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Developmental Biology 308 (2007) 54–67
DEVELOPMENTAL BIOLOGY
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Ciliary margin transdifferentiation from neural retina is controlled by canonical Wnt signaling

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Received for publication 21 November 2006; revised 9 April 2007; accepted 30 April 2007
Available online 16 May 2007

Abstract

The epithelial layers of the ciliary body (CB) and iris are non-neural structures that differentiate from the anterior region of the eyecup, the ciliary margin (CM). We show here that activation of the canonical Wnt signaling pathway is sufficient and necessary for the normal development of anterior eye structures. Pharmacological activation of β-catenin signaling with lithium (Li^+) treatment in retinal explants in vitro induced the ectopic expression of the CM markers *Otx1* and *Msx1*. Cre-mediated stabilization of β-catenin expression in the peripheral retina in vivo induced a cell autonomous upregulation of CM markers at the expense of neural retina (NR) markers and inhibited neurogenesis. Consistent with a cell autonomous conversion to peripheral eye fates, the proliferation index in the region of the retina that expressed stabilized β-catenin was identical to the wild-type CM and there was an expansion of CB-like structures at later stages. Conversely, Cre-mediated inactivation of β-catenin reduced CM marker expression as well as the size of the CM and CB/iris. Aberrant CB development in both mouse models was also associated with a reduction in the number of retinal stem cells in vitro. In summary, activation of canonical Wnt signaling is sufficient to promote the development of peripheral eyecup fates at the expense of the NR and is also required for the normal development of anterior eyecup structures.

Keywords: Retina; Ciliary margin; Ciliary body; Wnt; β-catenin; Li^+; Stem cell; Transgenic; Msx1; BMP4

Introduction

Eye development is driven by a series of inductive interactions involving the neuroectoderm, mesoderm and neural crest cells. Because of its accessibility and ease of manipulation the vertebrate eye is an excellent model system in which to study the patterning of complex organs and tissues in the central nervous system. The developing optic cup is partitioned into the central retina and a peripheral region called the ciliary margin (CM). Despite its neuroectoderm origin, the CM gives rise to two non-neural structures: the folded proximal ciliary epithelium (CE), the innermost layer of the ciliary body (CB), which secretes aqueous humor, and the distal iris (Smith et al., 2002). The neural crest mesenchyme contributes to the connective tissue and muscles of the CB, and a subset of cells in the iris differentiate as smooth muscle cells that form the iris sphincter muscle. Thus the CE and iris represent the very rare examples of derivation of non-neural tissue from the neural ectoderm. Specification of the CM is an active area of research because aberrant function of the CE is associated with human eye diseases, such as glaucoma (reviewed by Civan and Macknight, 2004) and because the pigmented ciliary epithelium (PCE) contains retinal stem cell (RSC) pools in mammals (Ahmad et al., 2000; Coles et al., 2006; Tropepe et al., 2000).

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doi:10.1016/j.ydbio.2007.04.052

In lower vertebrates, including fish and amphibians, the distal tip of the retina is referred to as the ciliary marginal zone (CMZ) (also called the ora serrata) and is a neurogenic region from which new neurons are generated for incorporation into the retina that grows throughout the life of the animal (reviewed by Harris and Perron, 1998). Due to its extreme peripheral position, the CMZ in lower vertebrates is topologically analogous to the CB of the eye in mammals (reviewed by Perron and Harris, 2000). The eye of the post-hatch chick also contains progenitors that are located at the retinal margin that resembles the CMZ cells of fish and amphibians (Fischer and Reh, 2000). Although RSC can be isolated from the adult rodent CB (Ahmad et al., 2000; Coles et al., 2006; Tropepe et al., 2000), this structure is not considered to be neurogenic in vivo (Moshiri and Reh, 2004).

Development of the peripheral region of the eye is regulated by adhesion molecules (Inagaki et al., 2005), signaling molecules and transcription factors (Davis-Silberman et al., 2005; Hsieh et al., 2002; Kubota et al., 2004; Pressman et al., 2000). Although information regarding the specification of these peripheral structures is limited, lens-derived signals and BMP signaling have been implicated as positive factors in CB development in the mouse eye (Thut et al., 2001; Zhao et al., 2002). In chick retina, *BMP4* and *BMP7* are expressed at an appropriate time and place to regulate CE and iris muscle formation (Jensen, 2005). Expression of several components of the Notch receptor pathway, including *Notch2* and the ligand, *Jagged* (Bao and Cepko, 1997), has also been reported in the anterior region of the vertebrate eye, implicating a role of the Notch pathway in this region.

Members of the Wnt signaling family are also good candidates for signaling molecules that pattern the peripheral retina. Wnts are secreted cysteine-rich glycoproteins that play key roles in the development of many regions of the vertebrate embryo and in adult tissue (reviewed by Ciani and Salinas, 2005). Wnt ligands signal through the Frizzled receptors and activate a number of distinct intracellular signaling cascades including the canonical Wnt/β-catenin pathway, the planar cell polarity pathway, the Wnt/Ca^{2+} pathways and a pathway that regulates spindle orientation and asymmetric cell division (reviewed by Huelsken and Birchmeier, 2001; and by Kohn and Moon, 2005). Activation of the canonical Wnt pathway results in the stabilization and accumulation of cytoplasmic β-catenin, which is otherwise phosphorylated in a protein complex containing glycogen synthesis kinase (Gsk)-3β and targeted for degradation. Stabilized β-catenin translocates to the nucleus and associates with TCF/Lef (T cell specific transcription factor and lymphoid enhancer-binding factor) transcription factors and other co-factors to activate target gene transcription (reviewed by Brembeck et al., 2006; and by Widelitz, 2005). Controlling the levels of β-catenin is essential in transducing canonical Wnt signaling and thus increasing β-catenin levels by inhibiting negative regulators or introducing activating mutations in β-catenin is sufficient in most cases to activate this pathway (reviewed by Brembeck et al., 2006).

Increasing evidence has pointed to Wnts as potential extrinsic regulators in the context of CM development in the eye. Previous studies have shown that *Wnt2b* (formerly known as *Wnt13*) is expressed in the retinal pigment epithelium (RPE) overlying CM and the expression of several additional components of the Wnt pathway is also detected in overlapping or exclusive patterns in this region in several species (Cho and Cepko, 2006; Liu et al., 2006; Van Raay et al., 2005; and reviewed by Van Raay and Vetter, 2004). In the frog eye, the canonical Wnt signaling pathway has been shown to regulate neural potential by driving progenitor proliferation and inducing proneural gene expression (Van Raay et al., 2005). In contrast, increased Wnt signaling in the chick eye at the optic vesicle stage, either through expression of the *cWnt2b*, a canonical Wnt ligand, or constitutively active β-catenin, has been shown to inhibit neuronal differentiation and to promote the development of peripheral eye structures including the CMZ, CB and iris (Cho and Cepko, 2006; Kubo et al., 2003). At later stages ectopic *cWnt2b* expression promotes proliferation and blocks neurogenesis in the chick retina (Kubo et al., 2005). In the mouse retina, activation of *TCF/Lef-LacZ*, a canonical Wnt reporter transgene, is observed in the prospective CM at the optic vesicle and eyecup stages and at later stages persists in the non-pigmented CE and iris as well as in discrete subsets of neurons in the neural retina (NR) (Liu et al., 2003, 2006). Based on the pattern of Wnt reporter gene activation in the peripheral eye, we hypothesized that canonical Wnt signaling controls the development of the CM and its non-neural derivatives in the murine eye.

One prediction of this hypothesis is that the level of canonical Wnt pathway activation would regulate the size of the CM. We show here that exposure to lithium (Li^{+}), a well-characterized activator of the canonical Wnt pathway, induced the ectopic expression of CM markers and increased the size of the CM in embryonic retinal explants. Cre-mediated conditional expression of stabilized β-catenin in the peripheral retina in vivo induced a cell autonomous expansion of the CM at the expense of the NR. Conversely, Cre-mediated inactivation of β-catenin reduced CM gene expression and resulted in aberrant CB development. Aberrant CB development in both mouse models was also associated with a marked reduction in the number of RSCs in vitro. These findings implicate β-catenin-mediated signaling as an instructive signal in the specification and development of peripheral eye fates in the murine retina.

Results

Retinal explants represent a loss-of-function model with respect to canonical Wnt signaling in the developing ciliary margin

The partitioning of the eyecup into CM and NR regions is apparent as early as E12.5, as evidenced by the expression of *Otx1* and *Msx1* in the distal tip of the eyecup (Martinez-Morales et al., 2001; Monaghan et al., 1991). To investigate the role of canonical Wnt signaling in CM development, we monitored CM specific gene expression and *TCF/Lef-LacZ* reporter gene activation over time in retinal explants from E14.5 *TCF/Lef-LacZ* transgenic mice. Because the lens has been shown to induce the expression of CM genes (Thut et al., 2001), contact

between the lens and retina was maintained in these cultures (Fig. 1A). At time 0 of the culture the pattern of TCF/Lef-LacZ reporter activity and the expression of CM marker and Wnt pathway genes in the explants was identical to that observed in vivo (Fig. 1B and data not shown). By 15 h of culture, however, TCF/Lef-LacZ reporter activity in the CM region in the explants was reduced compared with the equivalent stage in vivo and was almost abolished by 27 h of culture (Fig. 1C). The *Otx1*$^{+}$ CM region adjacent to the lens was identifiable in explants cultured for 3 days (Fig. 1B). Thus, explanting the retina to culture is associated with a reduction in canonical Wnt pathway activation.

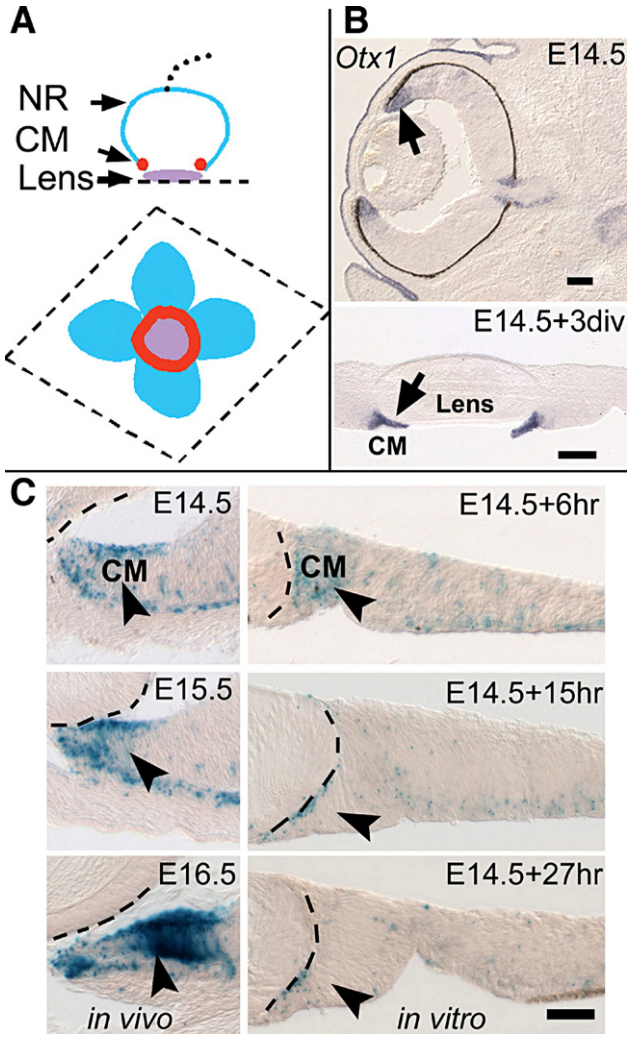

Fig. 1. Wnt reporter and CM marker gene expression in retinal explants. (A) Diagram of retinal explant procedure. Eyecups from embryonic mice, dissected free of the RPE and the sclera, were opened at the optic nerve region and flattened onto filters with lens intact and cultured in serum-free explant media. (B) ISH for CM marker gene *Otx1* at E14.5 (top panel) and in retinal explants cultured for 3 days (bottom panel). Arrows indicate expression of *Otx1* in the CM of the embryonic eye and the explants. (C) X-gal staining in the eyes of *TCF/Lef-LacZ* reporter mice at E14.5, E15.5 or E16.5 (in vivo, left panel) and in E14.5 explants cultured for 6 h, 15 h or 27 h (in vitro, right panel). Dashed lines indicate the lens. Arrowheads indicate β-gal reporter activity in the CM and its decay in explants by 15 h in culture ($n=5$ explants per time point). CM, ciliary margin; NR, neural retina. Scale bars: 100 μm in panel B, 50 μm in panel C.

Pharmacological stabilization of β-catenin in retinal explants induces a rapid change in gene expression that is characteristic of CM development

We investigated whether Wnt signaling could be reactivated in explants and its effects on retinal development by treating explants with Li^{+}, a well-known activator of the β-catenin-dependent canonical Wnt pathway. Li^{+} activates this pathway by inhibiting Gsk-3β activity, which results in stabilization of β-catenin protein levels and enhanced β-catenin-dependent transcriptional activity (Hedgepeth et al., 1997; Klein and Melton, 1996; Stambolic et al., 1996). As we have shown previously, Li^{+} induced a rapid induction of reporter gene activity in *TCF/Lef-LacZ* retinal explants, demonstrating the efficacy of Li^{+}-induced β-catenin-dependent transcription in this model (Liu et al., 2006). The Li^{+} response is maximal from 12 to 24 h and, therefore, we chose the 24 hour time point to compare the expression of the *TCF/Lef-LacZ* reporter gene, previously reported Wnt target genes, CM and NR specific genes in control and Li^{+}-treated explants. Li^{+} upregulated reporter activity throughout the explants, and it increased *Otx1* expression in the CM and induced ectopic *Otx1* expression in the adjacent NR region of the explants (Fig. 2A). The upregulation of mRNA for *Otx1* and *Msx1*, an additional CM marker, in Li^{+}-treated explants was confirmed by Q-PCR analysis (Fig. 2B; *Msx1*: 3.15 ± 0.7712 fold increase relative to control, $n=4$ independent experiments of pooled explants per treatment). Conversely, Li^{+} induced a marked downregulation of *Math3*, a proneural gene, *Crx*, a marker of committed photoreceptor cells, *Pax6* and *Delta-like I* (*Dll1*) expression (Figs. 2A and B). Therefore, short-term activation of β-catenin signaling in retinal explants is associated with a gain of CM identity and reduced neurogenesis.

Targeted expression of stabilized β-catenin in vivo promotes CM development

Because the effects of Li^{+} on TCF/Lef-LacZ reporter expression in explants were transient, we turned to a Cre-loxP transgenic system to target constitutive expression of stabilized β-catenin to the retina. We crossed α-*Cre* transgenic mice, where Cre activity is restricted to the peripheral retina during embryogenesis (Fig. 3B) (Marquardt et al., 2001) with conditional β-catenin knockin mice (*Catnb*$^{+/lox(ex3)}$) (Harada et al., 1999), in which Cre-mediated excision of floxed exon 3 removes the Gsk-3β phosphorylation site (α-*Cre;Catnb*$^{+/lox(ex3)}$, referred to as *βcat*^{act}) resulting in constitutive expression of a stabilized form of β-catenin that is transcriptionally active. Activation of canonical Wnt signaling in this line was assessed by crossing in the *TCF/Lef-LacZ* reporter transgene (Mohamed et al., 2004) (referred to as *βcat*^{act}*-LacZ*). We observed a marked increase in TCF/Lef-LacZ reporter activity (Fig. 3G) and expression of Wnt target genes, *Lef1* (Fig. 3H) and *BMP4* (Supplementary Fig. 1F) (Baker et al., 1999; Filali et al., 2002; Hovanes et al., 2001; Kim et al., 2002) in Cre^{+} cells in the peripheral retina of the *βcat*^{act}*-LacZ* mice compared with control littermates that did not express the conditional β-catenin

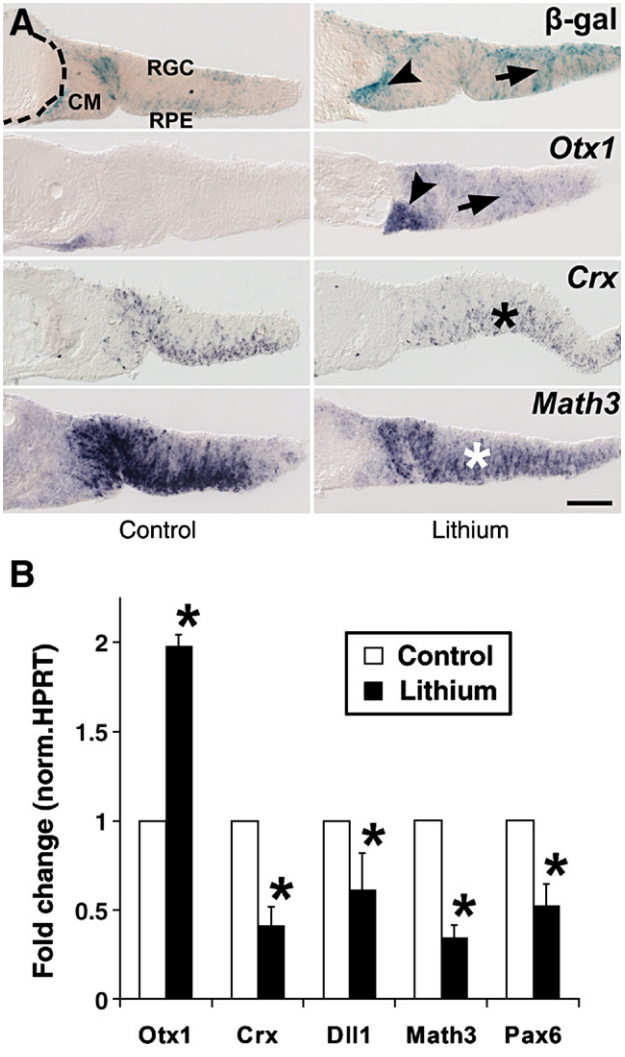

Fig. 2. Li^+ induced changes in gene expression in retinal explants. (A) X-gal staining and ISH for the indicated genes in E14.5 explants from *TCF/Lef-LacZ* mice cultured under control conditions or in the presence of Li^+ for 24 h. Expression of β-gal ($n=6$) and *Otx1* ($n=3$) was upregulated in the CM (arrowheads), and in the NR (arrows) of Li^+-treated explants. Black and white asterisks indicate the Li^+-induced downregulation of *Crx* ($n=7$) and *Math3* ($n=2$) expression, respectively. Dashed line indicates the position of the lens. CM: ciliary margin; RGC: retinal ganglion cell layer; RPE: retinal pigment epithelium. Scale bars: 100 μm. (B) Quantitative RT-PCR analysis (normalized to Hprt) for the indicated genes on RNA extracted from retinal explants ($n=6$ independent explant pools per treatment). Results are presented as mean fold change±SD. *$p<0.001$. Statistical significance was evaluated by Student's *t*-test.

allele (*α-Cre;Catnb*$^{+/+}$*;TCF/Lef-LacZ*, referred to as *βcat*$^+$*-LacZ*). The effect was more pronounced in the nasal side of the retina compared with the temporal side, which is consistent with our previous observations (Wang et al., 2005); nonetheless, Cre^+ cells on the temporal side of the eye exhibited identical changes in gene expression and reporter activation (Figs. 3G, H and data not shown). Thus, α-Cre targeted expression of stabilized β-catenin induces cell autonomous activation of canonical Wnt signaling in the peripheral retina.

The peripheral retina of E14.5 *βcat*^{act}*-LacZ* mice was disorganized, contained cellular rosettes and lacked a clear separation between the RGC and neuroblast layers compared with retinas from *βcat*$^+$*-LacZ* mice (Figs. 3A and E). The localization of Cre^+ cells was also altered in the *βcat*^{act}*-LacZ* mice—they appeared to be clumped in the very distal region of the eyecup in the *βcat*^{act}*-LacZ* retina, whereas they were distributed more evenly in the peripheral retina of the *βcat*$^+$*-LacZ* mice (Figs. 3B and F). Although the hyaloid vasculature had developed in the *βcat*^{act} eyes (Supplementary Fig. 2F), there was evidence of abnormal migration of mesenchymal cells into the vitreous (Supplementary Fig. 2E) and the eyes were reduced in size compared with *βcat*$^+$ mice (Figs. 3A and E). Examination of sagittal serial sections from *βcat*^{act} eyes at E11.5 did not reveal any abnormalities in the closure of the choroid fissure ($n=2$, data not shown). Thus, expression of stabilized β-catenin in the peripheral retina is associated with retinal abnormalities, microphthalmia and abnormal mesenchymal cell development in the eye, but not coloboma.

To identify the aberrant peripheral tissue in the *βcat*^{act} retina, we performed ISH with riboprobes specific for NR and CM markers. The expression of the NR markers *CyclinD1*, and *Crx* (Fig. 4) and *Sfrp2* and *Math3* (Supplementary Fig. 1) was downregulated in the Cre^+ region of the *βcat*^{act}*-LacZ* retina. IHC with RGC-specific antibodies confirmed the absence of RGCs in Cre^+ regions of the *βcat*^{act} retina and also revealed the disorganization of RGCs adjacent to Cre^+ region (Supplementary Fig. 3). In stark contrast, the expression of CM markers, including *Msx1*, *Otx1* (Fig. 4) and *CyclinD2* (Supplementary Fig. 1), was markedly increased in the *βcat*^{act}*-LacZ* retina. Examination of the *βcat*^{act}*-LacZ* retina at P7 revealed that the CB was expanded and disorganized (Fig. 5), which was consistent with the expansion of CM gene expression in these mice at embryonic stages. As the iris was not located in its normal position (Fig. 5), we stained the retinas at P7 for α-smooth muscle actin (SMA), a marker for the iris epithelium (Cho and Cepko, 2006; Zhao et al., 2002). In control littermates, SMA expression was detected in the iris epithelium as well as the folds of the CB, periocular mesenchyme and the cornea (Supplementary Fig. 4A). SMA^+ regions were present in the disorganized peripheral region of the retina of P7 *βcat*^{act} mice (Supplementary Figs. 4B and C). Because SMA staining was not restricted to the iris, the increase in SMA staining in the peripheral region of the *βcat*^{act} retina is consistent with the loss of NR and concomitant gain of CB and (or) iris identity.

These changes in NR and CM gene expression in the E14.5 *βcat*^{act} retina were restricted to the Cre^+ cells (Figs. 3 and 4), which raised the possibility that expression of activated β-catenin induced a cell autonomous fate switch from NR to CM. To determine how early this occurred, we examined gene expression in *βcat*^{act}*-LacZ* at E11.5. At this stage, eye size and the distribution of Cre^+ cells were comparable between *βcat*^{act}*-LacZ* and *βcat*$^+$*-LacZ* mice (Figs. 6A, B, E and F). Nonetheless, we detected TCF/Lef-LacZ reporter activation (Fig. 6G) and upregulation of *Msx1*, *Lef1* and *BMP4* (Figs. 6M, O and P) expression in the peripheral retina of *βcat*^{act}*-LacZ* mice. Activation of β-catenin signaling at E11.5 was also associated with the downregulation of *CyclinD1* (Fig. 6H) expression in

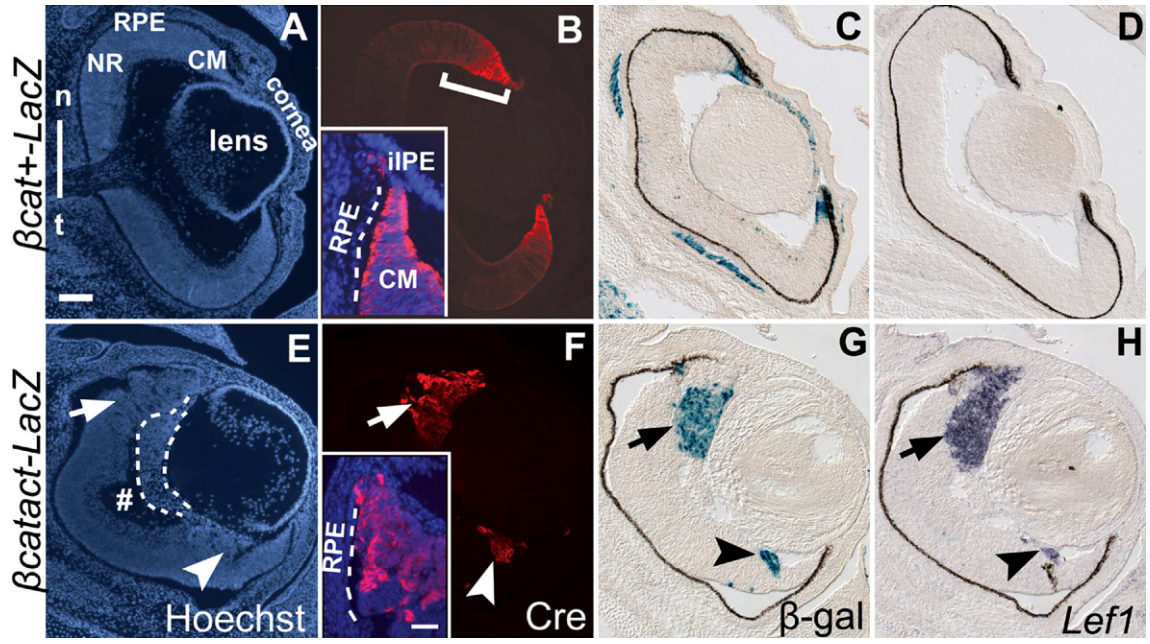

Fig. 3. Wnt reporter and Wnt target gene expression in the retinas of mice with targeted induction of stabilized β-catenin expression in the peripheral retina. (A, E) Hoechst nuclear staining, (B, F) IHC for GFP, (C, G) X-gal staining and (D, H) ISH for *Lef1* expression in serial transverse retinal sections from E14.5 *βcat*$^{+}$*-LacZ* (A–D) and *βcat*^{act}*-LacZ* (E–H) mice. (A, E) Hoechst staining reveals the reduced size of the vitreal compartment (# in panel E) and invasion of periocular mesenchyme (dashed lines in E) into the vitreous of the eyes of *βcat*^{act}*-LacZ* mice. The arrow in panel E indicates the rosettes in the CM corresponding to the *Cre* expressing region (arrow in panel F) in the *βcat*^{act}*-LacZ* mice. n/t indicates the nasal/temporal orientation of the eye section in this and all subsequent figures. (B, F) *Cre* expression, monitored by IHC for GFP expressed from the α-Cre transgene, was detected in the non-pigmented CM and the tip of the eyecup, the prospective iIPE in the eyes of *βcat*$^{+}$*-LacZ* mice, but not in the RPE overlying the CM in the control and the *βcat*^{act}*-LacZ* mice (inserts in panels B, F). (C, D, G, H) Activation of the *TCF/Lef-LacZ* reporter and upregulation of *Lef1* expression were detected in an identical pattern to *Cre* expression (arrows and arrowheads in E–H) ($n=4$ animals) in *βcat*^{act}*-LacZ* mice. CM, ciliary margin; iIPE, inner iris pigment epithelium; NR, neural retina; RPE, retinal pigment epithelium. Scale bars: 100 μm in panels A–H, 50 μm in inserts.

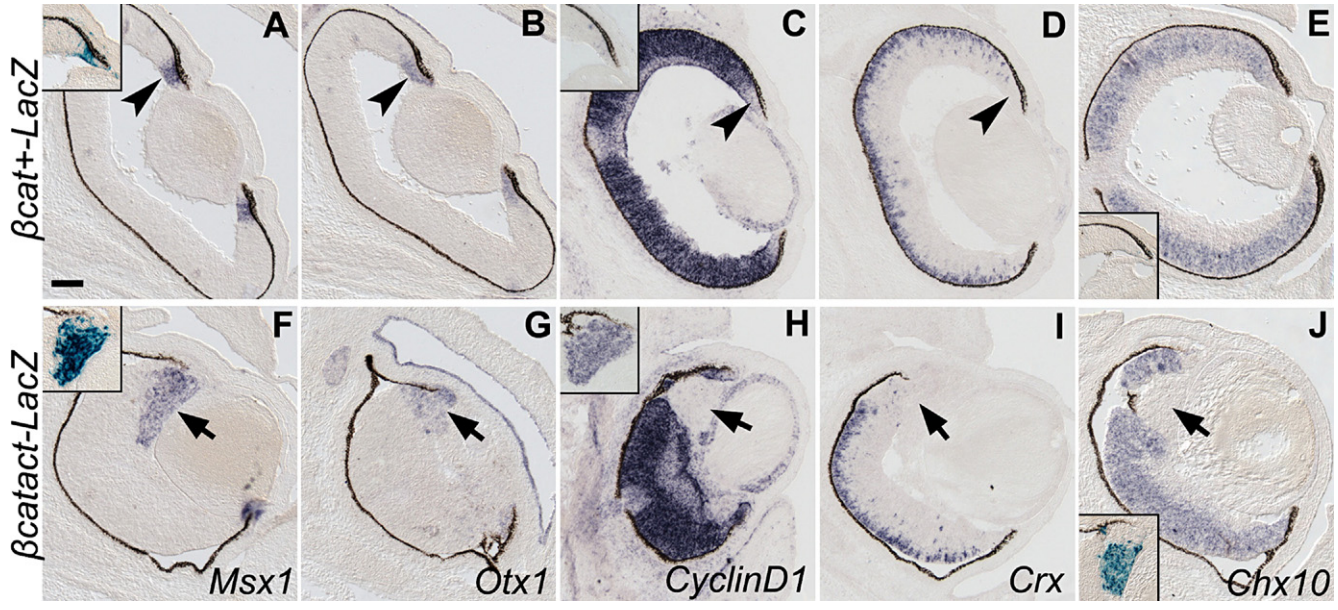

Fig. 4. Expression of CM and NR markers at E14.5 after targeted expression of stabilized β-catenin in the peripheral retina. ISH for *Msx1* (A, F), *Otx1* (B, G), *CyclinD1* (C, H), *Crx*, (D, I) and *Chx10* (E, J) in transverse retinal sections from E14.5 *βcat*$^{+}$*-LacZ* (A–E) or *βcat*^{act}*-LacZ* (F–J) mice. (A, B, F, G) Expansion of the *Msx1*$^{+}$ and *Otx1*$^{+}$ domains in the eye of *βcat*^{act}*-LacZ* ($n=4$) compared with *βcat*$^{+}$*-LacZ* control mice (compare arrowheads in panels A, B with arrows in panels F, G). (C–E, H–J) Reduction of *CyclinD1, Crx* and *Chx10* mRNA in the peripheral region of the *βcat*^{act}*-LacZ* retina (arrows in panels H–J) ($n=2$). Inserts show (A, E, F, J) β-gal activity and (C, H) *Lef1* expression in adjacent retinal sections, indicating activation of the Wnt signaling in the peripheral region of the *βcat*^{act}*-LacZ* retina. Scale bar: 100 μm.

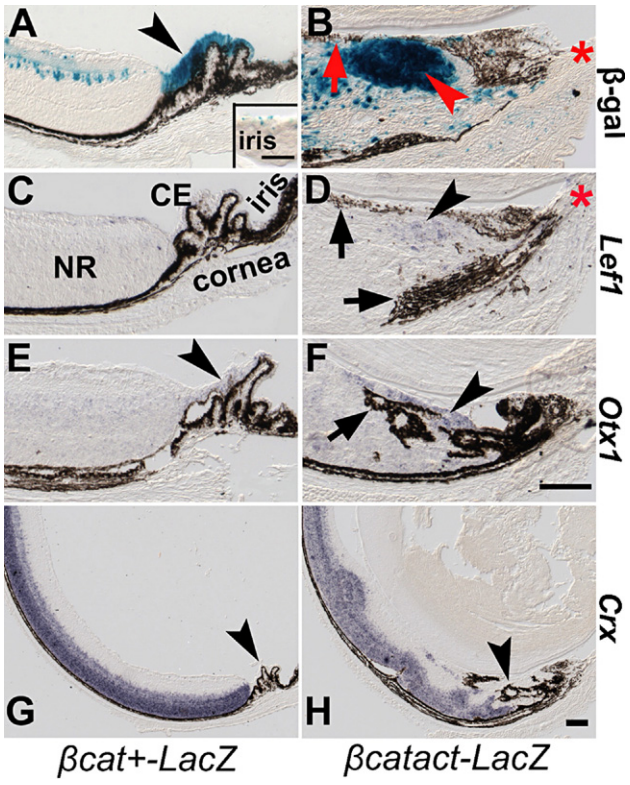

Fig. 5. Changes in Wnt reporter activation, gene expression and morphology in the peripheral retina of βcat^{act}-LacZ mice at P7. (A, B) X-gal staining and (C–H) ISH for Wnt target gene *Lef1*, CM marker gene *Otx1* and NR marker *Crx* in transverse retinal sections from P7 βcat^{+}-*LacZ* (A, C, E, G) or βcat^{act}-*LacZ* (B, D, F, H) mice. The *TCF/Lef-LacZ* reporter, which is activated in the non-pigmented CE in the control retina (arrowhead in A), is highly activated in the peripheral NR in the βcat^{act}-*LacZ* mice (arrowhead in B). Insert (A) shows a high magnification view of the iris, revealing *TCF/Lef-LacZ* reporter gene activation in a subset of cells in the iris of the control littermates. Arrows (B, D) indicate pigmented tissue and (F) ciliary process-like structure in the NR of the βcat^{act}-*LacZ* mice. Arrowheads in panels D, F indicate the induction of *Lef1* and *Otx1* expression in the βcat^{act}-*LacZ* mice ($n=2$). Arrowheads in panels G, H indicate the expanded and disorganized peripheral eye of a βcat^{act}-*LacZ* mouse at low magnification. Asterisks (B, D) indicate the absence of the iris in the eye of the βcat^{act}-*LacZ* mice. CE, ciliary epithelium; NR, neural retina. Scale bars: 100 μm in panels A–F and panels G, H, 25 μm in insert.

the Cre^{+} region of the βcat^{act}-*LacZ* retina. Thus, the initiation of CM development and inhibition of NR development in the βcat^{act}-LacZ retina were apparent at a stage when Cre^{+} cells were distributed in the prospective NR region of the eyecup.

Chx10 and Pax6 are homeodomain transcription factors that are expressed in progenitor cells throughout the developing eyecup, including the CM (Rowan et al., 2004; Walther and Gruss, 1991). Unexpectedly, the expression of both genes was markedly downregulated in the Cre^{+} region of the E11.5 and E14.5 βcat^{act}-*LacZ* retina (Figs. 4J, 6N, Supplementary Figs. 1J and 5B). Moreover, the patchy downregulation of *Chx10* and *Pax6* expression in some sections of βcat^{act}-*LacZ* retina completely overlapped with the pattern of *TCF/Lef-LacZ* reporter gene activation in adjacent sections (Figs. 6G, N, and Supplementary Fig. 5). Thus the CM marker profile that we observed in the β-catenin activated retina is associated with an atypical downregulation of *Pax6* and *Chx10* expression.

By E14.5 NR gene expression was not detected in cells that activated the *TCF/Lef-LacZ* reporter gene (Fig. 4), which is consistent with the possibility that expression of stabilized β-catenin converted all cells from NR to CM. Alternatively, it is possible that cells that expressed stabilized β-catenin, but that failed to downregulate NR genes, were eliminated by apoptosis. We did not, however, observe an increase in dying cells, as assessed by TUNEL staining, in the retinas of βcat^{act}-*LacZ* mice at E11.5 and E14.5 (data not shown). Finally, it is possible that cells expressing stabilized β-catenin that failed to convert to CM were undetectable because of silencing of the *TCF/Lef-LacZ* reporter transgene. This explanation is unlikely, however, as we did not detect Cre^{+} cells outside the region of the TCF/Lef-LacZ expressing domain in the βcat^{act}-*LacZ* retina. Therefore, our findings are consistent with the possibility that all of the Cre^{+} cells in the βcat^{act} retina were converted from an NR to CM fate.

To address the possibility that the expanded CM in βcat^{act} mice was due to the selective outgrowth of cells expressing stabilized β-catenin, we compared staining for Ki67, a proliferation marker, in βcat^{act} and control retinas. At E11.5, $Ki67^{+}$ nuclei were predominantly spindle shaped and the density of Ki67 staining in βcat^{+} retinas revealed a gradient of staining, with a high level in the center to a lower level in the periphery (Fig. 7B). The graded pattern of Ki67 staining in the eyes of the βcat^{act} mice was comparable to control littermates; however, the nuclei in the region expressing stabilized β-catenin had a rounded appearance (Fig. 7F, arrowhead). At E14.5, $Ki67^{+}$ cells in the βcat^{+} retina were highly compacted throughout the NR, while the CM region contained fewer and less intensely stained cells (Fig. 7D). The Cre^{+} region of the eye in the βcat^{act} mice, although highly disorganized, appeared to have fewer $Ki67^{+}$ cells than the central region, comparable to the CM region in the control retina (Fig. 7H). Quantification of the Ki67 labeling index in the retinas of control mice at E11.5, E14.5 and E15.5 revealed a reduction in the proportion of cycling cells in the CM compared with the neuroblast layer of the central retina (Fig. 7I), which is consistent with previous reports documenting lower proliferation rates in the peripheral region of the eyecup (Fischer and Reh, 2000; Kubota et al., 2004). Compared with the Cre^{-} central region of the βcat^{act} retina, the proportion of cycling cells in the Cre^{+} peripheral retina was reduced to the level found in the CM of wild-type littermates (Fig. 7I), indicating that the β-catenin-mediated expansion of CM marker expression is associated with acquisition of a CM-like proliferation rate. These data also show that selective outgrowth of CM progenitors is unlikely to account for the expansion of the CM region in the βcat^{act} mice.

CM development is reduced in the absence of β-catenin expression

To address the requirement for β-catenin in CM development, we examined the eyes of mice with a Cre-inducible conditional inactivation of the β-catenin gene (α-*Cre;Catnb*$^{flox/flox}$ referred to as βcat^{null}). At P10, the eyes of the βcat^{null} mice exhibited several abnormalities, including extensive rosetting,

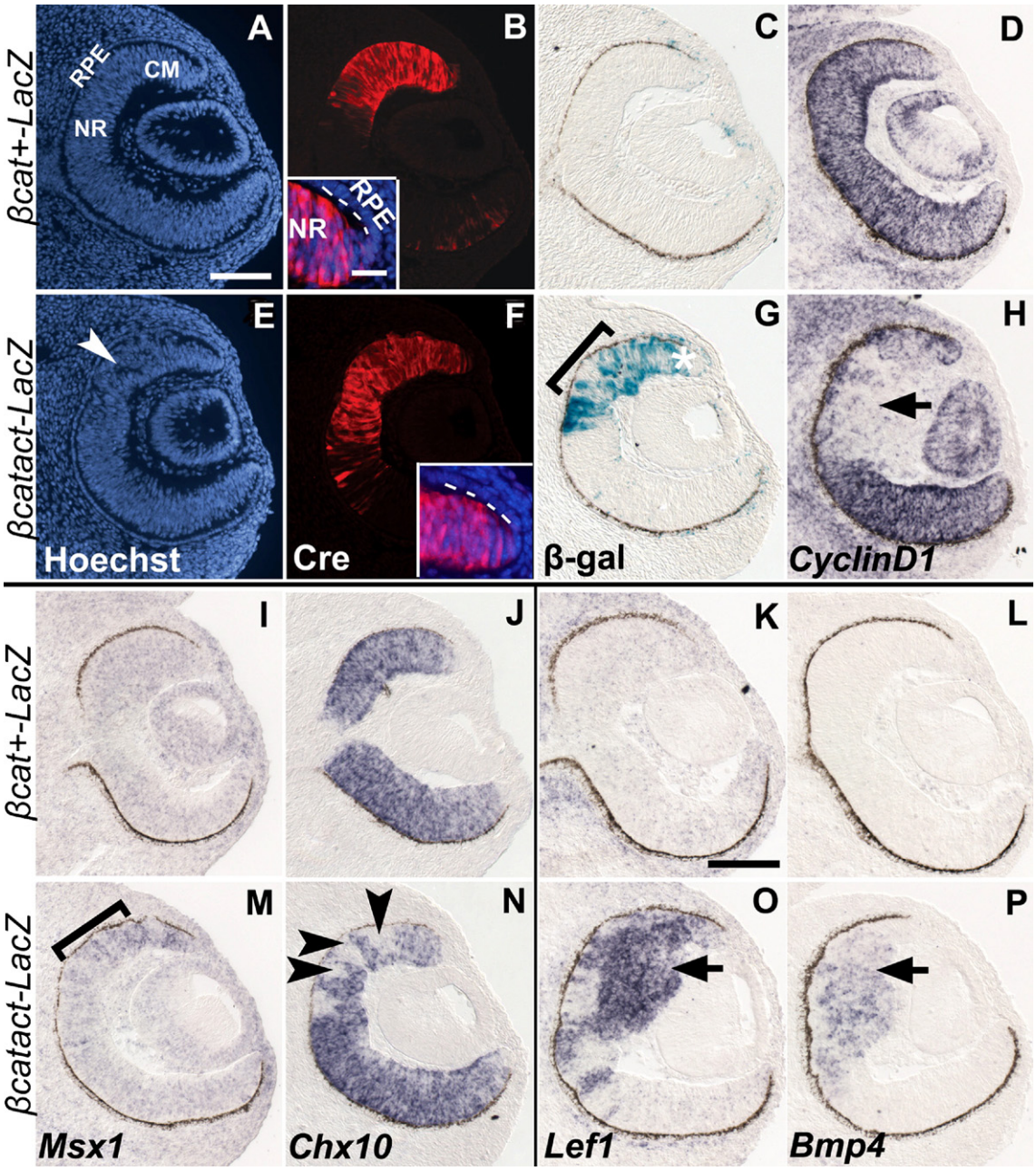

Fig. 6. Expression of CM and NR markers at E11.5 after targeted expression of stabilized β-catenin in the peripheral retina. (A, E) Hoechst nuclear staining, (B, F) IHC for GFP to identify Cre expression, (C, G) X-gal staining and ISH for (D, H) *CyclinD1*, (I, M) *Msx1*, (J, N) *Chx10*, (K, O) *Lef1* and (L, P) *Bmp4* expression in serial transverse retinal sections from E11.5 βcat^{+}-*LacZ* (A–D, I–L) or βcat^{act}-*LacZ* (E–H, M–P) retinas. Inserts (B, F) show a higher magnification view of Cre expression in the non-pigmented CM, but not the RPE overlying the CM of the control and β-catenin stabilized retina. Rosettes are present (arrowhead in E) in the βcat^{act}-*LacZ* retina at this stage. Induction of the *TCF/Lef-LacZ* reporter in the βcat^{act}-*LacZ* retina is observed in the Cre^{+} cells in the CM (asterisk in panel G) and NR (bracket in G) regions. Expression of the Wnt target gene *Lef1* was markedly upregulated (arrow in panel O) in the β-catenin activated regions. Note the reduction in *CyclinD1* mRNA (arrow in panel H) and the increase mRNA for CM markers *Msx1* (bracket in M) and *Bmp4* (arrow in panel P) in the peripheral region of the βcat^{act}-*LacZ* retina ($n=2$). (N) *Chx10* expression was lost in a patchy pattern (arrowheads in panel N) in the β-catenin stabilized region of the βcat^{act}-*LacZ* retina ($n=2$). Note the general structure and the size of the eyecup look normal in the βcat^{act}-*LacZ* mice at this stage. *Lef1* and *Bmp4* expression was examined in tissues different from those for other markers. CM, ciliary margin; NR, neural retina; RPE, retinal pigment epithelium. Scale bar: 100 μm in panels A–J, M and N and in panels K, L, O and P, 25 μm in inserts.

lens abnormalities and the absence of vitreous (data not shown). The CB of the βcat^{null} mice was also smaller in size and contained fewer folds (Fig. 8A). To determine whether the alterations in the CB were associated with changes in gene expression in the CM at earlier stages we performed ISH on βcat^{null} and wild-type littermates at E15. Compared with littermate control, the CM in the βcat^{null} retina was smaller, based on the reduced size of the *CyclinD1*$^{-}$ region of the peripheral retina and the reduction in *Msx1* expression (Fig. 8B). Thus, the development of the CM and CB is attenuated when β-catenin expression is lost in the peripheral eye.

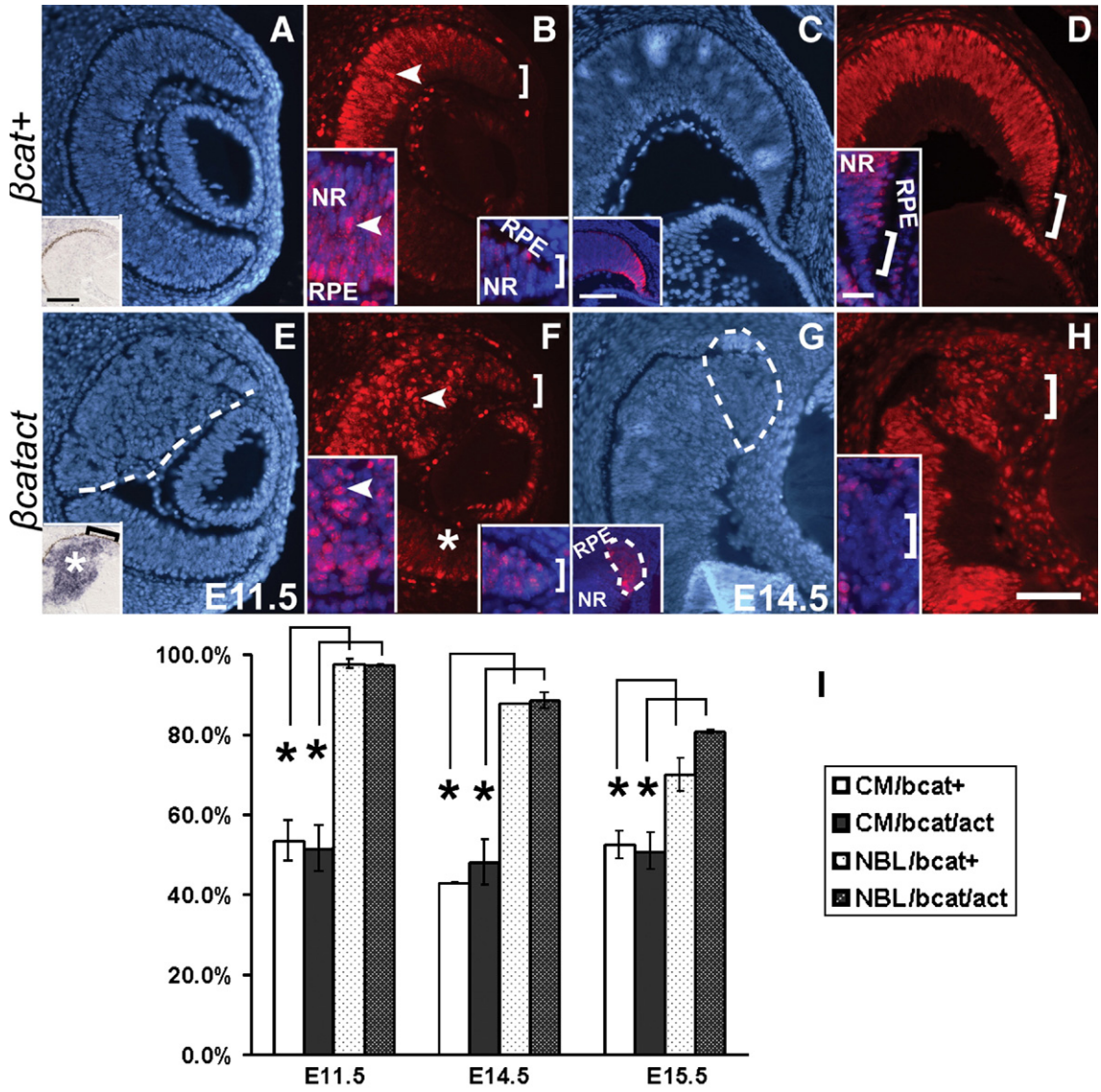

Fig. 7. Cell cycle marker expression in the retinas of embryonic βcat^{act} mice. (A, C, E, G) Hoechst nuclear staining and (B, D, F, H) IHC for Ki67, a proliferation marker, in transverse retinal sections of E11.5 and E14.5 βcat^{+} (A–D) and βcat^{act} (E–H) retinas. Inserts in (B, D, F, H) show high magnification images of Ki67 staining in the peripheral (brackets) or central (arrowheads) retina. *Lef1* (insert in A, E) or *Cre* expression (inserts in C, G) in adjacent sections defines the region of stabilized β-catenin expression in the βcat^{act} retina (dashed lines in panels E, G). (A, B, E, F) At E11.5, the density of $Ki67^{+}$ cells in the β-catenin stabilized region was comparable to control littermates (compare inserts in B, F), but the shape of the nuclei was markedly changed—spindle shaped in the controls (arrowheads in B) compared with round (arrows in F) in the region expressing stabilized β-catenin in the βcat^{act} mice ($n=2$). Note that in the region where stabilized β-catenin was not expressed in the βcat^{act} retina, the nuclear morphology was similar to control retinas (asterisk in panel F). (C, D, G, H) At E14.5, the density of $Ki67^{+}$ cells in the CM region of the βcat^{+} retina (bracket in panel D) and the β-catenin stabilized region (brackets in H) of the βcat^{act} retina was comparable ($n=2$). (I) Quantification and comparison of the labeling index for Ki67 in the CM and NBL of βcat^{+} versus βcat^{act} retinas at E11.5, E14.5 and E15.5. The data are the average±SD of the labeling indices from 4 CM and 4 NBL per genotype/stage with the exception of E15.5 βcat^{+} which was 12 CM and 12 NBL. $*p \leq 0.01$. Note in the βcat^{act} retina the CM is the Cre^{+} $Msx1^{+}$ region and the NBL counts were determined from the Cre^{-} region of the retina. Statistical significance was evaluated by Student's *t*-test. Error bars indicate SD. CM, ciliary margin; NBL, neural blast layer; NR, neural retina; RPE, retinal pigment epithelium. Scale bars: 100 μm in panels A–H, inserts in panels A, E and in panels C, G, 25 μm for inserts in panels B, D, F and H.

Effects of modulating β-catenin signaling on the RSC pool

The alterations in CB development in the βcat^{act} and βcat^{null} mice suggested the possibility that there should be effects on the RSC pool, which is derived from this region of the eye (Ahmad et al., 2000; Tropepe et al., 2000). To assay the in vivo RSC 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 (reviewed by Morshead and van der Kooy, 2001; Tropepe et al., 2000). The number of sphere forming cells was reduced by over 4 fold in the eyes of adult βcat^{null} mice compared with controls, consistent with the reduced size of the CB (117±5.1 spheres per eye in the wild-type ($n=8$ eyes) versus 25.6±8 spheres per eye in the βcat^{null} mice ($n=8$)). Genotypic analysis of the spheres derived from βcat^{null}

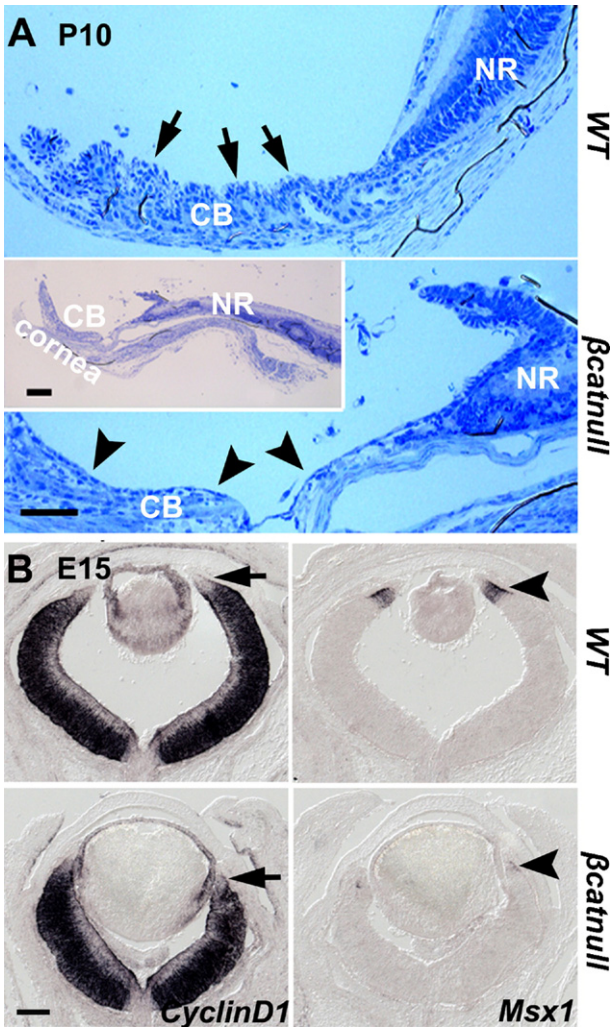

Fig. 8. Peripheral eye development in mice with targeted inactivation of β-catenin expression in the peripheral retina. (A) Histological analysis of retinal sections from P10 wild-type (WT) littermates and βcat^{null} mice. Insert shows a low magnification view of the eye from βcat^{null} mice. Arrows indicate the folds of the CB in the WT and arrowheads indicate the CB with markedly less folding in the βcat^{null} retina ($n=5$). (B) ISH for NR marker gene *CyclinD1* and CM marker gene *Msx1* expression in serial retinal sections from E15 WT littermates and βcat^{null} mice ($n=4$). Reduction in the size of the *CyclinD1*$^{-}$ domain (compare arrows), the expression level of *Msx1* and the size of *Msx1* expressing domain was apparent in the eye of βcat^{null} mice (compare arrowheads). CB, ciliary body; CM, ciliary margin; NR, neural retina. Scale bars: 50 μm in panel A, 100 μm for insert in panel A and for panel B.

eyes revealed that the floxed β-catenin allele was not recombined, indicating that the spheres developed from cells that had escaped Cre-mediated inactivation of the floxed β-catenin allele (data not shown). This finding is consistent with our observation of GFP^{-}, and hence Cre^{-}, regions in the dorsal and ventral eyes of α-*Cre* mice (Xu et al., 2007). The number of sphere forming cells was also reduced in the eyes of P10 and adult βcat^{act} mice compared with littermate controls (P10: 36 spheres per eye in βcat^{+} ($n=4$) versus 5.75 spheres per eye in βcat^{act} ($n=4$); adult: 50 spheres per eye in βcat^{+} ($n=2$) versus 4.5 spheres per eye in βcat^{act} ($n=2$)). The reduction in retinal sphere number in the βcat^{act} mice is difficult to reconcile with the increased size of the CB and previous reports implicating Wnt pathway activation in RSC self renewal (Kubo et al., 2003). Pax6 expression is required for RSC formation and growth (Xu et al., 2007), which raised the possibility that the reduction in RSC sphere number in the βcat^{act} could be secondary to a loss of *Pax6* expression in pigmented cells that expressed the stabilized β-catenin allele. However, Cre expression and an increase in TCF/Lef-LacZ reporter activation were not detected in the RPE overlying the CM in the embryonic βcat^{act} mice (Figs. 6F and Fig. 3F). Thus, the reduction in RSC spheres in vitro in the βcat^{act} model is unlikely to be caused by a primary defect in the pigmented cells of the CB, but instead could be secondary to abnormal development of peripheral eye structures.

Discussion

β-catenin signaling promotes the development of the CM and peripheral eye fates

We show here that activation of the canonical Wnt signaling pathway is sufficient and necessary for the development of the peripheral retina. We base our conclusions on the following observations. Pharmacological and genetic stabilization of β-catenin induces the expression of CM genes, downregulates the expression of NR genes and results in an expansion of peripheral eye structures. Expression of stabilized β-catenin in vivo was also associated with proliferation changes that were reminiscent of the lower rates of proliferation that have been documented in the CM (Fischer and Reh, 2000; Kubota et al., 2004). Conversely, Cre-mediated inactivation of β-catenin in vivo results in reduced CM gene expression and a reduction in the size of the CM and the folds of the CB.

The effects of acute and chronic β-catenin signaling that we document in the mouse retina are in excellent agreement with the findings of Cho and Cepko (2006), who also observed growth inhibition, suppression of NR marker expression and promotion of CB/iris cell fates following expression of stabilized β-catenin in the chick retina. Importantly, we show that in βcat^{act} transgenic mice these changes are autonomous to the β-catenin expressing cells and were observed at early stages when the eyecup was normal in size and where Cre^{+} cells were readily detected in the presumptive NR region. Since we did not observe an increase in cell death at two developmental stages or a selective increase in proliferation of Cre^{+} cells, we conclude that stabilized β-catenin drives CM specification by transdifferentiation of NR cells. However, formal proof of transdifferentiation will require genetic fate mapping studies.

Activation of stabilized β-catenin in the central retina with the *Six-Cre* transgene resulted in very similar retinal defects, including *Chx10* downregulation, microphthalmia and ectopic pigmented cells (Fu et al., 2006). It is possible that this phenotype represents the induction of a similar CM developmental program as we observed with the α-Cre driver, however, this will have to be substantiated with additional markers. Together with the induction of ectopic CM gene expression in Li^{+}-treated explants, these data suggest that the entire eyecup is competent to respond to β-catenin signaling by inhibiting neurogenesis

and becoming CM, at least at early stages. Clearly then, restricting activation of this pathway to the CM region is critical for proper partitioning of the eyecup into central NR and peripheral non-neural-fated CM, which is likely to be accomplished by restricting the location of the relevant ligand that activates this pathway.

The decay of Wnt reporter activation in retinal explants suggests that there is a loss of Wnt signals in these cultures, possibly from the RPE or periocular mesenchyme, which are removed from the explants. Wnt2b is an excellent candidate to mediate this signal as it is expressed in the RPE overlying the peripheral retina in the developing optic cup in chick and mice (Jasoni et al., 1999; Kubo et al., 2003; Liu et al., 2003). Expression of the cWnt2b ligand in chick has been shown to inhibit differentiation and to promote peripheral eye fates characteristic of the CMZ (Kubo et al., 2003, 2005) or more distal CB and iris (Cho and Cepko, 2006). In contrast, we have found that ectopic *Wnt2b* expression in mouse retinal explants does not activate the TCF/Lef-LacZ reporter and has no effect on gene expression (Liu and Wallace, unpublished observations). Interestingly, ectopic *Wnt2b* expression in the chick retina induced a milder phenotype compared with the expression of constitutively active β-catenin, which Cho and Cepko (2006) suggest is due to attenuation of ligand activity by extracellular antagonists, such as Sfrp2. Alternatively, our failure to induce CM development by ectopic *Wtn2b* expression in retinal explants could reflect a requirement for a synergistic signal that is missing in the explant cultures or that Wnt2b is not the relevant signal that induces β-catenin signaling in the mouse CM.

The requirement for canonical Wnt signaling in the specification and normal development of the peripheral eye

β-catenin inactivation in vivo is associated with a reduction in CM size and CM-specific gene expression (this study) and a reduction in the size of the CB (this study and Fu et al., 2006). Similarly, disruption of canonical Wnt signaling in the chick retina was shown to reduce peripheral gene expression and result in iris hypoplasia (Cho and Cepko, 2006). However, the CM and CB/iris still develop in these models, raising the question of whether β-catenin signaling is required for the specification of peripheral eye fates. The loss-of-function approach with the α-*Cre* transgene (this study and Fu et al., 2006) likely does not inactivate β-catenin early enough to block signaling in the optic vesicle stage, the earliest time point where this pathway has been shown to be activated in mouse and chick (Cho and Cepko, 2006; Liu et al., 2006). It is therefore possible that, prior to β-catenin inactivation with α-Cre, a sufficient level of Wnt signaling has been achieved to specify the CM. Nonetheless, our findings indicate that sustained β-catenin signaling is necessary for normal development of the CB and iris.

In addition to regulating transcription downstream of Wnt signaling, β-catenin also plays an important role in cell adhesion through its association with cadherins and α-catenin at the plasma membrane, however, the mechanisms that underlie its dual functions are still largely unknown (reviewed by Brembeck et al., 2006). Moreover, whether and how activated Wnt/β-catenin signaling affects the pool of β-catenin employed in cell–cell adhesion also remains to be clarified (reviewed by Brembeck et al., 2006). In addition to the downregulation of CM marker expression (in this study), the retinas of βcat^{null} mice exhibit severe cellular disorganization, which Fu et al. (2006) show is secondary to altered cell adhesion. It is thus possible that some of the retinal disorganization that we observed in the βcat^{act} mice, especially at later developmental stages, could also reflect changes in cell adhesion.

Canonical Wnt signaling and proliferation in the neural retina

There is considerable controversy with respect to the neurogenic and proliferative effects of the canonical Wnt pathway in the retina. In *Xenopus*, activation of this pathway is required for retinal neurogenesis (Van Raay et al., 2005), whereas in the chick and mouse retina it has been shown to inhibit neurogenesis (Cho and Cepko, 2006; Fu et al., 2006; Kubo et al., 2003, 2005; Nakagawa et al., 2003 and this study) and neuronal differentiation (Ouchi et al., 2005). Canonical Wnt pathway activation has been shown to stimulate growth in the frog, zebrafish and chick retina (Kubo et al., 2003, 2005; Van Raay et al., 2005; Yamaguchi et al., 2005), however, other studies in chick and mouse have reported either no effect or an inhibitory effect of this pathway on proliferation (Cho and Cepko, 2006; Ouchi et al., 2005). These discrepant effects of Wnt signaling could reflect species-specific differences or experimental differences in the timing, duration or intensity of Wnt signaling. It is also possible that there are regional and temporal differences in the developmental outcome of Wnt signaling in the developing eye, especially given that the outcome of Wnt signaling is temporally regulated in other tissues (Heiser et al., 2006). In this study, β-catenin signaling in the peripheral retina was associated with the acquisition of a proliferation rate that is characteristic of the normal CM. Moreover, this effect was observed at three different developmental stages, further reinforcing our conclusion that, in this context, activation of this signaling pathway is associated with reduced proliferation. The mechanistic basis for the effect of β-catenin signaling on proliferation is not known, but warrants further investigation.

β-catenin-induced Chx10 and Pax6 downregulation reflects an atypical developmental program

The pattern of gene expression in the peripheral eye of the βcat^{act} mice is consistent with conversion of the NR to a proximal CM fate, with the exception of the rapid downregulation of *Chx10* and *Pax6* expression, as both genes are normally expressed in the CM and its derivatives. This observation also raises the possibility that some of the effects that we observe in the βcat^{act} retina reflect a loss of function of these genes. Loss of *Chx10* is associated with increased pigmentation in the NR (Horsford et al., 2005; Rowan et al., 2004) and an increase in RSCs (Coles et al., 2006). It is unlikely, however that all of the βcat^{act} phenotypes reported in the present study are secondary to the loss of *Chx10* expression as RSC number was decreased

and we did not observe pigmentation changes in the embryonic retina of *βcat*^{act} mice. However, Fu et al. (2006) reported that the Chx10^{-} region in the embryonic retina in two different *βcat*^{act} mouse models contains scattered ectopic Mitf^{+} cells, which could account for the increase in pigmentation that is observed at later stages in this model. Haploinsufficiency of *Pax6* is associated with a reduction in the size of the CB and the iris (Davis-Silberman et al., 2005) and homozygous inactivation of *Pax6* in the peripheral retina results in the exclusive differentiation of amacrine cells (Marquardt et al., 2001). While the failure of iris development in the *βcat*^{act} retina could be secondary to loss of *Pax6* expression, these mice do not phenocopy all of the effects associated with loss of Pax6, as we observed that amacrine neuron differentiation is completely abrogated in the Cre^{+} region of the *βcat*^{act} retina (data not shown). Thus, the loss of *Pax6* expression in the *βcat*^{act} model results in a developmental outcome that is distinct from the *Pax6*^{null} retina. Finally, it is also possible that a Pax6^{-}Chx10^{-} cell lineage, which is present in the CM and CB, is induced or expanded by β-catenin signaling. This possibility is consistent with our observation that there are Chx10^{-} cells in the non-pigmented CE that express the *TCF/Lef-LacZ* reporter transgene (Liu and Wallace, unpublished observations).

Aberrant development of peripheral eye structures and microphthalmia in βcat^{act} *mice*

Ectopic expression of constitutively active β-catenin in chick resulted in a retina that resembled the CB as it was thin and folded multiple times (Cho and Cepko, 2006). Although the expression of CM markers is expanded in the *βcat*^{act} mice, this region of the retina does not fold like the CB and the eye is microphthalmic. Rather than promoting the full program of CB development, the primary effect of β-catenin signaling may be to establish the earliest peripheral eye fate, namely the blockade of neurogenesis that characterizes the CM region of the eyecup (see below). The failure of the converted region in the *βcat*^{act} mice to adopt the folded CB structure could be secondary to growth defects and (or) abnormal interactions with periocular mesenchyme as this tissue does play a role in anterior chamber and CB development (Pressman et al., 2000).

It has been suggested that the secretary function of the CE begins prior to the morphological maturation of the CB (reviewed by Hyer, 2004) and that signals from the developing CM are important for coordinating the growth and morphogenesis of the optic cup (reviewed by Beebe, 1986). The conversion of a large region of the NR to the slower growing CM and interference with the vascular supply due to the abnormal migration of mesenchyme into the vitreous could markedly reduce growth of the eyecup and ultimately perturb the CB developmental program.

Canonical Wnt signaling and the development of RSC

Altered development of the CM was also associated with changes in the number of CB-derived RSCs, as measured in a clonal sphere forming assay. In both gain and loss-of-function mouse models, the number of spheres was reduced. While this result is consistent with the smaller CB in the *βcat*^{null} mice, it is inconsistent with the increase in the size of the CB in the *βcat*^{act} mice. The reduction in sphere forming cells in the *βcat*^{act} could be secondary to the downregulation of *Pax6* expression in cells that express stabilized β-catenin as Pax6 is required for the sphere forming potential of RSCs (Xu et al., 2007). However, such a mechanism would require that the α-*Cre* transgene activates the stabilized β-catenin allele in the pigmented cells of the CB. We do not detect *Cre* expression in the embryonic retina in the region of the RPE that gives rise to the pigmented layer of the CB, although it is expressed in a subset of cells at the very tip of the eyecup, the prospective inner pigmented iris epithelium (Fig. 3B insert). However, we cannot rule out the possibility that activation of β-catenin in the pigmented CB at a later stage could inhibit RSC function as, in contrast to a previous report (Davis-Silberman et al., 2005), we have found evidence of Cre activity in some of the pigmented CB cells in the postnatal retina (Coles and van der Kooy, unpublished). It is possible that a precise level of β-catenin is required for the maintenance of RSCs, and that deviations from that level do not allow maximal maintenance of RSC numbers. Alternatively, it is also possible that the reduction in RSCs in the *βcat*^{act} mice is non-autonomous to the RPE and occurs because of aberrant CB development, abnormal interactions with periocular mesenchyme or inefficient dissociation prior to cell culture due to changes in the adhesive properties of the cells in the peripheral eye.

The role of BMP signaling in CB development

There is a considerable overlap in the target genes induced by BMP and Wnt signaling in the eye, which raises the question of how these two pathways might converge to pattern the CB. *BMP4* and *BMP7* are expressed in the CM and BMP signaling is required for CB development and for the maintenance of *Otx1*, *Msx1*, *BMP4* and *BMP7* expression in the CM (Zhao et al., 2002). While it has not been established whether *Otx1* is a direct target of BMP, it is required for CB development as this structure does not develop in *Otx1* null mice (Acampora et al., 1996). Our results indicate that *Otx1* might be a target of Wnt signaling as the expression of *Otx1* and activation of the TCF/Lef-LacZ reporter overlap in the optic vesicle and the optic cup (Liu et al., 2006), Li^{+} induces ectopic *Otx1* expression in retinal explants and *Otx1* expression is expanded in the *βcat*^{act} mice.

Msx1 is another potential effector of β-catenin and BMP signaling in the peripheral eye. *Msx* genes belong to a family of homeodomain transcriptional repressors that are involved in a number of developmental processes, including nervous system development (reviewed by Ramos and Robert, 2005) and the expression of *Msx* genes is frequently induced by BMP signaling (Liu et al., 2004). *Msx1* and *Msx2* are expressed in the CM (Monaghan et al., 1991), however, eye phenotypes in *Msx* null mice have not been described. Ectopic expression of *Msx1* has been shown to promote cell death and to inhibit neurogenesis and bHLH gene expression in the developing chick neural tube (Liu et al., 2004); ectopic *Msx2* expression is associated with increased cell death, as well as the expansion of

RPE/CM marker expression to the entire NR (Wu et al., 2003). Although BMP4 is required for the maintenance of *Msx2* expression in the optic vesicle (Furuta and Hogan, 1998) it may not be sufficient to induce *Msx* expression in the eyecup. We show here that increased *BMP4* expression does not induce the expression of CM markers in adjacent Cre^{-} cells in the βcat^{act} mice and BMP4 does not induce *Otx1* expression in retinal explants (data not shown). *Msx1* and *Msx2* have also been shown to be targets of Wnt signaling (Willert et al., 2002) and BMP and β-catenin signaling synergize to induce the expression of *Msx2* in ES cells (Hussein et al., 2003). Thus, it is possible that in the context of eye development these two signaling pathways converge to promote the expression of *Msx* genes and other CM markers, which in turn inhibit neurogenesis by repressing bHLH proneural gene expression.

Conservation of canonical Wnt signaling in the establishment of borders between neural and non-neural tissues

There are striking similarities in the role of Wnt and BMP signaling in establishing boundaries between neuroepithelium and secretary epithelium elsewhere in the brain. In the developing telencephalon, Wnt genes, including *Wnt2b*, are expressed in the cortical hem, a transient structure that forms the boundary between the hippocampus and the non-neural choroid plexus (Grove et al., 1998). Like the non-pigmented epithelium of the CB, cells of the prospective choroid plexus convert from a neuroepithelium to a single layer of cuboidal secretary epithelium and BMP and Wnt signaling play an important role in the development of this structure (Grove et al., 1998; Hebert et al., 2002).

Wnt signaling also appears to be playing a conserved role in controlling eye field specification in *Drosophila*. In the eye–antennal disc, *wg* expression is required to establish the border between the retina and adjacent head structures by antagonizing the expression of eye specification genes (Baonza and Freeman, 2002). Wg is also required to control neuronal differentiation in the *Drosophila* eye. Neuronal differentiation in the eye imaginal disc is propagated as a wave, the morphogenetic furrow, which travels across the eye disc in a posterior to anterior direction. *wg* expression at the lateral margins of the eye imaginal disc prevents lateral furrow initiation, thereby restricting furrow development to the posterior part of the disc (Treisman and Rubin, 1995). Thus, in flies and mammalian eyes, Wnt signaling is demarcating the boarder between neural and non-neural components of the eye.

Materials and methods

Transgenic mice

The α-*Cre* transgenic mice with a downstream GFP reporter tag were obtained from P. Gruss (Marquardt et al., 2001) and were maintained on a C57BL/6 background. The *TCF/Lef-LacZ* reporter transgenic mice were obtained from D. Dufort (Mohamed et al., 2004) and were maintained on a CD1 background. α-*Cre* mice were crossed with *TCF/Lef-LacZ* mice to generate α-*Cre;TCF/Lef-LacZ* mice. Mice heterozygous for a conditional β-catenin gain-of-function allele, in which exon 3 was flanked by loxP sites ($Catnb^{+/lox(ex3)}$) (Harada et al., 1999), were maintained on a C57BL/6 background. Heterozygous α-*Cre;TCF/Lef-LacZ* mice were crossed with $Catnb^{+/lox(ex3)}$ mice to generate α-*Cre;*$Catnb^{+/lox(ex3)}$ (referred to as βcat^{act}) or α-*Cre;*$Catnb^{+/lox(ex3)}$*;TCF/Lef-LacZ* (referred to as βcat^{act}*-LacZ*) mice. Control mice, simplified as wild-type mice, were α-*Cre;*$Catnb^{+/+}$ (referred to as βcat^{+}) or α-*Cre;*$Catnb^{+/+}$*;TCF/Lef-LacZ* (referred to as βcat^{+}*-LacZ*) littermates, respectively. We also generated βcat^{act} transgenic mice on a pure C57/BL6 background and identified 2 mice out of 7 βcat^{act} mice with CM defects in comparison to 100% ($n=11$) of βcat^{act} with C57/BL6xCD1 background that exhibited CM phenotypes as described in the present study, suggesting that the CM phenotypes are, to a certain extent, strain dependent. $Catnb^{flox/flox}$ mice, in which two loxP sites were flanking exons 2 and 6 (Huelsken et al., 2001), were obtained from J. Huelsken. $Catnb^{flox/flox}$ mice were crossed with α-*Cre* mice to generate α-*Cre;* $Catnb^{flox/flox}$ mice (referred to as βcat^{null}). Genotyping for the α-*Cre*, *TCF/Lef-LacZ* transgene, $Catnb^{+/lox(ex3)}$ and $Catnb^{flox/flox}$ allele was performed by PCR with the following primer pairs: α-*Cre* (forward (F) 5′-atgcttctgtccgtttgccg-3′ and reverse (R) 5′-cctgttttgcaggttcagcg-3′); *TCF/Lef-LacZ* (F 5′-cagtggcgtctggcggaaaacctc-3′ and R 5′-aaacaggcggcagtaaggcggtcgg-3′); $Catnb^{+/lox(ex3)}$ (F 5′-gacaccgctgcgtggacaatg-3′ and R 5′-gtggctgacagcagc-ttttctg-3′); $Catnb^{flox/flox}$ (F: 5′-act gcc ttt gtt ctc ttc cct tct g-3′; R: 5′-cag cca agg aga gca ggt gag g-3′).

In situ hybridization, immunohistochemistry, detection of β-galactosidase activity and histological analysis

Embryonic retina or retinal explants were prepared for ISH, IHC and detection of β-gal activity by X-gal staining as described previously (Dufort et al., 1998; Jensen and Wallace, 1997; Wallace and Raff, 1999). Note that to achieve a high level of β-gal activity, the fixation time in 4% paraformaldehyde was limited no longer than 3 h. For ISH, the following DIG-labeled antisense riboprobes were used: *BMP4* (a kind gift from J. Wozney), *Chx10* (a kind gift from R. McInnes), *Crx* (a kind gift from C. Cepko), *CyclinD1* (a kind gift from G. Peters), *CyclinD2* (a kind gift from N. Pringle), *Lef1* (a kind gift from J. Meeldijk), *Math3 (*a kind gift from T. Glaser), *Msx1* (a kind gift from Y.H. Liu), *Otx1* (a kind gift from M. Takahashi), *Pax6* (a kind gift from V. van Heyningen), *Pitx2* (a kind gift from P. Gage), *Sfrp2* (a kind gift from A. Rattner). For IHC, the following primary antibodies were used: goat polyclonal anti-Brn3B (Clone C-13, Santa Cruz Biotechnology); rabbit polyclonal anti-collagen IV (Biogenesis); rabbit polyclonal anti-GFP (Molecular Probes); mouse monoclonal anti-Ki67 (BD Bioscience); mouse monoclonal anti-smooth muscle actin (SMA) (clone 1A4, Sigma). All sections were viewed under a Zeiss Axioplan microscope and digital images were captured using an Axio Vision 2.05 (Zeiss) camera and processed with Adobe Photoshop®.

The labeling index for $Ki67^{+}$ cells in the CM and NR was quantified in retinal sections from βcat^{+} ($n=2$ eyes at E11.5 and E14.5, 6 eyes at E15.5) and βcat^{act} ($n=2$ eyes/age) mice that were stained for Ki67 and DAPI. The CM in the nasal and temporal sides of the eye was identified by the expression pattern of *Msx1* (control mice) and Cre and *Msx1* (βcat^{act} mice). The neural retina was defined as a region of the neuroblast layer (e.g. not including the RGC layer at E14.5 and E15.5) in the middle third region away from the optic nerve (control mice) and the Cre^{-} region of the retina (βcat^{act} mice). Data are the average±SD of the labeling indices from 4 CM and 4 NBL from each stage/genotype.

Histological analysis of βcat^{null} tissue was performed as described previously (Ren et al., 2000). Briefly, P10 mice were killed by cervical dislocation. The eyes were removed immediately and immersed in fixation solution containing 2% paraformaldehyde and 2.5% glutaraldehyde for overnight, dehydrated through a graded series of ethanol and embedded in Embed 812. Using a diamond knife on an ultramicrotome, 1 μm sections were made along the horizontal meridian. The sections were stained with toluidine blue.

Retinal explant culture

Retinal explants from E14.5 mice were established by removing the RPE and placing the globe with lens side down on a polycarbonate filter (pore size: 0.8 μm; Nucleopore). The globe was opened at the optic nerve head and the pieces of the retina (still attached to the lens) were flattened to the filter. Globes were dissected in MEM (Sigma). Explants were cultured under serum free conditions as described previously (Wang et al., 2005) and were treated with 20 mM LiCl or NaCl as control.

Quantitative RT-PCR

Quantitative RT-PCR (Q-PCR) analysis was carried out on E14.5 retinal explants cultured for 24 h after Li^+ treatment. The expression of *Crx*, *Dll1*, *Math3*, *Otx1* and *Pax6* was examined in 6 independent experiments and RNA samples were extracted pools from 5 to 7 explants. Superscript II reverse transcriptase reaction kit (Invitrogen) was used to synthesize first-strand cDNA according to the manufacturer's instruction. Q-PCR was performed on Mx4000 with the following cycling parameters: 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. Efficiency of the primer pairs was tested on P0 retinal RNA. Fold change of each gene was calculated by the 2^{-Ct} method adjusted with the amplification efficiency of each gene (Stratagene online introduction). The result was normalized to *HPRT* and *β-actin* and gave similar results. Statistical significance was evaluated by Student's *t*-test (Excel; Microsoft, Cupertino, WA). Sequences of the primer pairs: *Crx* (forward (F) 5′-caggtctggttcaagaatcg-3′ and reverse (R) 5′-acgagccttggtctgtgc-3′), *Dll1* (F 5′-cactgacccaatctgtctgc-3′ and R 5′-gatgcactcatcgcagtagc-3′), *HPRT* (F 5′-ctttgctgacctgctggatt-3′ and R 5′-ctttgctgacctgctggatt-3′), *Math3* (F 5′-accccgggaaagagaatc-3′ and R 5′-tgagagctcagacctttgtcc-3′), *Msx1* (F 5′-accacctggtcccttctctt-3′ and R 5′-gggctcatctcttgaagcac-3′), *Otx1* (F 5′-tcacagctggacgtgctc-3′ and R 5′-tgaaccaaacctggactctg-3′), *Pax6* (F 5′-cggcagaagatcgtagagc-3′ and R 5′-tggatacgttttcattgtccag-3′).

Sphere forming assay

The dissection and sphere formation culture were performed as previously described (Tropepe et al., 2000). Briefly, the PCE was dissected out and treated with Dispase (10 min at 37 °C), followed by treatment with a cocktail of 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase and 0.2 mg/ml kynurenic acid (Sigma) (15 min at 37 °C). The treated PCE were dissociated to single cells and plated in SFM + FGF2 (20 ng/ml) at 10 cell/μl on 24-well plate (500 μl/well) and incubated in 37 °C for 7 days followed by sphere counting. For both loss and gain-of-function mouse models, two independent experiments were performed and showed similar results.

Acknowledgments

We are grateful to Drs. P. Gruss, D. Dufort, and J. Huelsken for kindly providing us with transgenic mouse strains and Drs. J. Wozney, R. McInnes, C. Cepko, G. Peters, N. Pringle, J. Meeldijk, T. Glaser, Y.H. Liu, M. Takahashi, V. van Heyningen, P. Gage and A. Rattner for riboprobes. We thank Dr. Rod Bremner for critical reading and very helpful suggestions on this manuscript. We thank Brian McNeill for help in performing Q-PCR. This work was supported in part by grants from the Canadian Institutes of Health Research, NIH, the Foundation Fighting Blindness Canada and the Stem Cell Network of Canada. H. Liu was a recipient of a studentship from the Foundation Fighting Blindness Canada and University of Ottawa, Canada.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.04.052.

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