJ/orJ}$ compound mutant, that *Chx10* positive neural retinal cells can transdifferentiate to lightly pigmented RPE and lightly pigmented ciliary epithelial cells. However, the absence of heavily pigmented *Chx10* lineage cells in the *Chx10* mutant (Rowan *et al*., 2004) may indicate that the new stem cells reported in the present paper do not arise from transdifferentiated neural retina progenitors, but may arise nonautonomously from symmetric divisions of the stem cells, which invariably are heavily pigmented ciliary epithelial cells. In order to elucidate the mechanism more clearly, studies that monitor the stem cells through development will need to be performed. Also, it would be interesting to create double mutants where we would predict even more RSCs, however, there appears to be modifier genes on the different background strains of the two mutants that rescue the small-eye phenotype seen in the single mutants (D. J. Horsford, unpublished results). We suggest that the stem cell model would appear to explain better the several fold increase in RSCs demonstrated here in the presence of the dramatic small eye phenotypes of the mutant retinas.

In addition to the increased numbers of the surviving retinal progenitor cell types (RPE and neural retina progenitors; Fig. 6), there is a secondary progenitor cell effect in the two mutants. In wild-type mice, until postnatal day 4, there is a limited ability of some neural retinal cells to clonally produce progenitor spheres in culture that have a limited self-renewal ability. However, in the two small eye mutants, this timeline of clonal neural retina progenitor sphere proliferation is maintained until at least postnatal day 10. Sonic hedgehog (Shh) is a mitogenic factor that regulates retinal progenitor populations (Jensen & Wallace, 1997). Furthermore, mutant mice with a knockout of one allele of the Shh receptor (*patched+/–*; Moshiri *et al*., 2004) show an increase in the number of BrdU+ve cells in the peripheral neural retina and the nonpigmented ciliary epithelium, as well as a persistence of these proliferative progenitor cells until at least postnatal day 10. These proliferating progenitor cells may be the same population of proliferating neural retinal progenitor cells that we find persisting to postnatal day 10 in the *Chx10* and *Mitf* mutant mice. We suggest that these findings may result from a Shh or other signal in both of the mutant retinas that acts directly on retinal progenitors to extend their proliferative potentials. Because the BrdU+ve ciliary epithelial cells in the *patched* mutant are not found amongst the heavily pigmented cells, the sustained proliferative population in these mutant mice may include progenitor cells but not the stem cell population. Nevertheless, these neural retinal progenitor cells are not converted to stem cells in either the *Chx10* or the *Mitf* mutants, as they fail to manifest long-term self-renewal, a cardinal property of stem cells.

We hypothesize that the predominant mode of stem cell division during retinal development is asymmetric division, to give rise to either a stem cell and a neural retinal progenitor, or a stem cell and an RPE progenitor (Fig. 6). However, it must be noted that the population of specific progenitor cells affected by the mutations are not completely lost in either mutant mouse, only severely diminished. Therefore, at least some cells are still able to form both progenitor lineages, but at a much lower frequency compared to wild-type mice. According to this model, the loss of either the neural retina or the RPE populations feeds back (via a cell autonomous or more likely a nonautonomous signal) to the pigmented ciliary epithelial stem cell population to bias its RSCs to undergo more symmetrical divisions in which both of the progeny are stem cells. With more stem cells in each developing mutant eye, there are then more stem cells to divide asymmetrically and produce either more RPE progenitor cells or more neural retina progenitor cells.

Abbreviations

RPE, retinal pigmented epithelium, RSC, retinal stem cell.

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