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Hairy1 acts as a node downstream of Wnt signaling to maintain retinal stem cell-like progenitor cells in the chick ciliary marginal zone

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In the vertebrate retina, stem cell-like progenitor cells are maintained in a distinct region called the ciliary marginal zone (CMZ). Canonical Wnt signaling regulates the maintenance of the progenitor cells in the CMZ. However, its downstream molecular mechanisms have remained largely unclear. Here, we show that chick Hairy1, an established Notch signaling effector, mediates the Wnt-dependent maintenance of CMZ progenitor cells in chicken. Interestingly, unlike other developmental contexts in which Hes gene expression is regulated by Notch signaling, Hairy1 expression in the CMZ is regulated by Wnt signaling. Hairy1 is necessary and sufficient for the expression of a set of molecular markers characteristic of the CMZ, and Wnt2b fails to induce CMZ markers when Hairy1 activity is inhibited. Furthermore, microarray analysis identifies multiple Wnt-responsive transcription factors that activate Hairy1 expression. We thus propose that Hairy1 functions as a node downstream of Wnt signaling to maintain progenitor cells in the chick CMZ.

KEY WORDS: Retina, Stem cells, Wnt, Chick Hairy1 (c-hairy1), Ciliary marginal zone

INTRODUCTION

In the vertebrate retina, six types of neuron and one type of glia (Müller glia) are produced from a common multipotent progenitor cell according to intrinsic and extrinsic cellular cues (reviewed by Livesey and Cepko, 2001). The proliferative capacity of these progenitor cells depends on their location within the optic vesicles. In the central region of the retina, they proliferate for only a limited period to generate a relatively small number of progeny neurons and glia. By contrast, a distinct region of the peripheral optic vesicle between the ciliary epithelium and the neural retina, called the ciliary marginal zone (CMZ), contains stem cell-like progenitor cells that produce a much larger number of progenies compared with the central progenitor cells (reviewed by Moshiri et al., 2004; Perron and Harris, 2000). The number of cells produced from progenitor cells in the CMZ varies among individual vertebrate species (reviewed by Moshiri et al., 2004; Perron and Harris, 2000). In fish and frogs, they continue to divide throughout the lifetime to add new retina cells at the periphery, thus serving as bona fide retinal stem cells (Hollyfield, 1968; Jacobson, 1968; Johns, 1977). In the chicken retina, the CMZ incorporates BrdU even after hatching (Morris et al., 1976), and the late-proliferating cells can differentiate into multiple types of neuron, including amacrine and bipolar cells (Fischer and Reh, 2000). The CMZ of adult mice does not contain proliferating cells; however, retinal stem cells can be isolated in a neurosphere culture from the pigmented ciliary body, which is topographically equivalent to the CMZ (Tropepe et al., 2000). Hence, the stem cell-like feature of the CMZ progenitor cells is conserved among a wide range of vertebrate species.

The Wnt signaling pathway is a signal transduction cascade that plays key roles in a variety of developmental processes (reviewed by Logan and Nusse, 2004; Moon et al., 2002). Binding of Wnt ligand to its receptor frizzled triggers an intracellular signaling cascade that results in the transcriptional activation of target genes by β-catenin and the Tcf/Lef complex (reviewed by Logan and Nusse, 2004; Moon et al., 2002). The canonical Wnt pathway controls the differentiation of tissue-specific stem cells, including various epithelial tissues and the hematopoietic systems (reviewed by Blanpain et al., 2007; Clevers, 2006; Reya and Clevers, 2005). In the retina, Wnt2b is expressed in the anterior rim of the optic vesicles, which are neighboring stem cell-like progenitor cells in the CMZ (Cho and Cepko, 2006; Jasoni et al., 1999; Kubo et al., 2003; Liu et al., 2003; Zakin et al., 1998). In transgenic mice carrying a Wnt reporter cassette in which *lacZ* is expressed under the control of Wnt-responsive elements, reporter activity was observed in the peripheral part of the retina, including the CMZ (Liu et al., 2003; Liu et al., 2007). Similarly, Wnt activity is also observed in a peripheral region of the retina in zebrafish (Dorsky et al., 2002; Yamaguchi et al., 2005) and in the chicken embryo (Cho and Cepko, 2006). Functional relevance of the activation of Wnt signaling in the CMZ has been confirmed by a number of experiments (Cho and Cepko, 2006; Kubo et al., 2003; Kubo et al., 2005; Liu et al., 2007; Nakagawa et al., 2003). Wnt2b inhibits neuronal differentiation of retinal progenitor cells when overexpressed in ovo, and blocking its activity induces premature differentiation of the peripheral retina (Kubo et al., 2003). A soluble form of Wnt2b maintains the proliferative capacity and multipotency of progenitor cells in the CMZ in clonal cultures (Kubo et al., 2003). The central progenitor cells that normally remain undifferentiated for only a limited period of time continue to proliferate in a retinal explant that stably expresses Wnt2b, and these cells subsequently differentiate into neurons and glia upon blocking of the Wnt signaling (Kubo et al., 2005). All of these observations are consistent with the idea that Wnt signaling endows progenitor cells in the CMZ with a stem cell-like capacity distinct from the progenitor cells in the central retina. Wnt signaling has also been demonstrated to control both the differentiation of anterior eye structures, including the iris and the ciliary epithelium (Cho and Cepko, 2006) and the trans-differentiation of the neural retina into the ciliary margin (Liu et al.,

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2007). In the *Xenopus* retina, frizzled 5 promotes the neural potential of retinal progenitor cells rather than inhibiting their neuronal differentiation (Van Raay et al., 2005), although no Wnt ligand has so far been identified in this system.

Downregulation of proneural gene expression is essential for Wnt2b-induced inhibition of retinal progenitor cell differentiation (Kubo et al., 2005). Because the β-catenin and Tcf/Lef complex functions as a transcriptional activator, certain transcriptional repressors should function as Wnt signaling effector genes in the CMZ to suppress proneural gene expression. The Hes (hairy and enhancer of split) family of genes play essential roles to keep progenitor cells undifferentiated in a wide variety of tissues (reviewed by Davis and Turner, 2001; Fisher and Caudy, 1998; Kageyama et al., 2007; Ross et al., 2003). In the nervous system, the primary molecular action of the Hes family of proteins is to functionally antagonize proneural genes, such as the Atonal and Achaete-scute family of transcription factors (reviewed in Kageyama et al., 2005; Ross et al., 2003). In the retina, Hes family genes block neuronal differentiation (Hashimoto et al., 2006) and induce Müller cell fate (Furukawa et al., 2000; Hojo et al., 2000; Scheer et al., 2001; Takatsuka et al., 2004). It is widely accepted that the expression of Hes genes is directly regulated by the Notch signaling pathway through a transcriptional activator complex that consists of RBP-Jκ and the intracellular domain of the Notch receptor, which is cleaved upon binding of its ligand Delta (Artavanis-Tsakonas et al., 1999; Selkoe and Kopan, 2003; Yoon and Gaiano, 2005). However, Hes1 remains expressed in mice lacking the effector gene RBP-Jκ (de la Pompa et al., 1997). In addition, increasing evidence suggests that Hes genes can also be regulated independently of Notch signaling in particular regions of the nervous system, especially in a boundary of neuromere compartments (Baek et al., 2006; Geling et al., 2004; Kageyama et al., 2005; Kageyama et al., 2007). These observations support the idea that Notch signaling is not the sole regulator of the Hes family of genes. The upstream signaling that controls the expression of these Hes genes, however, remains largely unknown.

In this study, we show that chick Hairy1 (c-hairy1), one of the Hes genes, functions downstream of Wnt signaling to maintain the progenitor cells in the CMZ during the development of the chicken retina. Interestingly, unlike many other developmental contexts, Wnt signaling controls the expression of Hairy1 in the CMZ independently of Notch activity. In addition, Hairy1 overexpression inhibits neuronal differentiation and induces CMZ-specific markers in the central retina, which mimics the activity of Wnt2b overexpression. A dominant-negative form of Hairy1 suppresses the effect of Wnt signaling, which is to induce CMZ characteristics in the central retina. In addition, Wnt signaling activates multiple transcription factors that subsequently upregulate Hairy1 expression. Taken together, we propose that Hairy1 functions as an essential node downstream of Wnt signaling to maintain progenitor cells in the chick CMZ.

MATERIALS AND METHODS

Differential screening of putative Wnt-responsive genes

Isolation of cDNAs from single cells sorted by fluorescence-activated cell sorting (FACS) and subsequent differential screening was performed as previously described (Fujimura et al., 2006; Sone et al., 2007) with certain modifications. Briefly, cDNAs were synthesized from FACS-sorted single cells by the representative PCR, and Wnt-responsive cells were retrospectively identified based on Lef1 expression on Southern blots. Subtractive PCR was carried out using cDNAs derived from Lef1-positive and -negative cells following the manufacturer's instructions (PCR-Select cDNA Subtraction Kit; BD Biosciences). A plasmid library was constructed from the resultant PCR products and was differentially screened with probes prepared from original cDNAs. Clones that reacted with probes from Lef1-positive cells were selected for further analysis.

Immunohistochemistry and in situ hybridization

The antibodies used were the following: mouse anti-Hu (clone 16A11, Molecular Probes), mouse anti-BrdU (clone BU33, Sigma), rabbit anti-visinin (a kind gift from Dr Miki, Osaka University), rat anti-HA (clone 3F10, Roche), Cy3-conjugated anti-mouse IgG (Chemicon) and Alexa-fluor-488-conjugated anti-rabbit or rat IgG (Molecular Probes). The tissue explants or embryos were fixed for 1 hour at room temperature in 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose for 1 hour, embedded in OCT compounds and sectioned at a thickness of 10-15 μm. The sections were then permeabilized in 100% methanol for 5 minutes at –20°C and processed for standard immunostaining protocol. For BrdU staining, the sections were treated with 4N HCl for 30 minutes at room temperature. Confocal images were collected using an LSM510 microscope (Zeiss). For in situ hybridization, embryos were fixed in 4% paraformaldehyde overnight at 4°C. For collagen IX, GS, Hes5, Myc, Elk3, Lmo4 and Zic2, the cDNA fragments corresponding to the coding sequences or the 3′ UTR of the proteins were amplified by RT-PCR and subcloned into pCRII (Invitrogen), which were used to generate cRNA probes.

Vector construction

To make RCAS provirus vectors, cDNA sequences of each construct were initially subcloned into pSLAX13 (Kengaku et al., 1998) to attach 5′ untranslated leader sequences, and the cDNA fragments were subsequently subcloned into RCASBP (A/B) to generate provirus vectors. To generate RCAS frizzled 8-CRD, 293 cells were transfected with pJCH100 (Hsieh et al., 1999), and the cDNA encoding mouse Fzd8-CRD-Fc was isolated by RT-PCR using cDNAs from the transfectants as a template. RCAS HA-Hairy1-ΔW-VP16 construct was generated by fusing Hairy1 cDNA lacking the WRPW motif in-frame to a VP16 transactivation domain from pCS2+ NLS VP16AD (Muraoka et al., 2000), followed by subcloning into pSLAX-HA vector to add the HA epitope tag at its N terminus. To generate expression vectors for Myc, Elk3 and Lmo4, their coding sequences were amplified by PCR using corresponding FANTOM3 mouse cDNA clones as templates and were cloned into pCAG vector (Niwa et al., 1991). Mouse *Zic2* cDNA (Mizugishi et al., 2001) was subcloned into the pCAG vector. The vectors used were RCASBP (A) Wnt2b (a kind gift from Dr Izpisua-Belmonte), RCASBP (B) Wnt2b, RCASBP (A) Delta1 (a kind gift from Dr Henrique), RCASBP (A) Hairy1 (a kind gift from Dr Stern), RCASBP (A) Hes5, RCASBP (B) HA-Hairy1 ΔW VP16, and pCAG β-catenin (Takahashi et al., 2000).

Quantitative PCR

Quantitative PCR analysis was carried out on E6 retinal explants cultured for 24 hours after DAPT treatment. mRNA was extracted from each sample using a QuickPrep mRNA Purification Kit (GE Healthcare), and cDNAs were then synthesized from mRNAs by using oligo (dT) primer and ReverTra Ace (TOYOBO) according to the manufacturer's instructions. Quantitative PCR was performed in accordance with the manufacturer's instructions, using SYBR Green PCR Master Mix and ABI7900HT (Applied Biosystems). The amount of Hairy1 expression was normalized to that of GAPDH. Data are presented as the mean ± s.d. of triplicate quantifications.

Luciferase assay

To obtain the *Hairy1* promoter sequence, chicken BAC library (Geneservice) was screened using Hairy1 coding sequence as a probe and positive clone (50-I15) was purchased from Geneservice. Putative promoter sequence corresponding to the regions from –2 kb to +2 relative to the putative transcription start site was amplified by PCR and cloned into promoter-less luciferase vector pGL3-basic vector. Embryonic day 1.5 (E1.5) chicken optic vesicles were in ovo electroporated with reporter plasmid (2.5 μg/μl), expression vectors (2.5-5 μg/μl) and pRL-tk (Promega) (0.125 μg/μl) as an internal standard to normalize transfection efficiency. Twenty-four hours after electroporation, eyes were dissected, lysed and assayed for luciferase activity (Toyo Ink). To generate the mutant Hairy1 2

kb reporter (–2 kb mut-Luc), all of putative Tcf-binding sites (5′-ATCAAAG-3′, 5′-ATCAAAC-3′, 5′-GTTTGTT-3′, 5′-AACAAAC-3′) were mutated into sequences to which Tcf does not bind (5′-GCCAAAG-3′, 5′-GCCAAAC-3′, 5′-GTTTGGC-3′, 5′-GCCAAAC-3′) by a PCR-based method.

Microarray analysis

Total RNA was purified from E6.5 retinas transfected either with Wnt2b-, Delta- or mock-expressing RCAS plasmid using Trizol reagent (Invitrogen). Microarray analysis using GeneChip Chicken Genome Array (Affymetrix) was performed according to the manufacturer's protocols. Data were analyzed using GCOS (Affymetrix) and dChip (Li and Wong, 2001). To explore candidates for new Wnt target genes, genes with higher expression in Wnt2b-expressing retina than control or Delta1-expressing retinas were selected for further analysis. The array data are available at GEO (http://www.ncbi.nlm.nih.gov/geo/) through Accession number GSE15382.

In ovo electroporation

In ovo retinal electroporation was carried out as previously described (Kubo et al., 2003). Briefly, optic vesicles of E1.5 retinas were electroporated with expression plasmids and were incubated at 38.5°C until they reached appropriate stages. To study cell proliferation, 100 μl of BrdU (5 mM) was injected into the yolk before harvesting.

Retinal explant cultures

The retinal explant cultures were performed as previously described (Kubo et al., 2005). Briefly, the retina from E4.5 or E6.5 chicken embryos were dissected out, and the surrounding mesenchyme as well as the retinal pigment epithelium was removed. The explants thus prepared were placed in wells of non-coated 24-well dishes (Iwaki, Japan) with 1 ml of culture medium (1:1 mixture of DMEM and Ham's F12 supplemented with 10% FCS), and the dishes were rotated at a speed of 60 rpm on a rotary shaker. Under this culture condition, Müller cell marker GS, which normally is not expressed until late developmental stages, was observed as early as 3 days in vitro. To block the Notch signaling pathway, a γ-secretase inhibitor, DAPT {N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester; Calbiochem}, was added to the culture medium at a concentration of 2 μM.

RESULTS

Identification of Rdh10 as a molecular marker that specifically labels CMZ progenitor cells

We have previously proposed that Wnt signaling regulates the maintenance of retinal stem cells in the CMZ (Kubo et al., 2003; Kubo et al., 2005; Nakagawa et al., 2003). However, all the currently available CMZ markers are also expressed in the iris/ciliary epithelium, raising a possibility that Wnt signaling promotes differentiation of these non-neural anterior eye structures, as proposed by other groups (Cho and Cepko, 2006; Liu et al., 2007). To clarify exactly which cell type Wnt signaling regulates, we first attempted to isolate Wnt-responsive genes and examine their expression pattern in the retina. To isolate Wnt-responsive genes, we utilized the single-cell PCR-based subtraction method (Brady and Iscove, 1993; Dulac and Axel, 1995) (Fig. 1A). cDNAs were synthesized from dissociated single cells derived from the peripheral region of E5 retina, and the Wnt-responsive cells were retrospectively identified by the expression of Lef1, which is known to be upregulated in cells that receive canonical Wnt signaling (Kengaku et al., 1998; Kubo et al., 2003; Schmidt et al., 2000). We then carried out subtractive PCR (Fujimura et al., 2006) using cDNAs derived from Lef1-positive and -negative cells to isolate differentially expressed genes. We identified four genes specifically expressed in the peripheral region of E7.5 retina, which could further be classified into two categories. One group was expressed in a broad area of the most peripheral retina, which gives rise to iris and ciliary epithelium. These genes included connexin 43 (Musil et al., 1990) and opticin (Frolova et al., 2004), which coincided with the expression of iris/ciliary epithelium marker collagen IX (Fig. 1B,D). The other group was expressed exclusively in a distinct intermediate region of the peripheral retina between the presumptive iris/ciliary epithelium and the neural retina. These genes included retinol

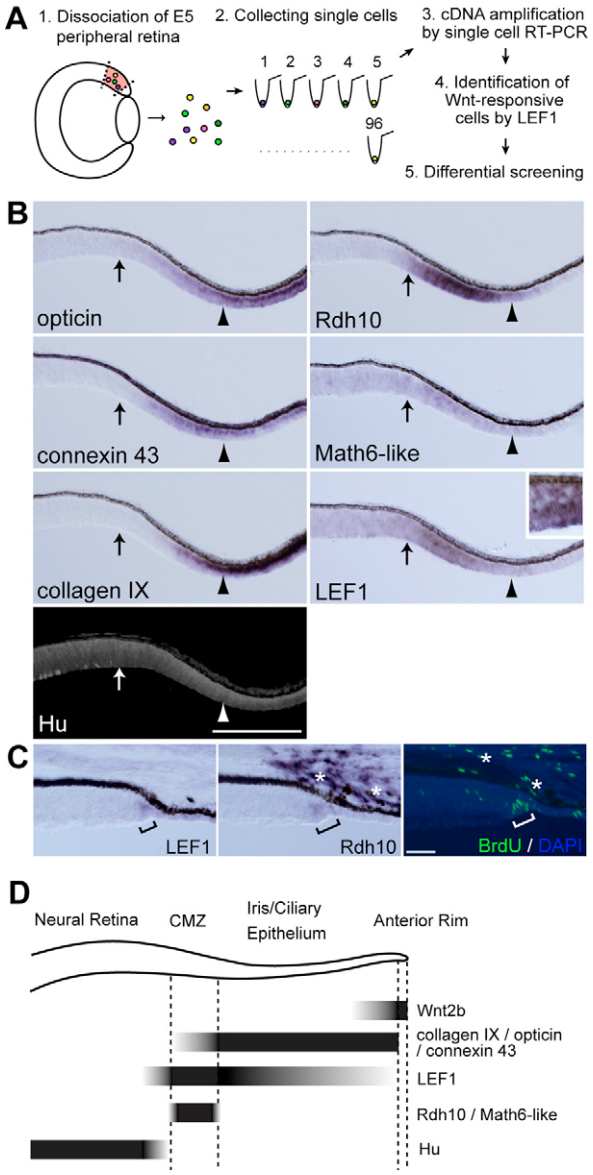

Fig. 1. Subdomains of peripheral retinas can be identified by the expression of Wnt-responsive genes. (**A**) The screening strategy used to isolate Wnt-responsive genes. (**B**) Expression patterns of Wnt-responsive genes that label a subdomain of the peripheral retinas. Adjacent sections of E7.5 retinas were hybridized with antisense probes to detect expression of genes (as listed in the panels), except for Hu, which was detected by immunostaining. Arrows and arrowheads indicate the putative border between the neural retina/CMZ and the CMZ/ciliary epithelium, respectively. Note the mosaic expression of Lef1 in the CMZ (inset). (**C**) Expression patterns of Lef1, Rdh10 and BrdU-incorporated cells in E16 retinas. Adjacent sections were stained for each marker. Brackets show the CMZ that contains proliferating cells. Asterisks indicate the non-retinal expression of Rdh10 or BrdU incorporation in the extraocular mesenchymal cells. (**D**) Expression patterns of the marker genes in the peripheral retinas. Subdomains of the anterior eye structures can be identified by the marker expression. Scale bars: 200 μm in B; 50 μm in C.

dehydrogenase 10 (Rdh10), putative chicken homolog of Math6, and Lef1 (Fig. 1B,D). At E16, when the histogenesis of the retina is almost complete, Rdh10 and Lef1 were expressed in the CMZ, which could be identified by morphological criteria and by the incorporation of BrdU (Fig. 1C). These results indicate that the subdomains of the peripheral region of the retina can be distinguished by means of these molecular markers as early as E7.5, before the emergence of distinct morphological features of the anterior eye structures.

Wnt signaling is required for the formation of the CMZ as well as the iris/ciliary epithelium

We then asked if Wnt signaling could induce the expression of these molecular markers in the central region of the retina, which normally does not express these markers. On the one hand, as previously reported (Cho and Cepko, 2006; Kubo et al., 2003; Kubo et al., 2005), Wnt2b overexpression inhibited neuronal differentiation in the central retina, as revealed by the decrease in retinal neurons positive for Hu (a marker for ganglion and amacrine cells) and visinin (a marker for photoreceptor cells) (Fig. 2A). On the other hand, collagen IX, connexin43 and opticin were ectopically upregulated in the central region of Wnt2b-expressing retinas, suggesting that Wnt signaling induces iris/ciliary epithelium cell fate (Fig. 2A,B). Wnt2b overexpression also induced the ectopic expression of CMZ markers Rdh10 and Math6-like, suggesting that Wnt signaling can promote cell fates specific to the CMZ as well as the iris/ciliary epithelium (Fig. 2A,B). We next carried out loss-of-function experiments by overexpressing the soluble form of the frizzled 8 extracellular domain (Fzd8-CRD), which inhibits the Wnt signaling pathway by sequestering Wnt proteins (Hsieh et al., 1999; Nakagawa et al., 2003). Overexpression of Fzd8-CRD caused a remarkable reduction in the size of non-neural anterior eye structures, as evident from the shortening of the Hu- or visinin-free region (Fig. 2C). In these retinas, the size of both the iris/ciliary epithelium and CMZ was severely decreased, as revealed by the reduction of the expression of collagen IX and Rdh10, respectively

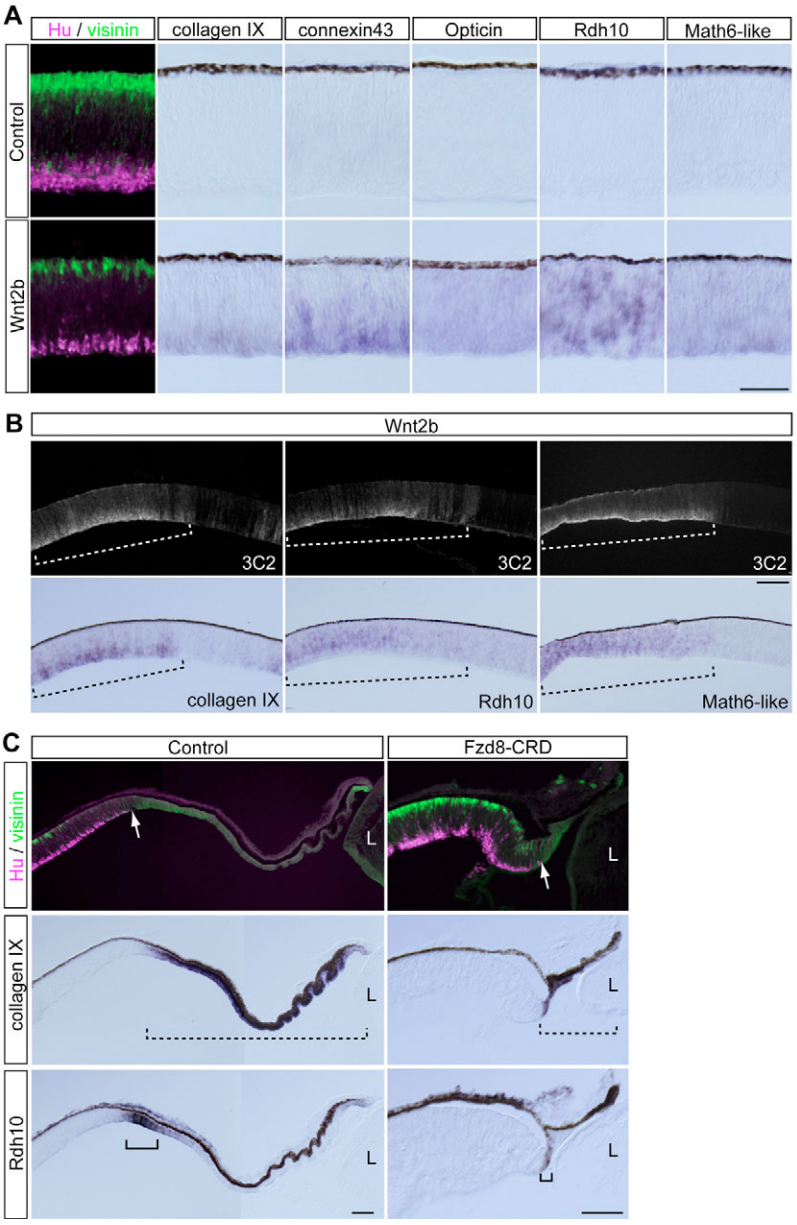

Fig. 2. Wnt2b is necessary for the formation of both iris/ciliary epithelium and CMZ. (**A**) Effect of Wnt2b overexpression in the central retina. Retinas were electroporated with control (top row) and Wnt2b-expressing (bottom row) RCAS provirus, and adjacent sections of E7.5 retinas stained for each marker (as listed at the top). Wnt2b overexpression ectopically induced the expression of both iris/ciliary epithelium and CMZ markers in the central retina. (**B**) Lower magnification of the peripheral retina expressing Wnt2b. The peripheral markers were expressed in RCAS-infected regions detected by 3C2 antibody (dashed bracket). (**C**) Functional blocking of Wnt signaling by Fzd8-CRD. Retinas were electroporated with control and Fzd8-CRD-expressing RCAS provirus, and adjacent sections of the peripheral region of E7.5 retinas stained for markers. The sizes of the iris/ciliary epithelium (dashed bracket) as well as of the CMZ (bracket) were dramatically reduced upon inhibition of Wnt signaling. Note the different sizes of the scale bars in C. Arrows indicate the most peripheral region of differentiated neurons. L, lens. Scale bars: 50 μm in A; 100 μm in B,C.

(Fig. 2C). These results suggest that Wnt2b signaling is both necessary and sufficient for the formation of the CMZ as well as the iris/ciliary epithelium.

Hairy1 expression in the CMZ is regulated by Wnt signaling but not by Notch signaling

Downregulation of proneural genes is crucial for the Wnt-mediated maintenance of the undifferentiated state of progenitor cells (Kubo et al., 2005). Hes genes are well-established effectors of Notch signaling and inhibit neuronal differentiation by functionally antagonizing the proneural genes (reviewed by Davis and Turner, 2001; Fisher and Caudy, 1998; Kageyama et al., 2007; Ross et al., 2003). Although Wnt inhibits neuronal differentiation independently of Notch signaling (Kubo et al., 2005), the anti-neurogenic activity of Hes genes made themselves good candidates as downstream effectors of Wnt signaling. These observations prompted us to examine the expression patterns of Hes genes to test the possibility that they might mediate Wnt signaling to maintain progenitor cells in the CMZ. At least two Hes family genes, Hairy1 and Hes5, were expressed in the chicken retina (Nelson and Reh, 2008). Hairy1 was predominantly detected in the putative CMZ of E5.5 retinas, although weak expression was also observed in the central retinas (Fig. 3A,B). By contrast, Hes5 is expressed in the central region of the retina, which coincided with the expression of Notch1 (Fig. 3A,B). Because Notch1 is absent in the CMZ (Fig. 3B), Hairy1

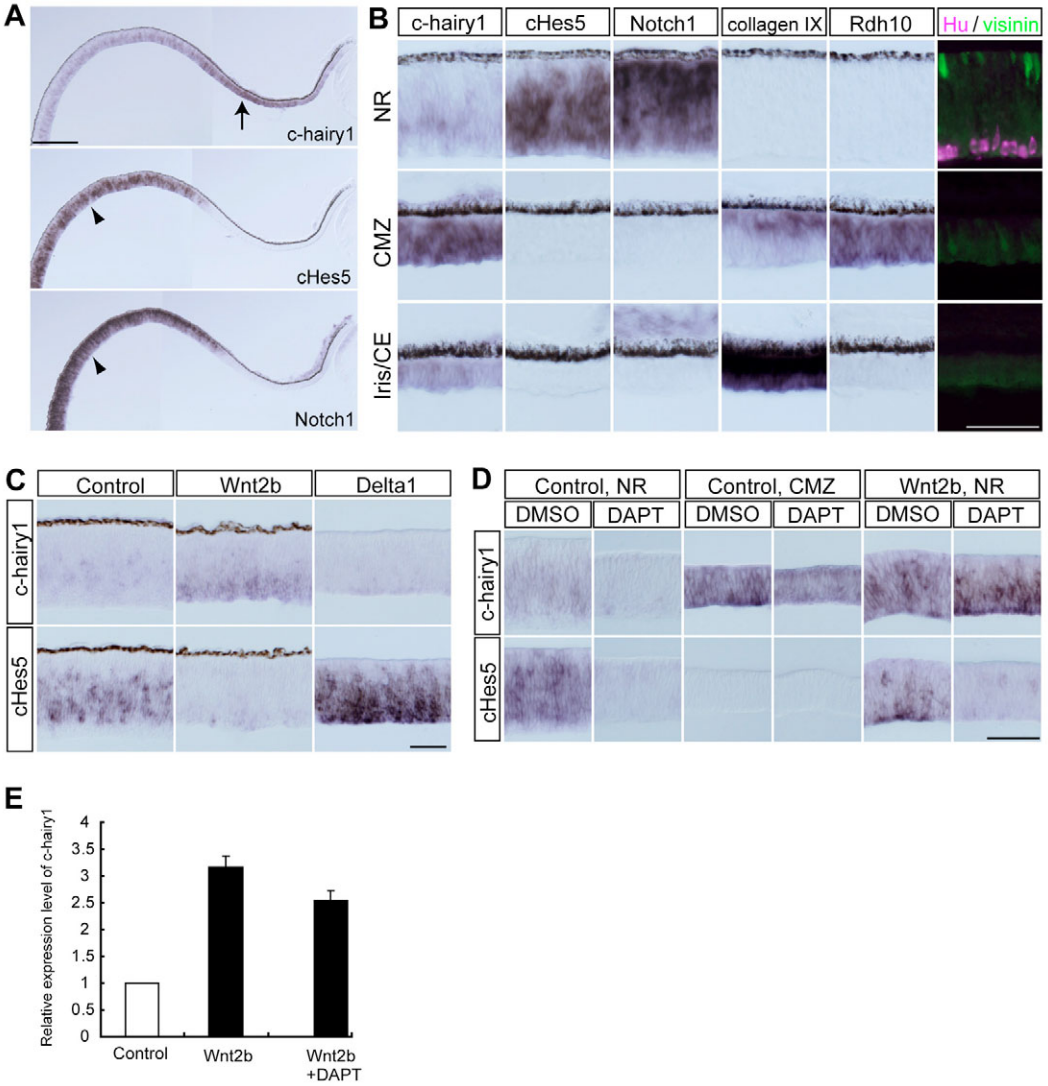

Fig. 3. Expression of Hairy1 in the CMZ is controlled by Wnt2b but not by Notch signaling. (**A**) Expression patterns of Hairy1, Hes5 and Notch1 in the peripheral retina at E5.5. Adjacent sections were hybridized with each probe. Hairy1 is expressed in the putative CMZ (arrow), where Notch1 is not expressed. Arrowheads indicate the expression of Hes5 and Notch1 in the neural retina. (**B**) Higher-magnification images from A-C showing expression of each gene (as listed at the top) in particular regions of the optic vesicles. (**C**) Expression patterns of Hairy1 and Hes5 in the central region of an E6.5 retina electroporated with control, Wnt2b- or Delta1-expressing RCAS provirus. Wnt2b overexpression upregulated Hairy1, whereas overexpression of Delta1 did not. By contrast, Hes5 expression was upregulated by Delta1, but not by Wnt2b. (**D**) Effect of DAPT treatment on Hairy1 and Hes5 expression in retinal explants. The explants were prepared from the central region (NR) or peripheral region (CMZ) of the E4.5 retina and cultured for 24 hours in the absence (DMSO) or presence of DAPT, a Notch-signaling inhibitor. In the right-hand two columns, E6.5 retinas electroporated with Wnt2b-expressing RCAS provirus were used. (**E**) Quantitative PCR analysis of *Hairy1* mRNA expression in retinal explants (n=3). Data are presented as the mean±s.d. of triplicate quantifications. Iris/CE, iris/ciliary epithelium; NR, neural retina. Scale bars: 100 μm in A; 50 μm B-D.

expression in this region was thought to be controlled independently of Notch signaling. To test whether Hairy1 expression in the CMZ was regulated by Wnt signaling, we overexpressed Wnt2b and examined the expression of Hairy1 in the central region of the retina at E6.5, which normally expresses low levels of this molecule. Overexpression of Wnt2b induced prominent expression of Hairy1 in the central retina (Fig. 3C), whereas Hes5 expression was inhibited (Fig. 3C). The same results were obtained by overexpressing a stabilized β-catenin (F.K. and S.N., unpublished observation). We then examined effects of Notch activation on the expression of Hairy1 and Hes5 in the central retina by overexpressing Delta1 (Henrique et al., 1997). In contrast to Wnt2b overexpression, Delta1 upregulated Hes5 expression but not that of Hairy1 (Fig. 3C). These results suggest that Hairy1 is positively regulated by Wnt signaling independently of Notch, whereas Hes5 expression is under the control of Notch signaling. To further confirm this possibility, we prepared retinal explants and treated them with a γ-secretase inhibitor, DAPT, a potent inhibitor of the Notch signaling pathway (Geling et al., 2002). As previously reported (Nelson et al., 2006; Nelson et al., 2007), the DAPT treatment severely inhibited cHes5 expression as well as weak Hairy1 expression in the retinal explants prepared from the central retina (Fig. 3D), suggesting that Notch signaling was efficiently blocked by the drug treatment (Fig. 3D). However, endogenous Hairy1 expression in the CMZ explants was not affected by the DAPT treatment, suggesting that the expression is independent of Notch signaling (Fig. 3D). In addition, upregulation of Hairy1 by Wnt2b overexpression was still observed in explants treated with DAPT (Fig. 3D,E), suggesting that Wnt signaling does not require Notch activity to induce Hairy1 expression in the central retina.

Hairy1 inhibits neuronal differentiation of retinal progenitor cells and ectopically induces CMZ progenitor markers in the central retina

Because Wnt2b controls Hairy1 expression, we speculated that Hairy1 might function as an effector of Wnt signaling during the maintenance of progenitor cells in the CMZ. We first examined whether Hairy1 can mimic the effect of Wnt2b overexpression, which inhibited neuronal differentiation and induced ectopic CMZ markers in the central retina. In Hairy1-expressing retinas, the number of neurons expressing Hu or visinin was reduced compared with the control retinas (Fig. 4A), similar to that observed with Wnt2b overexpression (Fig. 2A). Hes5 overexpression also reduced the number of neurons, although its effect was less clear compared with that of Hairy1 (Fig. 4A,B). We then examined differentiation of glial cells in the retinal explants. GS expression was inhibited in the Hairy1-expressing retinal explants, whereas it was enhanced in the Hes5-expressing retina (Fig. 4A). These data suggest that, like Wnt2b, Hairy1 inhibits both neuronal and glial cell differentiation, whereas Hes5 promotes glial cell differentiation, as has been reported previously for Notch activation (Furukawa et al., 2000; Hojo et al., 2000; Scheer et al., 2001; Takatsuka et al., 2004).

To further determine the cell types induced by Hairy1, we investigated the expression of collagen IX and Rdh10 at E7.5. Hairy1 overexpression induced ectopic expression of Rdh10 in the central region of the retina, whereas expression of collagen IX was not affected (Fig. 4C). These results suggest that Hairy1 can specify the CMZ but not the iris/ciliary epithelium. We also examined cellular proliferation by BrdU incorporation. At E9.5, only 5% of the cells incorporated BrdU in the control retina (Fig. 4D,E). Hairy1 overexpression increased the number of BrdU-positive cells up to

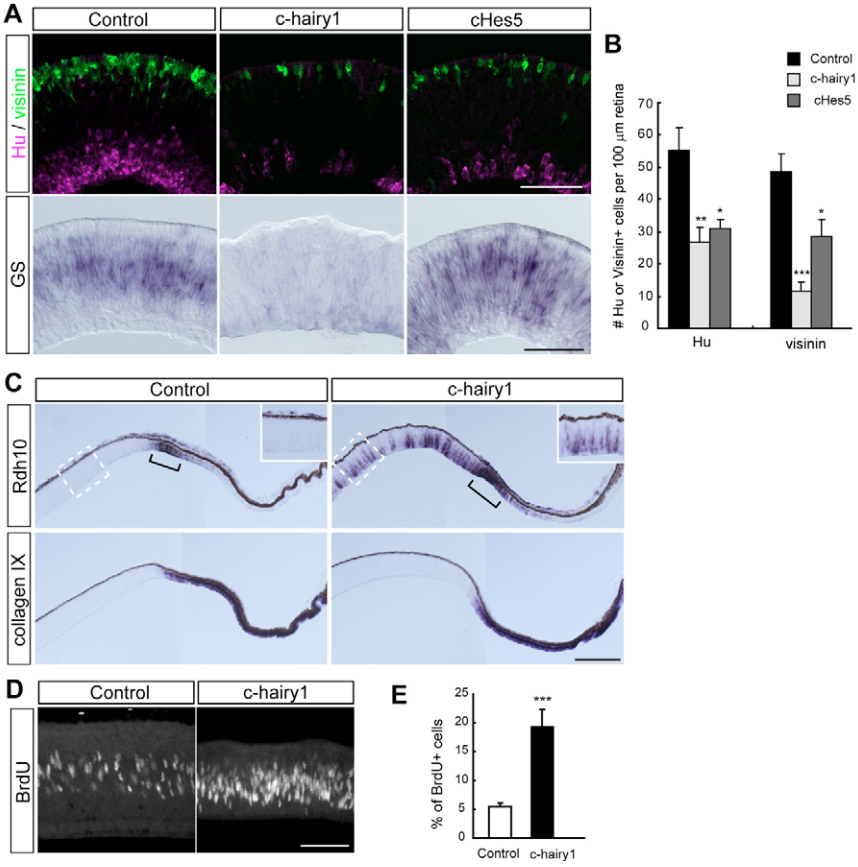

Fig. 4. Overexpression of Hairy1 induces molecular markers characteristic of CMZ progenitor cells in the central retina. (**A**) Expression patterns of Hu/visinin and glutamine synthetase (GS) in retinal explants electroporated with control, Hairy1- or Hes5-expressing RCAS provirus. Hairy1 inhibited both neuronal and glial cell differentiation, whereas Hes5 promoted glial cell differentiation. (**B**) Number of Hu- and visinin-positive cells found per 100 μm width on sections of the retina electroporated with each construct (*n*=4). (**C**) Expression pattern of the CMZ marker Rdh10 and of the iris/ciliary epithelium marker collagen IX in control and Hairy1-overexpressed retina. Note that Hairy1 induced the CMZ marker but not the iris/ciliary epithelium marker. The brackets indicate the putative CMZ region. The insets in C show higher-magnification images of the central region of the retina indicated by the dashed box. (**D**) BrdU incorporation in E9.5 retina electroporated with control and Hairy1-expressing RCAS provirus. More proliferating cells were observed in Hairy1-overexpressing retina. (**E**) Percentage of BrdU-positive cells in the control and Hairy1-expressing retinas (*n*=7). **P*<0.05, ***P*<0.01, ****P*<0.0001 versus control. Scale bars: 50 μm in A,D; 200 μm in C.

fourfold (Fig. 4D,E), suggesting that it endows progenitor cells in the central retina with prolonged proliferative properties similar to those of progenitor cells in the CMZ.

Hairy1 is required for the maintenance of the CMZ progenitor cells

We next investigated the effect of functionally blocking Hairy1 on the maintenance of progenitor cells in the CMZ. To perform the loss-of-function experiments, we utilized a dominant-negative form of Hairy1 (HA-Hairy1-ΔW-VP16) (Fig. 5A), in which the WRPW motif was replaced with the transactivation domain of VP16 (Bae et al., 2005; Bally-Cuif et al., 2000; Shinga et al., 2001; Solter et al., 2006). In the embryos expressing HA-Hairy1-ΔW-VP16, visinin and Hu were ectopically expressed in the peripheral retina at E7.5 (Fig. 5E). These effects were not observed in the central retina, which normally does not express Hairy1 (Fig. 5B,D), supporting the specificity of the dominant-negative molecule (see Fig. S1 in the supplementary material). The expression of Rdh10 (Fig. 5F,F′,G,G′), as well as the number of proliferating cells incorporating BrdU (Fig. 5H-J), was dramatically reduced in the retina overexpressing the dominant-negative molecule, suggesting that Hairy1 is required for the maintenance of an undifferentiated state of progenitor cells in the CMZ.

To further test if Hairy1 acts as an effector gene that acts downstream of Wnt signaling, we carried out a semi-epistatic analysis by co-overexpressing Wnt2b and HA-Hairy1-ΔW-VP16. Notably, cells expressing HA-Hairy1-ΔW-VP16 could not maintain an undifferentiated state and differentiated into neurons expressing Hu or visinin (Fig. 6). HA-Hairy1-ΔW-VP16 also suppressed the ability of Wnt2b to induce Rdh10 expression and maintain proliferative cells in the central retina (Fig. 6). We then examined if Hairy1 overexpression could rescue the effect of Fzd8-CRD that inhibits Wnt signaling in the peripheral retina (Fig. 2C). Fzd8-CRD induced ectopic expression of Hu or visinin; however, Hairy1-expressing cells did not express these neuronal markers (Fig. 7). In addition, expression of Rdh10 was restored in the Fzd8-CRD-expressing marginal retina by Hairy1 overexpression (Fig. 7). All of these observations are consistent with the idea that Hairy1 is a major effector gene of Wnt signaling in the retina.

The *Hairy1* promoter is activated by the Wnt/β-catenin pathway through divergent Wnt-responsive factors

Finally, to elucidate the mechanisms of the Wnt-dependent regulation of Hairy1 expression, we examined whether Wnt signaling directly regulates *Hairy1* promoter activity. We identified four consensus Tcf-binding sites located in the *Hairy1* promoter sequence (–2 kb/+2) (Fig. 8A). Therefore, a genomic region containing these sequences was cloned into a luciferase reporter plasmid and subsequently electroporated into the E1.5 optic vesicle. When canonical Wnt signaling was activated by the constitutively active form of β-catenin (Takahashi et al., 2000), Hairy1 –2 kb-Luc reporter activity was stimulated approximately by sevenfold (Fig. 8B). Blocking the canonical pathway by a dominant-negative Lef1 (ΔLef1) (Kengaku et al., 1998) significantly suppressed the reporter activation induced by β-catenin (Fig. 8B), indicating that this activation depends on the canonical β-catenin/Tcf pathway. To test if the Wnt signaling pathway directly activated the *Hairy1* promoter or not, we generated a mutant reporter construct, in which all of the putative Tcf-binding sites were mutated (–2 kb mut-Luc) (Fig. 8A). However, the mutant reporter still responded to β-catenin, indicating that these Tcf-binding sites are dispensable for β-catenin-dependent promoter activation (Fig. 8B).

To further dissect the mechanisms by which the *Hairy1* promoter is activated by canonical Wnt/β-catenin signaling, we hypothesized that Wnt signaling induces expression of certain transcription factors that in turn activate Hairy1 expression. We thus searched for Wnt-responsive genes by performing a microarray analysis using RNAs prepared from E6.5 retina transfected either with Wnt2b-, Delta- or mock-expressing RCAS plasmid. We made use of our observation that Hairy1 expression depends on Wnt signaling but not on Notch signaling, and therefore screened for genes that are highly expressed in Wnt2b-expressing retina but are only weakly expressed in control or Delta-expressing retina. Among candidate genes, which contained Lef1, Hairy1 and Rdh10 (see Fig. S2 and Table S1 in the supplementary material), we focused on genes encoding transcription factors. We examined the expression patterns of these candidate genes by in situ hybridization at E5.5. Among ten

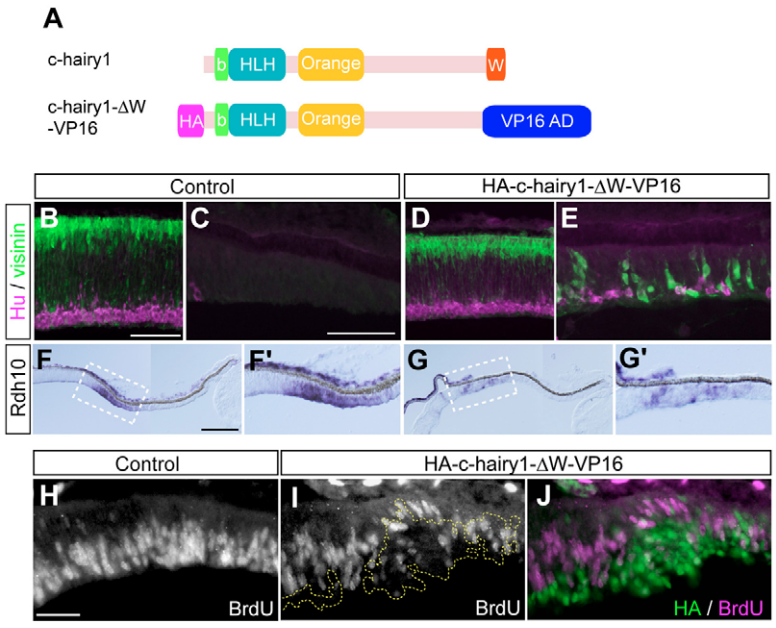

Fig. 5. Interfering with Hairy1 abrogates the CMZ characteristics. (**A**) Domain organization of chick Hairy1 and its dominant-negative derivative HA-Hairy1-ΔW-VP16. b, basic domain; VP16AD, VP16 activation domain; W, WRPW motif. (**B-G′**) Expression pattern of visinin (green in B-E), Hu (magenta in B-E) and Rdh10 (F-G′) in the central region (B,D) or CMZ (C,E,F-G′) of E7.5 retina electroporated with control (B,C,F,F′) and HA-Hairy1-ΔW-VP16-expressing (D,E,G,G′) RCAS provirus. F′ and G′ show a magnified view of the boxed regions from F and G, respectively. The dominant-negative Hairy1 induced ectopic expression of Hu/visinin and downregulated Rdh10 in the CMZ. (**H-J**) BrdU incorporation in the CMZ electroporated with control (H) and Hairy1-ΔW-VP16-expressing (I,J) RCAS provirus. The dotted yellow line in I indicates the area expressing the Hairy1-ΔW-VP16 construct as revealed by anti-HA immunostaining. Most of the cells in the CMZ uniformly incorporated BrdU in the control retina (H), whereas the majority of the cells expressing Hairy1-ΔW-VP16 did not incorporate BrdU (I,J). Scale bars: 200 μm in B-E; 50 μm in F-H.

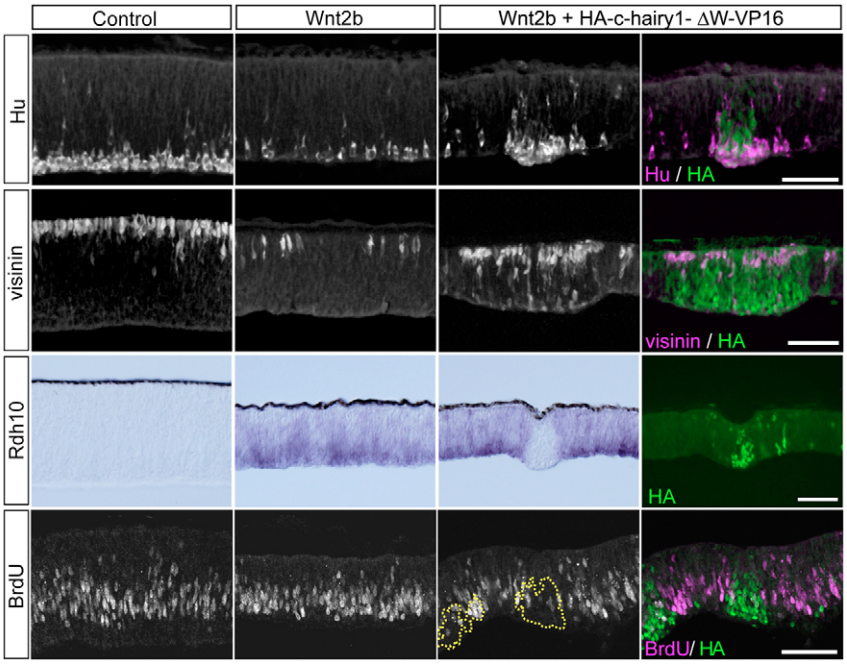

Fig. 6. Wnt2b fails to induce CMZ-specific marker expression in the central retina upon blocking of Hairy1 activity. Expression patterns of Hu, visinin and Rdh10 and distribution of BrdU-incorporated cells in E7.5 retina electroporated with control, Wnt2b- or both Wnt2b- and HA-Hairy1-ΔW-VP16-expressing RCAS provirus. Electroporated cells that expressed HA-Hairy1-ΔW-VP16 were labeled by anti-HA immunostaining (green). Note that the dominant-negative Hairy1 suppressed the effect of Wnt2b, which is to inhibit neuronal differentiation, induce Rdh10 and promote proliferation in the central retina. The dotted yellow line in K shows the areas that contain cells expressing the dominant-negative molecule. Scale bars: 50 μm.

genes analyzed, Myc (HLH-leucine zipper transcription factor), Elk3 (Net/Sap-2/Erp, Ets transcription factor), Lmo4 (LIM domain only 4), and Zic2 (zinc-finger transcription factor) were predominantly expressed in the CMZ, which was similar to the Hairy1 expression. However, Elk3 and Lmo4 were expressed in the presumptive iris/ciliary epithelium as well as in the CMZ (Fig. 8C). We then examined whether these four candidate genes activate the *Hairy1* promoter. Strikingly, overexpression of Myc and Elk3 significantly activated the *Hairy1* 2 kb promoter (Fig. 8D). We subsequently examined if endogenous Hairy1 expression is also induced by these candidate genes. The overexpression of Elk3, Lmo4 and Zic2 ectopically induced the endogenous Hairy1 expression in the central retina, where Hairy1 is not normally expressed (Fig. 8E-L). These results indicate that the *Hairy1* promoter receives inputs from multiple transcription factors that are responsive to Wnt signaling.

DISCUSSION

We have previously demonstrated that Wnt signaling maintains progenitor cells in the CMZ by downregulating proneural genes in a Notch-independent manner (Kubo et al., 2005). In this study, we identified Hairy1 as an essential effector gene of Wnt signaling, supported by the following observations. First, Hairy1 expression is predominantly observed in the CMZ, and is highly induced in the central retina upon activation of Wnt signaling. Second, overexpression of Hairy1 mimics the effect of Wnt2b and induces the expression of CMZ-specific markers in the central retina. Third, blocking Hairy1 function leads to ectopic neuronal differentiation in the CMZ. Fourth, Wnt2b fails to induce CMZ progenitor cells in the central retina when Hairy1 activity is blocked. Fifth and finally, promoter analysis revealed that Wnt signaling activates Hairy1 expression by controlling the expression of multiple downstream transcription programs.

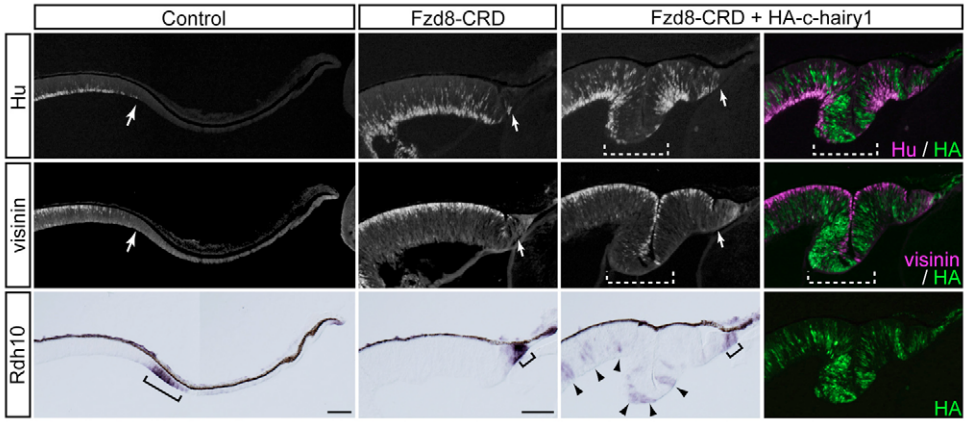

Fig. 7. Hairy1 rescues ectopic neuronal differentiation induced upon blocking of Wnt signaling. Expression patterns of Hu, visinin and Rdh10 in E7.5 retina electroporated with control, Fzd8-CRD- or both Fzd8-CRD- and HA-Hairy1-expressing RCAS provirus. Electroporated cells that expressed HA-Hairy1 were labeled by anti-HA immunostaining (green). Note that the Hairy1-expressing cells do not express Hu or visinin, but express Rdh10 (arrowheads). The large dashed brackets show Hairy1-expressing areas and the small black brackets show the endogenous Rdh10 expression in the CMZ. The arrows indicate the most peripheral region of differentiated neurons. Scale bars: 100 μm.

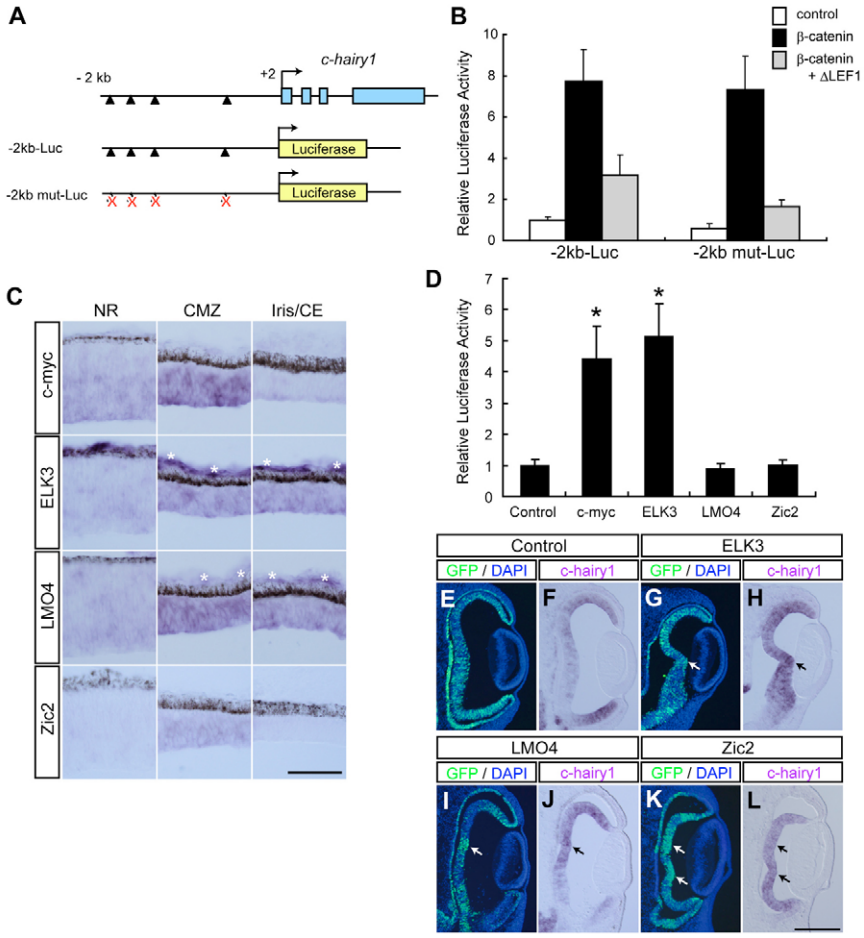

Fig. 8. The *Hairy1* promoter is regulated by Wnt signaling via Wnt-responsible factors. (**A**) The *Hairy1* promoter region and reporter constructs. Blue boxes represent exons. An ~2 kb region upstream of a putative transcription start site (–2 kb-Luc) was used for the lucifersae assay. Four putative Tcf-binding sites found in the *Hairy1* promoter region (black triangles) were mutated in the –2 kb mut-Luc reporter construct. (**B**) Effects of canonical Wnt signaling on *Hairy1* promoter activation. Reporter constructs and expression vectors under the control of the CAG promoter were co-electroporated into E1.5 retina as indicated and the luciferase activity measured 24 hours later. The luciferase activity from the –2 kb-Luc reporter with the mock electroporation control is represented as 1. Both Hairy1 –2 kb-Luc and –2 kb mut-Luc reporters were activated by constitutively active β-catenin, and this activation was suppressed by ΔLef1. (**C**) Expression patterns of Wnt-responsive genes identified by the microarray analysis. Adjacent sections of E5.5 retinas were hybridized with antisense probes for *Myc*, *Elk3*, *Lmo4* and *Zic2*. Asterisks show the non-retinal signal in the extraocular mesenchymal cells. (**D**) Effects of the candidate genes on *Hairy1* promoter activation. Hairy1 –2 kb-Luc reporter and expression vectors were co-electroporated at E1.5 as indicated and luciferase activity was measured 24 hours after electroporation. The luciferase activity with the mock electroporation control is represented as 1. **P*<0.05 versus control. (**E-L**) Effects of Elk3, Lmo4 and Zic2 overexpression on endogenous Hairy1 expression. E1.5 optic vesicles were electroporated either with control (E,F), Elk3 (G,H), Lmo4 (I,J) or Zic2 (K,L) together with a GFP expression plasmid, and adjacent sections were analyzed for GFP and by DAPI staining (E,G,I,K) and *Hairy1* mRNA expression (F,H,J,L) at E3.5. In the embryos electroporated with Elk3, Lmo4 and Zic2, endogenous Hairy was ectopically expressed in the central retina (arrows). Iris/CE, iris/ciliary epithelium; NR, neural retina. Scale bars: 50 μm in C; 200 μm in L.

In contrast to the widely accepted notion that the Notch signaling pathway plays a major role in regulating the transcription of Hes genes (Artavanis-Tsakonas et al., 1999; Selkoe and Kopan, 2003; Yoon and Gaiano, 2005), Hairy1 expression was controlled by Wnt signaling without Notch activity in the chicken retina. However, Hes5 was tightly regulated by Notch signaling in the central retina, where active neurogenesis is taking place. Although these two Hes genes belong to the same family, there were functional differences between Hairy1 and Hes5; Hairy1 overexpression induced the CMZ marker Rdh10, whereas Hes5 overexpression increased the number of cells expressing the Müller cell marker glutamine synthetase. Therefore, expression of the two Hes family genes are differentially regulated in a retinal-region-specific manner, at least in the chicken retina, and this might be responsible for the differential properties of the retinal progenitor cells in each part of the optic vesicle. According to this hypothesis, progenitor cell differentiation in the retina could be explained in the following way (Fig. 9). In the CMZ, Wnt signaling controls Hairy1 expression, which endows the progenitor cells with a prolonged proliferative capacity that is characteristic of this region. As they proliferate, their progenies are displaced to the central region, where Wnt signaling is no longer available. In the central region, progenitor cell differentiation is controlled by Notch via Hes5, which transiently inhibits neuronal differentiation while making an appropriate number of neurons (Artavanis-Tsakonas et al., 1999; Selkoe and Kopan, 2003; Yoon and Gaiano, 2005). The progenitor cells that continue to receive Notch signaling finally give rise to Müller cells after a certain number of cell divisions, as has been proposed previously (Furukawa et al., 2000; Hojo et al., 2000; Scheer et al., 2001; Takatsuka et al., 2004).

We found that Hairy1 expression is activated by multiple Wnt-responsive genes; Myc and Elk3 induced *Hairy1* promoter activity in the luciferase assay, and Elk3, Lmo4 and Zc2 induced endogenous Hairy1 expression when overexpressed in ovo. Therefore, Wnt signaling in the CMZ presumably regulates divergent downstream transcription factors that converge to activate Hairy1 expression in the progenitor cells in the CMZ (Fig. 8). Myc strongly activated the *Hairy1* promoter in the luciferase assay, but did not induce endogenous Hairy1 expression when overexpressed in ovo. This is possibly due to higher sensitivity of the luciferase assay compared to in situ hybridization. However, Lmo4 and Zic2 did not activate Hairy1 reporter activity, but did induce the endogenous Hairy1 expression. These two factors may act through regulatory elements located outside of the –2 kb promoter region we used. It remains to be elucidated how these factors mediate the Wnt-dependent Hairy1 expression; namely, how they interact with each other and whether they act directly or indirectly on *Hairy1* promoter

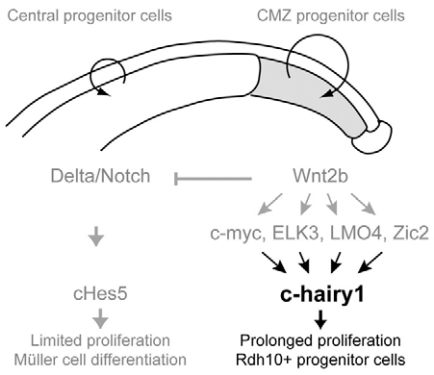

Fig. 9. Model of region-specific control of progenitor cell differentiation in the retina. Two independent signaling pathways, Wnt and Notch, operate in the retina. On the one hand, Wnt signaling maintains prolonged proliferation of progenitor cells in the CMZ through Hairy1. On the other hand, Notch signaling induces Müller cell differentiation through Hes5 after a limited round of cell division of central progenitor cells. Note that Hairy1 functions as a node to integrate multiple Wnt-responsive transcription factors, Myc, Elk3, Lmo4 and Zic2. See text for details.

sequences. Whatever the case, we propose Hairy1 as a node to integrate multiple transcriptional inputs downstream of Wnt signaling in the progenitor cells in the CMZ.

It has recently been proposed that Wnt signaling promotes iris/ciliary epithelium fate rather than controlling the undifferentiated state of progenitor cells in the CMZ based on the observation that constitutive β-catenin activation induces the expression of molecular markers specific to the anterior eye structures (Cho and Cepko, 2006; Liu et al., 2003). At the time of this study, however, no molecular marker was available that could distinguish progenitor cells in the CMZ from the iris/ciliary epithelium, leaving the possibility that Wnt signaling is also involved in the maintenance of the stem cell-like progenitor cells in the CMZ. We isolated Rdh10 when screening for Wnt-responsive genes and found that it is specifically expressed in the CMZ but not in the iris/ciliary epithelium. Of note, Rdh10 is the first molecular marker that distinguishes progenitor cells in the CMZ from centrally located progenitor cells, which are fundamentally different in terms of their proliferative capacity. The number of cells expressing Rdh10 was greatly reduced upon blocking of Wnt signaling by Fzd8-CRD, suggesting that Wnt signaling is required for the maintenance of the CMZ as well as for the formation of the iris/ciliary epithelium. Although Rdh10 is not functionally involved in the maintenance of progenitor cells in the CMZ (F.K. and S.N., unpublished), identification of a CMZ-specific marker may facilitate further studies on the retinal stem cells. It should be noted that the expression patterns of Lef1, Rdh10 and Math6 were mosaic in the CMZ, and not all the cells expressed these molecular markers. Therefore, the CMZ is not a structure that contains a homogenous stem cell population, but a structure that contains multiple cell types at different progenitor stages. It would be intriguing to examine if there is any special microenvironment or 'niche' surrounding the presumptive retinal stem cells expressing these CMZ-specific markers.

In the *Xenopus* retina, frizzled 5 signaling potentiates committed retinal neural precursor cells to become retinal neuron by inducing Sox2 expression (Van Raay et al., 2005). Consistent with this idea, frizzled 5 is not expressed in the CMZ but is expressed in the central region of the retina in chicken and mice, where active neurogenesis is taking place (Burns et al., 2008; Fuhrmann et al., 2003; Kubo et al., 2003). Because frizzled 4 is expressed in the peripheral part of the retina (Fuhrmann et al., 2003; Kubo et al., 2003), different subtypes of frizzled may mediate distinct differentiation programs. Alternatively, the Wnt signaling pathway plays different roles depending on each vertebrate species, as has been proposed in a recent study (Burns et al., 2008). In either case, Wnt/β-catenin signaling may function at multiple steps of cellular differentiation during the development of the optic vesicle.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/11/1823/DC1

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