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The Proliferation and Expansion of Retinal Stem Cells Require Functional *Pax6*

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Abstract

Retinal stem cells (RSCs) exist as rare pigmented ciliary epithelial cells in adult mammalian eyes. We hypothesized that RSCs are at the top of the retinal cell lineage. Thus, genes expressed early in embryonic development to establish the retinal field in forebrain neuroectoderm may play important roles in RSCs. *Pax6*, a paired domain and homeodomain-containing transcription factor, is one of the earliest genes expressed in the eye field and is considered a master control gene for retinal and eye development. Here, we demonstrate that *Pax6* is enriched in RSCs. Inactivation of *Pax6* *in vivo* results in loss of competent RSCs as assayed by the failure to form clonal RSC spheres from the optic vesicles of conventional *Pax6* knockout embryos and from the ciliary epithelial cells of adult *Pax6* conditional knockout mice. *In vitro* clonal inactivation of *Pax6* in adult RSCs results in a serious proliferation defect, suggesting that *Pax6* is required for the proliferation and expansion of RSCs.

Keywords

retinal stem cell; ciliary epithelia; Pax6

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Introduction

Mammalian adult retinal stem cells (RSCs) have been isolated from the pigmented ciliary epithelia (PCE) of mouse (Tropepe et al., 2000) and human eyes (Coles et al., 2004). Although quiescent *in vivo*, RSCs persist in the PCE and proliferate to form clonal sphere colonies *in vitro* in the absence of exogenous growth factors, exhibiting the cardinal stem cell properties of self renewal and multipotentiality. However, little is known about the molecular mechanisms underlying the generation, proliferation and differentiation of these RSCs.

In fish and amphibians, many retinal precursor cells are located in the ciliary margin zone (CMZ) and continuously add new neurons and Muller glial cells to the retina in adult animals (Johns, 1977; Wetts and Fraser, 1988). Cells in CMZ are spatially ordered with respect to the development, with stem cells being most peripheral and differentiating retinal progenitor cells (RPCs) more central in the CMZ. Correspondingly, only the early genes in retinal development, e.g. *Xrx1*, *Pax6*, *XSix3*, are expressed in the most peripheral stem cell compartment. Genes expressed later in the development in RPCs are expressed more centrally in the CMZ (Perron et al., 1998). The genes, expressed early to establish the eye field, including *ET*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *tll* and *Six6*, have been shown to be critical to retinal development (Zuber et al., 2003). Mutations in these genes result in malformation of the retina and eye or no eyes (Andreazzoli et al., 1999; Hanson et al., 1994; Hill et al., 1991; Jordan et al., 1992; Lagutin et al., 2003; Mathers et al., 1997; Porter et al., 1997; Quiring et al., 1994; Yu et al., 2000). Misexpression of these genes can induce ectopic retinal or eye tissues (Andreazzoli et al., 1999; Bernier et al., 2000; Chow et al., 1999; Halder et al., 1995; Loosli et al., 1999; Mathers et al., 1997; Oliver et al., 1996; Zuber et al., 2003). All of these studies suggest that the early expressed eye field genes may have important functions in the regulation of retinal stem cells. However, the roles of these genes in RSCs have not been fully studied, especially in mammalian RSCs.

To study the molecular mechanisms underlying the generation, proliferation and differentiation of mammalian RSCs, we hypothesized that RSCs are at the top of the retinal cell lineage, and that genes expressed early in embryonic development to establish the retinal field may play important roles in RSCs. *Pax6*, a paired domain and homeodomain containing transcription factor, is one of the earliest genes expressed in the eye field. It is considered a master control gene for retinal and eye development (Gehring, 1996; Ton et al., 1991; Walther and Gruss, 1991). Misexpression of *Pax6* induces ectopic eye structures in *Drosophila* and *Xenopus* (Chow et al., 1999; Halder et al., 1995). Conversely, loss of function of *Pax6* results in aniridia and Peter's anomaly in humans (Hanson et al., 1994; Jordan et al., 1992), small eye phenotype in mouse (Hill et al., 1991) and eyeless in *Drosophila* (Quiring et al., 1994). In *Pax6* homozygous mutant mice, retinal development arrests at an early primitive optic vesicle stage (Grindley et al., 1995; Hill et al., 1991). *Pax6* mutant optic vesicles have reduced proliferation coupled with precocious adoption of a generic neuronal fate, rather than a specific retinal neuron fate (Philips et al., 2005), suggesting that *Pax6* regulates the timing of neurogenesis and retinal specific neuron differentiation in the developing retina. In a conditional knockout mouse model, inactivation of *Pax6* in the retina restricted retinal progenitor cell (RPC) differentiation entirely to amacrine cells (Marquardt et al., 2001), suggesting that *Pax6* is required for the multipotent state of RPCs. These reports have provided evidence for the importance of *Pax6* in retinal development. However, the role(s) of *Pax6* in RSCs, especially in the adult RSCs, have not been directly illustrated. We employed both the conventional and conditional *Pax6* knockout mouse models (Marquardt et al., 2001; St-Onge et al., 1997) to present direct evidence that *Pax6* is required for the proliferation and expansion of RSCs.

Experimental Procedures

Animals

The Z/EG reporter mice (Novak et al., 2000) were provided by Drs. Andras Nagy and Corrine Lobe. The *Pax6^{LacZ/LacZ}* (St-Onge et al., 1996; St-Onge et al., 1997) and α -Cre;*Pax6^{loxP/loxP}* mice (Marquardt, 2003; Marquardt et al., 2001) kindly were provided by Dr. Peter Gruss.

Immunohistochemistry

Pax6, Syntaxin (HPC-1), Rhodopsin (1D4) and nestin antibodies were purchased from Developmental Hybridoma Bank, Sigma and Chemicon respectively. Immunohistochemistry was performed as previously described (Tropepe et al., 2000).

RNA preparation and RT-PCR

Total RNA was isolated using RNeasy kit (Qiagen). RT-PCR was performed using OneStep RT-PCR kit (Qiagen). Primer sequences: *Pax6*: Fw1:5'-TCACAGCGGAGTGAATCAGC-3' and Rev1:5'-TATCGTTGGTACAGACCCCCTC-3'; Fw2:5'-CGGAGTGAATCAGCTTGGT-G-3' and Rev2:5'-GTTGGTACAGACCCCCTCGG-3'. GAPDH: Fw:5'-TGCACCACCAACTGCTTAGC-3' and Rev: 5'-TGGATGCAGGGATGATGTTTC-3'. 18s rRNA: Fw:5'-GTAACCCGTTGAACCCATT-3'; Rev: 5'-CCATCCAATCGGTAGTAGCG-3'. 50 ng of total RNA or 1/4 of the RNA from a single sphere was used in the first round *Pax6* amplification with Fw1/Rev1. The reaction program was 50 °C for 30 minute for RT, 95 °C for 15 minutes to inactivate RT, followed by 25 cycles of 95°C for 30 seconds, 60 °C for 1 minute, and 72 °C for 1 minute and then 72 °C for 10 minutes. 2 μ l of 1:10 dilutant of the first round amplification product was used in *Pax6* nested PCR with primers Fw2/Rev2. The primers encompass the alternatively spliced exon5a (Glaser et al., 1992; Ton et al., 1991; Walther and Gruss, 1991). The amplicons are 367 base pairs (bp) (without exon5a) and 409 bp (with exon5a).

Embryonic day (E)14 and adult RSC primary culture

The dissection and primary culture were performed as described previously (Tropepe et al., 2000). Briefly, for adult RSC isolation: adult mice were sacrificed by cervical dislocation. Eyes were removed and placed in oxygenated artificial cerebral spinal fluid (aCSF: 124mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 2mM CaCl₂, 26 mM NaHCO₃, and 10 mM D-glucose, pH 7.4). Each eye was bisected and the ciliary body was isolated as a thin strips and placed in dispase (Invitrogen) for 10 minutes at 37°C, followed by trypsin/hyaluronidase treatment [1.33 mg/mL trypsin (Sigma), 0.67 mg/mL hyaluronidase (Sigma) and 0.2 mg/mL kynurenic acid (Sigma) in aCSF modified with high Mg²⁺ (3.2 mM MgCl₂) and low Ca²⁺ (0.1 mM CaCl₂)] at 37°C for 15 minutes. Ciliary epithelial cells were scraped off from the strips and collected in serum free medium (SFM) with trypsin inhibitor (1 mg/mL Roche Diagnostics). The cells were triturated 60 times to a single cell suspension. Cells were resuspended in SFM with FGF2 (10 ng/mL) and heparin (2 μ g/mL), plated at a low clonal density (10 cells/ μ L) and incubated at 37°C in a CO₂ (5%) incubator (Thermo Electron Co) for 7 days.

For E14 embryos: the eyes were carefully removed and placed in dispase (Invitrogen) at 37°C for 30 seconds. RPE and neural retina (NR) were separated, collected in SFM and triturated about 60 times to achieve a single cell suspension. The cells were spun down and resuspended in SFM with FGF2 (10 ng/mL) and heparin (2 μ g/mL). The NR cells were plated at 10 cells/ μ L. All RPE cells from each animal were plated in 4 wells of a 24-well plate and the total numbers of RSCs were assessed as the total number of RSC spheres formed. In *Pax6* homozygous knockout (*Pax6^{LacZ/LacZ}*) embryos, retinal development was aborted at the primitive optic vesicle stage, which consisted of a thin epithelial sheet, without the thickening

of the inner neuroretinal layers and the differentiation of the RPE layer. To evaluate the RSC status in these *Pax6*^{LacZ/LacZ} embryos, each entire primitive optic vesicle carefully was dissected, dissociated into single cells, and plated in 4 wells of a 24-well plate. Cells were incubated at 37°C in a CO₂ (5%) incubator for 7 days.

Three and four independent experiments were performed at the adult and E14 time points, respectively.

Fluorescent Activated Cell Sorting (FACS)

The ciliary epithelial cells of adult *Pax6*^{LacZ/wt} or wild type littermates were isolated and dissociated into single cells in 200µL of SFM. The cells were separated into two aliquots; one was treated with 100µM of a fluorescent β-gal substrate: 5'-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG) (Invitrogen), the other was used as negative control. Both aliquots were incubated at 37 °C for 25'. The cells were spun down and resuspended in 500µl of SFM+FGF2 (10 ng/ml)+ heparin (2 mg/ml). Propidium iodide (PI) (1.5µM) was added to assay for live/dead cells. Total live cells were sorted by the green fluorescence from breakdown product of CMFDG, plated at clonal density (<10 cells/µl) and incubated at 37 °C for 1 week.

Retroviral infection

Bi-cistronic pMXIE-Cre-EGFP virus was constructed by inserting a nlsCre cassette in front of the IRES-EGFP in a replication-incompetent retroviral construct pMXIE-EGFP (Hitoshi et al., 2002). Ciliary epithelial cells from adult *Pax6*^{flox/flox} or *Pax6*^{wt/wt} animals were isolated and dissociated to single cells and plated in fibronectin-coated 24-well plates at 10 cells/ul. Retrovirus (pMXIE-Cre-EGFP or pMXIE-EGFP) was added 18-24 hours after plating (m.o.i.=10). The infected cells (GFP+) were counted under the fluorescent microscope (Olympus) every 24 hours. For differentiation, differentiation medium (SFM+FGF2+heparin +1% Fetal Bovine Serum (FBS)) was added 24 hours after viral infection and was changed every 4 days. After 21 days, cells were fixed with 4% paraformaldehyde. Immunostaining with the amacrine cell marker, HPC-1, or the rod photoreceptor marker, 1D4, was performed.

Results

Pax6 is expressed in the adult retinal stem cell niche- the ciliary epithelia

To study the role of *Pax6* in RSCs, we first asked how *Pax6* is expressed in the adult RSC niche – the pigmented ciliary epithelia. It has been shown that *Pax6* is expressed in the ciliary epithelia and the epithelia of the iris (Marquardt et al., 2001). By immunohistochemistry and confocal microscopy, *Pax6* was detected in both layers of the ciliary epithelia (Fig. 1A). However, *Pax6* is expressed at a much higher level in the outer layer (arrows in Fig. 1A), where pigmented RSCs are found (Tropepe et al., 2000), than in the inner layer (arrowheads in Fig. 1A). *Pax6* expression was further confirmed by RT-PCR on RNA isolated from ciliary epithelia and clonally derived RSC spheres from adult mice (Fig. 1B). As controls, *Pax6* expression also was assessed and detected in adult neural retina (NR), forebrain neural stem cell (NSC) spheres (Reynolds and Weiss, 1996), embryonic stem (ES) cell-derived NSC spheres (ESDS) (Tropepe et al., 2000; Tropepe et al., 2001) and ES cells. The primers in the RT-PCR assays encompass the alternatively spliced exon5a (Glaser et al., 1992; Ton et al., 1991; Walther and Gruss, 1991). Although both forms of *Pax6* transcripts, with or without exon5a (+5a or -5a), were expressed, the ratios of -5a transcript to +5a transcript were different in different tissues (Fig 1C). As in the neural retina, the long transcript with exon5a (+5a) was predominant in RSCs and in the ciliary epithelium (Fig 1 B & C), while the short transcript (-5a) was predominant in NSC spheres. The ESDS and undifferentiated ES cells expressed almost exclusively the short form. The alternatively spliced exon5a encodes a 14 amino acid insertion in the paired

domain, which changes the binding specificity of the paired domain (Epstein et al., 1994; Glaser et al., 1992) and may contribute to the different functions of Pax6 in different tissues (Azuma et al., 2005; Dominguez et al., 2004).

Selection for Pax6 enriches for retinal stem cells

To further investigate if Pax6 is expressed in RSCs and its role as a possible intrinsic regulating factor for RSCs, we employed the knockout/knockin mouse model (St-Onge et al., 1997), in which the *Pax6* gene, from the start codon through the entire paired domain, was replaced by β -galactosidase (β -gal) gene. Therefore, the β -gal expression is under the control of the endogenous *Pax6* promoters and can be used as a marker for *Pax6* expression. In the heterozygous *Pax6*^{LacZ/wt} ciliary epithelial cells, the wild type allele provides the *Pax6* function, while the mutant allele with the β -gal gene provides a marker for *Pax6* expression. Taking advantage of this, the ciliary epithelial cells from adult *Pax6*^{LacZ/wt} mice were treated with a fluorescent β -gal substrate CMFDG and were sorted into three non-overlapping portions by the intensity of their fluorescence: R1, R2 and R3 (Fig.2 A&B). Subsequent clonal RSC sphere assays showed that cells with low or no *Pax6* expression (R1 and R2) did not give rise to any RSC spheres (Fig.2 A&B and table I). Only the cells with high *Pax6* expression (R3) gave rise to clonal RSC spheres at a frequency of 1/76, at least a 6.6-fold enrichment of RSCs, compared to the 1/500 frequency seen in non-sorted ciliary epithelial cells (Tropepe et al., 2000). This result suggests that high levels of *Pax6* expression are seen in RSCs and that selection for *Pax6* enriches for RSCs. The power of enrichment of RSCs by high levels of *Pax6* expression might be underestimated here, because the heterozygous *Pax6*^{LacZ/wt} cells used in this experiment have only one functional *Pax6* allele. We have observed a 56% reduction of sphere formation in the adult *Pax6*^{LacZ/wt} ciliary epithelial cells (fig. 2C) and a similar reduction in E14 embryonic heterozygous RSCs (Fig. 2D), compared to the wild type. Therefore, we predict a higher RSC enrichment in the wild type cells based on their *Pax6* expression.

Inactivation of Pax6 function in vivo results in loss of competent retinal stem cells at embryonic day 14 (E14)

To study the role of *Pax6* in the early development of RSCs, we performed clonal RSC sphere assays on embryonic day (E) 14 *Pax6*^{LacZ} mice (Fig.2D). At this stage in wild type mice, RSCs reside in the peripheral retinal pigmented epithelium (RPE), which includes the presumptive pigmented ciliary epithelia where the adult RSCs reside (Tropepe et al., 2000). In *Pax6*^{LacZ/LacZ} embryos, retinal development arrests at the early optic vesicle stage (Grindley et al., 1995; Hill et al., 1991). To evaluate the total RSC population of these embryos, we harvested the entire rudimentary optic vesicles from *Pax6*^{LacZ/LacZ} embryos and performed clonal RSC sphere assays. No RSC spheres were derived from *Pax6*^{LacZ/LacZ} optic vesicle cells. In *Pax6*^{LacZ/wt} heterozygote, we found a 58% reduction in the number of RSC spheres compared to the wild type. These results strongly suggest that loss of *Pax6* function led to a complete loss of competent RSCs in *Pax6* knockout embryos and that a decreased level of *Pax6* in the heterozygote resulted in a reduction in RSC population. Thus, *Pax6* is required for the generation of clonal RSC spheres.

The RSC spheres are composed by a few stem cells, but the majority of the cells in the spheres are progenitor cells. The failure to form RSC spheres could be a result of defects in either the stem cells or the progenitor cells or both. At E14, the neural retina contains RPCs, which can proliferate and form primary PRC spheres, which, however, cannot be clonally passaged because of their limited self-renewal ability (Tropepe et al., 2000). A clonal RPC sphere assay on the heterozygote and wild type controls showed a slight, but not significant decrease in the numbers of RPC from the heterozygote when compared to the wild type [3.6 \pm 0.7, (n=12) in the heterozygote versus 4.9 \pm 1.0 (n=9) per 5,000 cells in the wild type, $p=0.27$ (Kruskal-

Wallis test)]. This suggests that the effect of inactivation of *Pax6* on clonal RSC sphere formation can not be explained fully by its effect on RPCs and that inactivation of *Pax6* may have a direct effect on the proliferation of RSCs.

Pax6 is required for RSC sphere formation in adult animals

The *Pax6*^{LacZ/LacZ} conventional knockout mice die soon after birth. Therefore, to directly study the role of *Pax6* in RSCs in adult mice, we employed the α -Cre; *Pax6*^{loxP/loxP} conditional knockout mouse model (Marquardt et al., 2001), in which the endogenous *Pax6* gene was replaced by a modified *Pax6* allele, *Pax6*^{loxP}, in which exons 4-6 are flanked by two loxP sites. The expression of Cre recombinase is under the control of the *Pax6* retinal specific regulatory element, α promoter (Kammandel et al., 1999), which targets the expression of *Pax6* to the peripheral neuroretina, ciliary epithelia and the iris. A RSC sphere assay on the adult conditional knockout mice (Fig.3A) revealed that there were 23% ($p < 0.05$) and 72% ($p < 0.05$) decreases in the numbers of clonal RSC spheres derived from the heterozygote and conditional knockout, respectively, compared to wild type. This suggests that inactivation of *Pax6* results in a negative effect on the competence of RSCs to form RSC spheres and possibly the maintenance of RSCs in adult animals.

It has been shown that the α -promoter activity has a dorsal expressional gap at E13.5 (Kammandel et al., 1999; Marquardt et al., 2001). If this expression gap persists in the adult mice, Cre recombinase in the α -Cre transgenic animals would fail to be expressed in the gap region, and hence, *Pax6* function wouldn't be inactivated in the gap. Therefore, we hypothesized that the spheres from the α -Cre; *Pax6*^{loxP/loxP} mice might have been derived from the RSCs that escaped Cre excision. To confirm the α -promoter controlled expression in adult mice, we generated α -Cre; Z/EG double transgenic mice. The Z/EG line (Novak et al., 2000) is a Cre reporter line: Cre excision removes the lacZ gene and activates the expression of the second reporter, enhanced green fluorescent protein (EGFP). The α -Cre; Z/EG double transgenic mice showed that the α promoter not only has the dorsal expression gap, but also a ventral gap in the adult mice that extended into the adult peripheral retina and ciliary epithelia (Fig. 3B). To further confirm our hypothesis that the residual spheres from the α -Cre; *Pax6*^{loxP/loxP} mice were derived from the RSCs that have escaped Cre excision, we performed RT-PCR on the RNA samples from these spheres. Indeed, all of the spheres derived from the α -Cre; *Pax6*^{loxP/loxP} mice were actually *Pax6* positive (figure 3C). Therefore, no RSC spheres were derived from true *Pax6* knockout ciliary epithelial cells in adult mice, suggesting that *Pax6* is required for clonal RSC sphere formation.

Inactivation of Pax6 inhibits the proliferation of retinal stem cells

To study the mechanism(s) of *Pax6* on the maintenance of RSCs, we infected *Pax6*^{loxP/loxP} and wild type adult ciliary epithelial cells *in vitro* with a bi-cistronic Cre-EGFP retrovirus (pMXIE-Cre-EGFP) (Fig.4A), which expresses both Cre recombinase and EGFP in the infected cells. Since, only RSCs in the adult ciliary epithelial cells divide and only the dividing cells can be infected with retrovirus, we could use EGFP expression to trace the infected clonal RSCs in which *Pax6* function is inactivated by the Cre recombinase. Four days after viral infection, the infected cells still retained a retinal precursor phenotype (Fig. 4B). However, the average number of progeny in each GFP positive RSC clone derived from a single RSC was reduced by 65% in Cre infected *Pax6*^{loxP/loxP} clones compared to the Cre infected wild type (*Pax6*^{wt/wt}) RSC clones (Fig.4C). 91% of the Cre virus-infected *Pax6*^{loxP/loxP} RSC clones had only one or two cells/clone (Fig.4D). Only one of these *Pax6*^{-/-} clones had 4 GFP-positive cells, and none of the clones contained more than 4 GFP positive cells. In contrast, 43% of the wild type infected clones had more than 4 progeny, including 2 clones (~7.6%) with 10 or more GFP positive cells. We did not observe decreases in RSC clone size when using the control retrovirus (pMXIE-EGFP) in *Pax6*^{loxP/loxP} or *Pax6*^{wt/wt} RSCs (4.8 +/- 0.6 and 4.1 +/- 0.5 cells/

clone, respectively). The Cre-EGFP virus-infected RSCs survived as well as the control virus (pMXIE-EGFP) infected cells. Indeed, we observed more clones in the pMXIE-Cre-GFP infected $Pax6^{loxP/loxP}$ cells than in the wild type cells (total numbers of clones observed: in wild type cells: n=35 with the Cre-EGFP virus and n=65 with the EGFP virus; in $Pax6^{loxP/loxP}$ cells: n=55 with the Cre-EGFP virus and n=26 with the EGFP virus). Therefore, the smaller number of cells per clone in pMXIE-Cre-EGFP infected Pax6 mutant RSCs cannot be due to more cell death of RSCs. The best explanation for this result is that inactivation of *Pax6* inhibits the proliferation of RSCs.

Previously, Marquardt et al showed that Pax6 inactivation results in the exclusive generation of amacrine cells in the retina (Marquardt et al., 2001). To determine the function of *Pax6* on fate determination in adult RSCs and their progeny, we differentiated retrovirus-infected RSC colonies on laminin coated slides. The results showed that inactivation of *Pax6* by retroviral expression of Cre-EGFP in $Pax6^{loxP/loxP}$ RSCs resulted in differentiation almost exclusively into amacrine cells using syntaxin as a marker (97.2 +/- 2.8% versus 12.8 +/- 1.1% in wild type infected RSC colonies) (Table 2 and figure 4E), consistent with the previous observation by Marquardt et al in α -Cre;*Pax6*^{loxP/loxP} animals in vivo. In contrast, only 0.95 +/- 0.9% of infected cells are rhodopsin (1D4) positive photoreceptors amongst the $Pax6^{loxP/loxP}$ cells compared to 21.8% +/- 1.7% in wild type infected RSC colonies. Our data suggest that inactivation of *Pax6* produces two effects: inhibiting the proliferation of RSCs, and separately, directing the differentiation of RSC progeny to an amacrine cell fate.

Discussion

Mammalian adult RSCs exist as rare pigmented ciliary epithelial cells. They are quiescent in vivo, but they persist through their adult lives, maintaining their capability to self renew and differentiate into mature retinal cell types (Coles et al., 2004; Tropepe et al., 2000). Previously, we demonstrated that the numbers of retinal progenitors negatively regulate the numbers of RSCs by a cell non-autonomous mechanism in vivo (Coles et al., 2006; Tropepe et al., 2000). Here, we reveal directly that *Pax6* is required for the proliferation and thus the expansion of RSCs. In vivo, this serious proliferation defect in $Pax6^{-/-}$ RSCs would preclude the expansion of the first RSCs by symmetrical divisions in the embryonic optic cup, as well as inhibit the expansion of the retinal progenitor populations through asymmetric proliferation of RSCs.

Our result showed that *Pax6* is highly expressed in the retinal stem cell niche- the pigmented ciliary epithelia. Previously, *Pax6* has been shown to be expressed in the ciliary epithelia (Das et al., 2005; Marquardt et al., 2001). By immunostaining and confocal microscopy, the present report reveals that Pax6 expression is not equal in the two layers of the ciliary epithelia (Fig 1). Pax6 is expressed at a much higher level in the outer layer than the inner layer of the ciliary epithelia. The inner layer of the ciliary epithelia is nonpigmented and is continuous with the neural retina, the outer layer is pigmented and continuous with the RPE. It is the outer pigmented layer where the RSCs reside (Tropepe et al., 2000). Therefore, the results suggest that high level Pax6 expression co-exists with RSCs in the outer pigmented ciliary epithelia. Our FACS sorting and subsequent clonal sphere assay further showed that only the cells with the highest levels of Pax6 expression can give rise to RSC spheres, suggesting that Pax6 is highly enriched in RSCs and that high levels of Pax6 expression are hallmarks of the retinal stem cells.

Clonal RSC sphere assays on both the embryonic retina of the conventional knockout mice and adult retina of the conditional knockout mice revealed that *Pax6* expression is not only a hallmark of the retinal stem cells, but also that *Pax6* is required for their stemness or competence to form RSC spheres (Fig 2D & 3A). In *Pax6* homozygous knockout ($Pax6^{LacZ/LacZ}$) embryos, retinal development is arrested at a primitive optic vesicle stage. Although some RPE markers

are expressed in these primitive optic vesicles, no fully differentiated RPE is developed in the *Pax6* knockout embryo (Grindley et al., 1996; Baumer et al., 2003; Philips et al., 2005). However, the mere absence of the pigment could not be the reason for the loss of RSC competence. Previously, we have shown that similar numbers of RSC spheres can be derived from pigmented and non-pigmented animals (Tropepe et al., 2000).

Furthermore, the experiments employing *in vitro* inactivation of *Pax6* by the Cre-EGFP retroviral infection showed that the underlying mechanism is a requirement for *Pax6* in the proliferation and expansion of retinal stem cells (Fig 4). Our *in vitro* inactivation of *Pax6* experiment supports that the inability of adult retinal stem cells to form spheres is the result of a decrease in their ability to proliferate. Previously, Marquardt et al showed a reduced proliferation of RPCs in the *Pax6* conditional knockout retina at E12.5-18.5 (Marquardt et al., 2001). While we were preparing this manuscript, Philips et al (Philips et al., 2005) also reported reduced proliferation of RPCs in *Pax6*^{-/-} optic vesicle at E10.5. We believe that *Pax6* plays roles in both the proliferation and differentiation of RSCs and RPCs. Our present study is the first report on the function of *Pax6* in retinal stem cells and our results uncovered a novel aspect of *Pax6* function that it plays important roles in the proliferation of retinal stem cells, other than in the retinal progenitor cells.

Our finding that *Pax6* is required for the expansion of RSCs contrasts with the function of *Pax6* in the cortical neural stem cells, where inactivation of *Pax6* results in increased proliferation of neural stem cells and overexpression of *Pax6* in cortical precursors led to decreased proliferation (Estivill-Torrus et al., 2002; Haubst et al., 2004; Heins et al., 2002). However, in neural progenitor cells, *Pax6* appeared to have similar functions as in retinal progenitors (Philips et al., 2005) to promote proliferation by preventing the progenitor cells from exiting the cell cycles and premature differentiation (Quinn et al., 2006). *Pax6* carries multiple functional domains. It is suggested that the selective use of the paired domain (PD and PD5a) and homeodomain of the gene may contribute to the region-specific differences of *Pax6* function (Dominguez et al., 2004; Haubst et al., 2004; Heins et al., 2002). It appears that both the paired domain and the homeodomain are important for the regulation of proliferation and cell fate determination in the retina (Favor et al., 2001; Haubst et al., 2004), while the homeodomain plays no role in these functions in the telencephalon (Haubst et al., 2004). The two different isoforms of paired domains, PD and PD5a, also impose different effects to the overall function of *Pax6* in different tissues. With the insertion of exon 5a, PD5a appears to enhance the proliferation rather than the cell fate specification in both the telencephalon in mouse (Haubst et al., 2004) and the eyes in vertebrates (Azuma et al., 2005; Singh et al., 2002) and in *Drosophila* (Dominguez et al., 2004). The relative predominant expression of the +5a transcript encoding PD5a in the RSCs and retina (Fig 1) may, to some extent, explain the proliferative deficits we observed in the RSCs in the mouse models, because both isoforms (PD & PD5a) of the *Pax6* are inactivated in these mouse models (Marquardt et al., 2001; St-Onge et al., 1997). The cross-interactions between PD/PD5a (Epstein et al., 1994; Epstein et al., 1994), homeodomain (Mishra et al., 2002; Singh et al., 2000) and the C terminus of *Pax6* protein (Singh et al., 2001; Singh et al., 2002) modulate the overall functions of *Pax6* in different tissue at different developmental stages. More detailed studies on these cross-interactions and the target genes of *Pax6* and *Pax6*(+5a) will be critical to the full understanding of the different functions of *Pax6* in the regulation of the proliferation and cell fate determination in different tissues.

In our *in vitro* inactivation experiment, under stem cell culture condition (non-differentiating), the RSCs with *Pax6* inactivated by the Cre recombinase retain retinal precursor phenotype and continue to express nestin. Under differentiating condition for 21 days, however, they differentiated almost exclusively to an amacrine cell phenotype characterized by the expression of amacrine cell specific marker, syntaxin, consistent with the observation by Marquardt et al

(2001) in the conditional knockout retina that inactivation of *Pax6* result in loss of the multipotency of the RPCs in the developing neural retina, biasing the differentiation of RPCs almost exclusively to amacrine cells.

In *Sey/Sey* (*Pax6*^{-/-}) embryos, most of the eye field transcription factors, e.g. *Rx1*, *Six3*, *Six6* and *Lhx2* are expressed in the rudimentary optic vesicle (Jean et al., 1999; Mathers et al., 1997; Oliver et al., 1995; Porter et al., 1997). Other reports show that retinal markers, such as *Tyrp2*, *Mitf* and *CHX10* are expressed (Baumer et al., 2003; Grindley et al., 1995) in *Pax6* mutants, arguing that RSCs and progenitor cells can be formed in the absence of *Pax6*. The present data suggest that even if the first RSCs are generated in the embryonic optic vesicle, the presence of a serious proliferation deficit in *Pax6*^{-/-} RSC will limit the expansion of the RSC pool by decreasing symmetrical divisions and limit the increase of the RPC pool by decreasing the asymmetrical division of RSCs.

The present study reveals that an intrinsic factor, *Pax6* is required for and acts cell autonomously in retinal stem cells. Other early genes involved in eye field specification, e.g. *ET*, *Rx1*, *Six6*, *Six3*, *Lhx2* and *tll* (Hollemann et al., 1998; Jean et al., 1999; Li et al., 1997; Mathers et al., 1997; Oliver et al., 1995; Porter et al., 1997; Zuber et al., 2003) also may play important roles in retinal stem cells. It will be important to study the different roles and interactions of these early genes in the generation, proliferation and differentiation of RSCs. Furthermore, searching for the upstream and downstream targets of these key players regulating RSCs will open new directions in the improved manipulation of these stem cells and their use in the treatment of retinal degeneration.

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References

- Andreazzoli M, Gestri G, Angeloni D, Menna E, Barsacchi G. Role of *Xrx1* in *Xenopus* eye and anterior brain development. *Development* 1999;126:2451–60. [PubMed: 10226004]
- Azuma N, Tadokoro K, Asaka A, Yamada M, Yamaguchi Y, Handa H, Matsushima S, Watanabe T, Kohsaka S, Kida Y, Shiraishi T, Ogura T, Shimamura K, Nakafuku M. The *Pax6* isoform bearing an alternative spliced exon promotes the development of the neural retinal structure. *Hum Mol Genet* 2005;14:735–45. [PubMed: 15677484]
- Baumer N, Marquardt T, Stoykova A, Spieler D, Treichel D, Ashery-Padan R, Gruss P. Retinal pigmented epithelium determination requires the redundant activities of *Pax2* and *Pax6*. *Development* 2003;130:2903–15. [PubMed: 12756174]
- Bernier G, Panitz F, Zhou X, Hollemann T, Gruss P, Pieler T. Expanded retina territory by midbrain transformation upon overexpression of *Six6* (*Optx2*) in *Xenopus* embryos. *Mech Dev* 2000;93:59–69. [PubMed: 10781940]
- Chow RL, Altmann CR, Lang RA, Hemmati-Brivanlou A. *Pax6* induces ectopic eyes in a vertebrate. *Development* 1999;126:4213–22. [PubMed: 10477290]
- Coles BL, Angenieux B, Inoue T, Del Rio-Tsonis K, Spence JR, McInnes RR, Arsenijevic Y, van der Kooy D. Facile isolation and the characterization of human retinal stem cells. *Proc Natl Acad Sci U S A* 2004;101:15772–7. [PubMed: 15505221]
- Coles BL, Horsford DJ, McInnes RR, van der Kooy D. Loss of retinal progenitor cells leads to an increase in the retinal stem cell population in vivo. *Eur J Neurosci* 2006;23:75–82. [PubMed: 16420417]
- Das AV, James J, Rahnenfuhrer J, Thoreson WB, Bhattacharya S, Zhao X, Ahmad I. Retinal properties and potential of the adult mammalian ciliary epithelium stem cells. *Vision Res* 2005;45:1653–66. [PubMed: 15792841]

- Dominguez M, Ferres-Marco D, Gutierrez-Avino FJ, Speicher SA, Beneyto M. Growth and specification of the eye are controlled independently by Eyegone and Eyeless in *Drosophila melanogaster*. *Nat Genet* 2004;36:31–9. [PubMed: 14702038]
- Epstein J, Cai J, Glaser T, Jepeal L, Maas R. Identification of a Pax paired domain recognition sequence and evidence for DNA-dependent conformational changes. *J Biol Chem* 1994;269:8355–61. [PubMed: 8132558]
- Epstein JA, Glaser T, Cai J, Jepeal L, Walton DS, Maas RL. Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing. *Genes Dev* 1994;8:2022–34. [PubMed: 7958875]
- Estivill-Torres G, Pearson H, van Heyningen V, Price DJ, Rashbass P. Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. *Development* 2002;129:455–66. [PubMed: 11807037]
- Favor J, Peters H, Hermann T, Schmahl W, Chatterjee B, Neuhauser-Klaus A, Sandulache R. Molecular characterization of Pax6(2Neu) through Pax6(10Neu): an extension of the Pax6 allelic series and the identification of two possible hypomorph alleles in the mouse *Mus musculus*. *Genetics* 2001;159:1689–700. [PubMed: 11779807]
- Gehring WJ. The master control gene for morphogenesis and evolution of the eye. *Genes Cells* 1996;1:11–5. [PubMed: 9078363]
- Glaser T, Walton DS, Maas RL. Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 gene. *Nat Genet* 1992;2:232–9. [PubMed: 1345175]
- Grindley JC, Davidson DR, Hill RE. The role of Pax-6 in eye and nasal development. *Development* 1995;121:1433–42. [PubMed: 7789273]
- Halder G, Callaerts P, Gehring WJ. Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. *Science* 1995;267:1788–92. [PubMed: 7892602]
- Hanson IM, Fletcher JM, Jordan T, Brown A, Taylor D, Adams RJ, Punnett HH, van Heyningen V. Mutations at the PAX6 locus are found in heterogeneous anterior segment malformations including Peters' anomaly. *Nat Genet* 1994;6:168–73. [PubMed: 8162071]
- Haubst N, Berger J, Radjendirane V, Graw J, Favor J, Saunders GF, Stoykova A, Gotz M. Molecular dissection of Pax6 function: the specific roles of the paired domain and homeobox in brain development. *Development* 2004;131:6131–40. [PubMed: 15548580]
- Heins N, Malatesta P, Ceconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Gotz M. Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci* 2002;5:308–15. [PubMed: 11896398]
- Hill RE, Favor J, Hogan BL, Ton CC, Saunders GF, Hanson IM, Prosser J, Jordan T, Hastie ND, van Heyningen V. Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* 1991;354:522–5. [PubMed: 1684639]
- Hitoshi S, Alexson T, Tropepe V, Donoviel D, Elia AJ, Nye JS, Conlon RA, Mak TW, Bernstein A, van der Kooy D. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev* 2002;16:846–58. [PubMed: 11937492]
- Holleman T, Bellefroid E, Pieler T. The *Xenopus* homologue of the *Drosophila* gene *tailless* has a function in early eye development. *Development* 1998;125:2425–32. [PubMed: 9609825]
- Jean D, Bernier G, Gruss P. Six6 (*Optx2*) is a novel murine Six3-related homeobox gene that demarcates the presumptive pituitary/hypothalamic axis and the ventral optic stalk. *Mech Dev* 1999;84:31–40. [PubMed: 10473118]
- Johns PR. Growth of the adult goldfish eye. III. Source of the new retinal cells. *J Comp Neurol* 1977;176:343–57. [PubMed: 915042]
- Jordan T, Hanson I, Zaletayev D, Hodgson S, Prosser J, Seawright A, Hastie N, van Heyningen V. The human PAX6 gene is mutated in two patients with aniridia. *Nat Genet* 1992;1:328–32. [PubMed: 1302030]
- Kammandel B, Chowdhury K, Stoykova A, Aparicio S, Brenner S, Gruss P. Distinct cis-essential modules direct the time-space pattern of the Pax6 gene activity. *Dev Biol* 1999;205:79–97. [PubMed: 9882499]

- Lagutin OV, Zhu CC, Kobayashi D, Topczewski J, Shimamura K, Puelles L, Russell HR, McKinnon PJ, Solnica-Krezel L, Oliver G. Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. *Genes Dev* 2003;17:368–79. [PubMed: 12569128]
- Li H, Tierney C, Wen L, Wu JY, Rao Y. A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. *Development* 1997;124:603–15. [PubMed: 9043075]
- Loosli F, Winkler S, Wittbrodt J. Six3 overexpression initiates the formation of ectopic retina. *Genes Dev* 1999;13:649–54. [PubMed: 10090721]
- Marquardt T. Transcriptional control of neuronal diversification in the retina. *Prog Retin Eye Res* 2003;22:567–77. [PubMed: 12892642]
- Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P. Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* 2001;105:43–55. [PubMed: 11301001]
- Mathers PH, Grinberg A, Mahon KA, Jamrich M. The Rx homeobox gene is essential for vertebrate eye development. *Nature* 1997;387:603–7. [PubMed: 9177348]
- Mishra R, Gorlov IP, Chao LY, Singh S, Saunders GF. PAX6, paired domain influences sequence recognition by the homeodomain. *J Biol Chem* 2002;277:49488–94. [PubMed: 12388550]
- Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 2000;28:147–55. [PubMed: 11105057]
- Oliver G, Loosli F, Koster R, Wittbrodt J, Gruss P. Ectopic lens induction in fish in response to the murine homeobox gene Six3. *Mech Dev* 1996;60:233–9. [PubMed: 9025075]
- Oliver G, Mailhos A, Wehr R, Copeland NG, Jenkins NA, Gruss P. Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* 1995;121:4045–55. [PubMed: 8575305]
- Perron M, Kanekar S, Vetter ML, Harris WA. The genetic sequence of retinal development in the ciliary margin of the *Xenopus* eye. *Dev Biol* 1998;199:185–200. [PubMed: 9698439]
- Philips GT, Stair CN, Young Lee H, Wroblewski E, Berberoglu MA, Brown NL, Mastick GS. Precocious retinal neurons: Pax6 controls timing of differentiation and determination of cell type. *Dev Biol* 2005;279:308–21. [PubMed: 15733660]
- Porter FD, Drago J, Xu Y, Cheema SS, Wassif C, Huang SP, Lee E, Grinberg A, Massalas JS, Bodine D, Alt F, Westphal H. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development* 1997;124:2935–44. [PubMed: 9247336]
- Quinn JC, Molinek M, Martynoga BS, Zaki PA, Faedo A, Bulfone A, Hevner RF, West JD, Price DJ. Pax6 controls cerebral cortical cell number by regulating exit from the cell cycle and specifies cortical cell identity by a cell autonomous mechanism. *Dev Biol*. 2006
- Quiring R, Walldorf U, Kloter U, Gehring WJ. Homology of the eyeless gene of *Drosophila* to the Small eye gene in mice and Aniridia in humans. *Science* 1994;265:785–9. [PubMed: 7914031]
- Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 1996;175:1–13. [PubMed: 8608856]
- Singh S, Chao LY, Mishra R, Davies J, Saunders GF. Missense mutation at the C-terminus of PAX6 negatively modulates homeodomain function. *Hum Mol Genet* 2001;10:911–8. [PubMed: 11309364]
- Singh S, Mishra R, Arango NA, Deng JM, Behringer RR, Saunders GF. Iris hypoplasia in mice that lack the alternatively spliced Pax6(5a) isoform. *Proc Natl Acad Sci U S A* 2002;99:6812–5. [PubMed: 11983873]
- Singh S, Stellrecht CM, Tang HK, Saunders GF. Modulation of PAX6 homeodomain function by the paired domain. *J Biol Chem* 2000;275:17306–13. [PubMed: 10747901]
- St-Onge L, Furth PA, Gruss P. Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res* 1996;24:3875–7. [PubMed: 8871571]
- St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P. Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* 1997;387:406–9. [PubMed: 9163426]
- Ton CC, Hirvonen H, Miwa H, Weil MM, Monaghan P, Jordan T, van Heyningen V, Hastie ND, Meijers-Heijboer H, Drechsler M, et al. Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell* 1991;67:1059–74. [PubMed: 1684738]

- Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der Kooy D. Retinal stem cells in the adult mammalian eye. *Science* 2000;287:2032–6. [PubMed: 10720333]
- Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 2001;30:65–78. [PubMed: 11343645]
- Walther C, Gruss P. Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* 1991;113:1435–49. [PubMed: 1687460]
- Wetts R, Fraser SE. Multipotent precursors can give rise to all major cell types of the frog retina. *Science* 1988;239:1142–5. [PubMed: 2449732]
- Yu RT, Chiang MY, Tanabe T, Kobayashi M, Yasuda K, Evans RM, Umesono K. The orphan nuclear receptor Tlx regulates Pax2 and is essential for vision. *Proc Natl Acad Sci U S A* 2000;97:2621–5. [PubMed: 10706625]
- Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. Specification of the vertebrate eye by a network of eye field transcription factors. *Development* 2003;130:5155–67. [PubMed: 12944429]

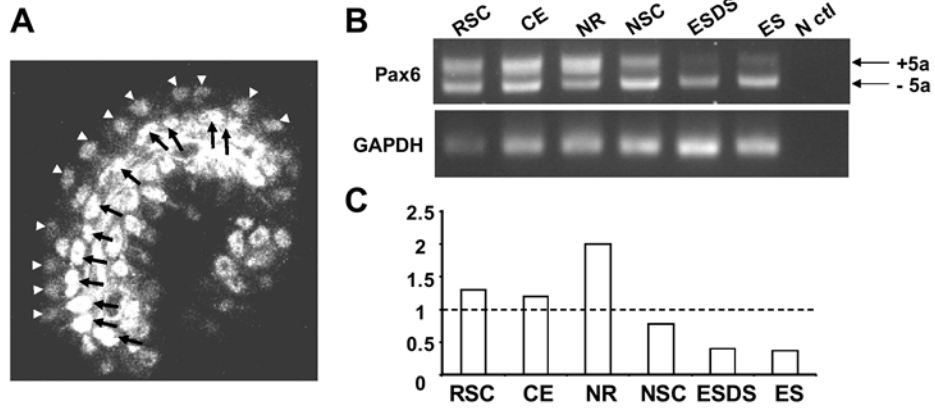


Fig 1. *Pax6* is expressed in the adult ciliary epithelia and RSC spheres

A. confocal image of *Pax6* immunostaining of a ciliary process; Anti-*Pax6* Monoclonal antibody stains the nuclei of the positive cells. White arrowheads show the less intensely stained the ciliary epithelial cells of the inner layer; black arrows indicate the more intensely stained ciliary epithelial cells in the outer layer. The arrowheads and arrows mark the epithelial cells on one side of the ciliary process. B. RT-PCR of *Pax6* gene expression. *Pax6* is detected in clonally derived RSC spheres (RSC) and ciliary epithelia (CE) of adult mice. N ctl: negative control (amplification without reverse transcription). Amplicons encompass the alternatively spliced exon 5a (Ton et al., 1991;Walther and Gruss, 1991). The amplification products with or without alternatively spliced exon 5a were labeled as +5a and -5a. C. The ratio of the +5a and -5a transcripts normalized by the control housekeeping gene GAPDH.

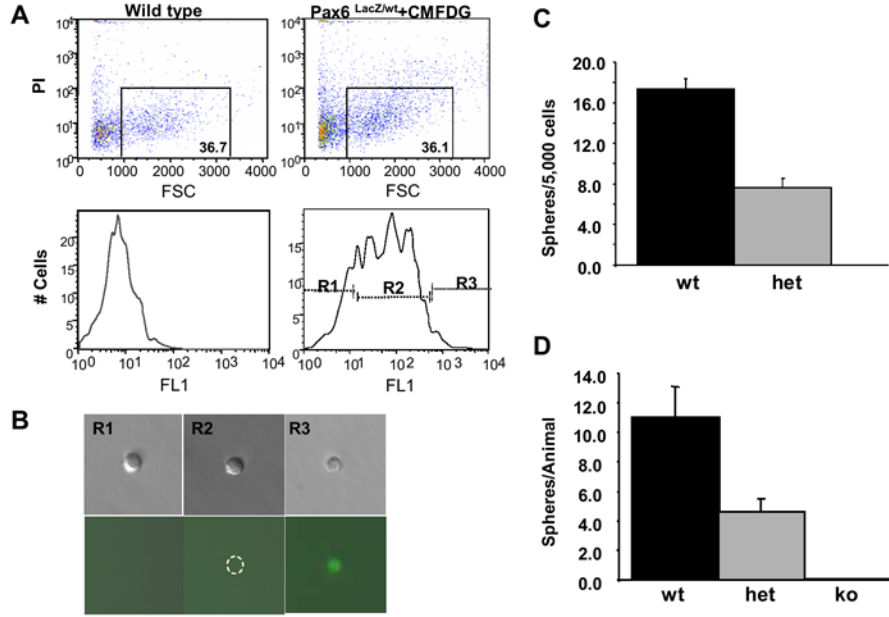


Fig 2. *Pax6* is enriched in retinal stem cells and required for the generation of clonal RSC spheres

A. (top) Profiles of the non-treated wild type and CMFDG-treated adult *Pax6^{LacZ/wt}* cells sorted by propidium iodide (PI) and forward scattered count (FSC). The rectangles show the live single cells. The numbers in the rectangles are percentages of live cells. (bottom) Profiles of the live single cells sorted by the CMFDG reaction elicited fluorescence. R1, R2 and R3 indicate the three non-overlapping portions we collected for the clonal RSC sphere assay in table I; **B.** Examples of the cells from R1, R2 and R3 portions under light microscopy (top) and fluorescent microscopy (bottom). Note that only cells from R3 portion have significant fluorescence elicited from CMFDG/ β -galactosidase reaction; the dashed circle in the R2 bottom panel marks the R2 cell with very weak fluorescence; **C.** Clonal RSC sphere assays on adult *Pax6^{LacZ/wt}* (het) and wild type (wt) mice; **D.** Clonal RSC sphere assays on embryonic day 14 (E14) *Pax6^{LacZ}* mice. ko: *Pax6^{LacZ/LacZ}* (n=9). het: *Pax6^{LacZ/wt}* (n=16); wt: wild type littermates (n=13).

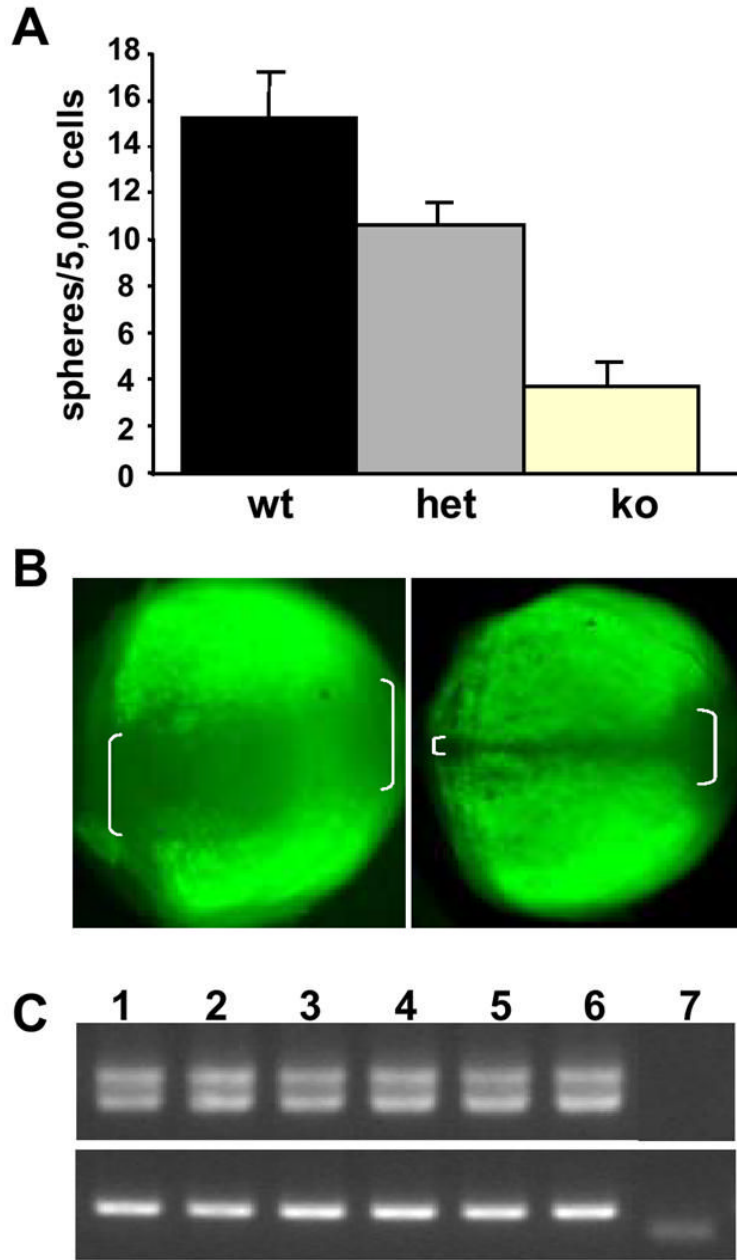


Fig 3. Pax6 is required for RSC sphere formation in adult animals

A. RSC sphere assays in the *Pax6* conditional knockout mouse model (Marquardt et al., 2001). ko: α -Cre +/-; *Pax6*^{loxP/loxP} (n=4). het: α -Cre +/-; *Pax6*^{loxP/wt} (n=4). wt: α -Cre -/-; *Pax6*^{wt/wt} (n=4). B. whole mount fluorescent images of adult α -Cre, Z/EG mouse eyes showing the α -promoter-driven expression has gaps (marked by white brackets) as seen from dorsal (left) and ventral (right) views of the retina. Anterior is on the left. C. RT-PCR of *Pax6* on the clonal RSC spheres derived from adult α -Cre; *Pax6*^{loxP/loxP} ciliary epithelial cells. The same primers, encompassing the alternatively spliced exon 5a were used. 1: wild type single RSC sphere; 2-6: RSC spheres derived from α -Cre; *Pax6*^{loxP/loxP} CE cells, 7: negative control (amplification without reverse transcription).

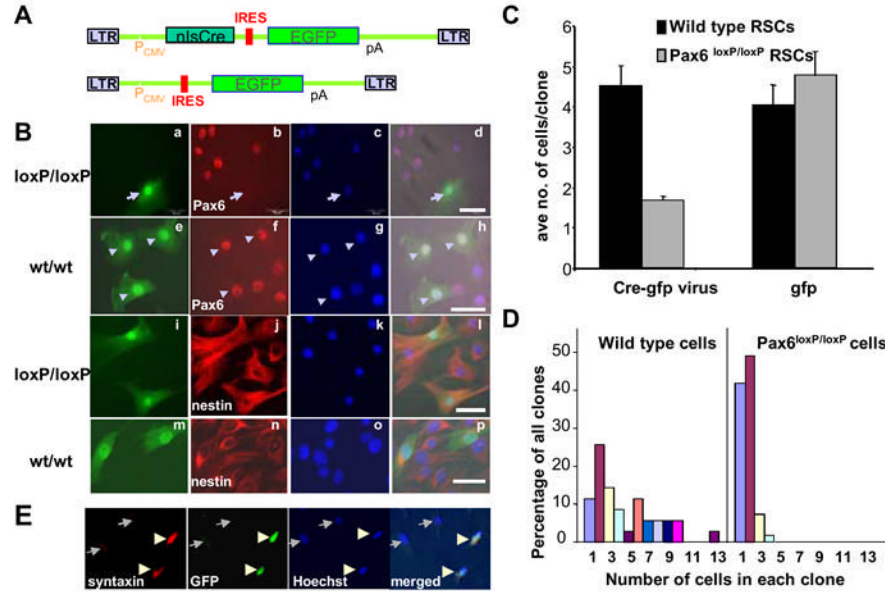


Fig 4. Inactivation of Pax6 inhibits the proliferation of RSCs in vitro

A. pMXIE-Cre-EGFP (top) and pMXIE-EGFP (bottom) retroviral constructs. P_{CMV}: CMV promoter; nlsCre: Cre recombinase with a nuclear localization signal (nls); EGFP: enhanced green fluorescent protein; IRES: internal ribosomal entry site; pA: polyA signal. B. Cre-EGFP retroviral infection of adult Pax6^{loxP/loxP} and wild type RSCs. a-h: Pax6 is inactivated in the Cre-EGFP infected Pax6^{loxP/loxP} RSCs (arrows in a-d), but not the wild type RSCs (arrowheads in e-h). a,e: GFP channel showing the infected cells; b,f: Pax6 immunostaining; c,g: Hoechst nuclear staining; d,h: merged images. i-p: RSCs infected with Cre-EGFP retrovirus retain progenitor identity with neural progenitor marker nestin expression. i,m: GFP channel showing the infected cells; j,n: immunostaining of nestin. k,o: Hoechst nuclear staining; l,p: merged images. GFP expression marks the infected RSCs and their progeny. C: Average number of progeny in each clone derived from single viral infected adult RSCs 4 days after retroviral infection in vitro. Data represent means +/- SEMs. D: Distribution of different sized clones derived from single virus-infected RSCs 4 days after Cre-EGFP retroviral infection of wild type or Pax6^{loxP/loxP} ciliary epithelial cells. E. Immunohistochemistry of differentiated RSCs from Pax6^{loxP/loxP} animals 21 day after pMXIE-Cre-EGFP viral infection with syntaxin monoclonal antibody. Arrowheads showed two infected cells, which are expressing the amacrine cell marker syntaxin. Arrows pointed two non-infected cells, which were syntaxin negative.

Table 1
Summary of the clonal RSC sphere assays after the FACS sorting

	R1	R2	R3
Cells Collected	5089	15,396	832
Spheres Formed	0	0	11
Frequency	0	0	1/76

Table 2
Differentiation assays on the pMXIE-Cre-EGFP retroviral infected RSCs

Cell type	Pax6 ^{Flox/flox}	Pax6 ^{wt/wt}
Amacrine cells (syntaxin positive)	97.2±2.8	12.8±1.1
Rod photoreceptors (1D4 positive)	0.95±0.9	21.8±1.7