Wnt2b inhibits differentiation of retinal progenitor cells in the absence of Notch activity by downregulating the expression of proneural genes

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Summary

During the development of the central nervous system, cell proliferation and differentiation are precisely regulated. In the vertebrate eye, progenitor cells located in the marginal-most region of the neural retina continue to proliferate for a much longer period compared to the ones in the central retina, thus showing stem-cell-like properties. Wnt2b is expressed in the anterior rim of the optic vesicles, and has been shown to control differentiation of the progenitor cells in the marginal retina. In this paper, we show that stable overexpression of Wnt2b in retinal explants inhibited cellular differentiation and induced continuous growth of the tissue. Notably, Wnt2b maintained the undifferentiated progenitor cells in the explants even under the conditions where Notch signaling was blocked. Wnt2b downregulated the expression of multiple proneural bHLH genes as well as Notch. In addition, expression of Cath5 under the control of an exogenous promoter suppressed the negative effect of Wnt2b on neuronal differentiation. Importantly, Wnt2b inhibited neuronal differentiation independently of cell cycle progression. We propose that Wnt2b maintains the naive state of marginal progenitor cells by attenuating the expression of both proneural and neurogenic genes, thus preventing those cells from launching out into the differentiation cascade regulated by proneural genes and Notch.

Key words: Wnt, retina, progenitor cells, stem cells, proneural gene, Notch, Delta, differentiation, cell cycle

Introduction

In the vertebrate retina, a single progenitor cell type gives rise to a whole spectrum of retinal cell types including six types of neurons and 1 type of glia, i.e. the Müller cell (reviewed by Livesey and Cepko, 2001). The proliferation and differentiation of progenitor cells are precisely regulated, and those cells located in the peripheral region of the neural retina proliferate for a longer period than the ones in the central region, which has long been known from classical autoradiography studies [in fish (Johns, 1977), in frog (Hollyfield, 1968; Jacobson, 1968), in chicken (Kahn, 1974) in mouse (Sidman, 1961)]. In the chicken neural retina, the marginal progenitor cells incorporate BrdU even after hatching, but they can differentiate into only limited neuronal cell types such as bipolar or amacrine cells (Fischer and Reh, 2000). Exogenous application of insulin and FGF2, however, induces production of retinal ganglion cells from those progenitor cells (Fischer et al., 2002), implying that the marginal progenitor cells are also potentially multipotent in the chicken retina and that their final cell fate is restricted by the microenvironment surrounding that region at later developmental stages.

Wnt family proteins are secreted signaling molecules that regulate various aspects of developmental processes (reviewed by Wodarz and Nusse, 1998). Wnt2b is expressed in the anterior rim of the optic vesicles, neighboring the marginal progenitor cells of the retina (Zakin et al., 1998; Jasoni et al., 1999). In mice, the canonical Wnt pathway that induces target gene transcription (reviewed by Wodarz and Nusse, 1998) is activated in the peripheral part of the retina, as was revealed by expression of a reporter gene under the control of Wnt-responsive promoter sequences (Liu et al., 2003). In chicken embryos, expression of Lef1 mRNA is upregulated when the canonical Wnt pathway is activated (Schmidt et al., 2000), and this tentative marker for the active state of Wnt signaling is highly expressed in the marginal, but not in the central, region of the neural retina (Kubo et al., 2003). Such observations suggest that the canonical Wnt signaling is operating in the marginal part of the optic vesicles where undifferentiated retinal progenitor cells reside. Wnt2b can inhibit the differentiation of the progenitor cells derived from the central retina both in vitro and in ovo (Nakagawa et al., 2003; Kubo et al., 2003). In addition, blocking of the Wnt signaling pathway by a dominant-negative form of Lef1, a downstream effector of the canonical Wnt signaling, induces premature neuronal differentiation in the marginal retina (Kubo et al., 2003). Furthermore, Wnt2b promotes prolonged proliferation of multipotent progenitor

cells in clonal cultures (Kubo et al., 2003). These observations indicate that Wnt2b is responsible for the maintenance of progenitor cells in the marginal retina; however, the precise molecular mechanism by which Wnt2b maintains progenitor cells in the undifferentiated state has remained an open question.

A number of proneural genes that encode basic helix-loop-helix (bHLH) transcription factors are expressed in the developing retina, and they control the differentiation of particular cell types in combination with other regulatory factors (reviewed by Vetter and Brown, 2001; Perron and Harris, 2000a; Kageyama et al., 1997). For example, forced expression of an atonal family transcription factor, Ath5, leads to an overproduction of retinal ganglion cells in various vertebrate species (Liu et al., 2001; Matter-Sadzinski et al., 2001; Kanekar et al., 1997), and this cell type is absent in *Ath5*-mutant animals (Kay et al., 2001; Brown et al., 2001; Wang et al., 2001). Other proneural genes that influence the production of specific neuronal cell types include *NeuroD* (Yan and Wang, 1998; Morrow et al., 1999; Hutcheson and Vetter, 2001; Inoue et al., 2002), *Ath3* (Perron et al., 1999; Tomita et al., 2000; Inoue et al., 2002), *neurogenin 2* (*Ngn2*) (Marquardt et al., 2001) and an acute-scute family transcription factor *Ash1* (Tomita et al., 1996; Brown et al., 1998; Tomita et al., 2000). The expression or activities of these proneural genes are regulated by various mechanisms depending on each gene or the developmental stage. The signal mediated by the Delta ligand and Notch receptor is an important factor that antagonizes the proneural gene function through a downstream effector gene called *hairy-related proteins* (*Hes*) (reviewed by Kageyama et al., 2000; Vetter and Brown, 2001; Perron and Harris, 2000a). In the vertebrate retina, downregulation of Notch expression by antisense oligonucleotide treatment increases the number of retinal ganglion cells (Austin et al., 1995), whereas persistent activation of Notch signaling by a constitutively active form of the receptor inhibits neuronal differentiation (Dorsky et al., 1995; Bao and Cepko, 1997; Austin et al., 1995). In addition, overexpression of Delta inhibits differentiation of retinal progenitor cells, and that of the dominant-negative form of the ligand leads to premature neuronal differentiation (Henrique et al., 1997; Dorsky et al., 1997; Ahmad et al., 1997). All of these experiments support the idea that the Delta-Notch signaling keeps retinal progenitor cells in an undifferentiated state by antagonizing the proneural gene cascade and that they differentiate into specific neurons once they escape this inhibitory signal.

In this study, we first compared the effect of Wnt2b and Delta on retinal progenitor cell proliferation and differentiation. We show that the overexpression of Wnt2b supported prolonged proliferation of retinal progenitor cells whereas that of Delta did not. We also demonstrate that Wnt2b inhibited neuronal differentiation even under the conditions where the Delta/Notch signaling pathway was blocked. Wnt2b downregulated mRNA expression of multiple proneural genes as well as that of *Notch1*. In addition, forced expression of Cath5 under the control of ubiquitous promoter neutralized the neural differentiation-inhibiting effect of Wnt2b. We propose that Wnt2b maintains progenitor cells by preventing them from entering the differentiation cascade regulated by Notch and proneural genes.

Materials and methods

In ovo electroporation of RCAS provirus vector or plasmid expression vector

Provirus plasmids or plasmid expression vectors were introduced into optic vesicles of stage 10/11 chicken embryos as previously described (Kubo et al., 2003). In some experiments, pCAG-EGFP or pCAG-EGFP fused with a nuclear localization signal (nls EGFP) was mixed with the plasmids at a final concentration of 0.5 μg/ μl to visualize electroporated cells. For double electroporation with two provirus/expression vectors, two provirus-vectors, RCASBP (A) and (B) or pCAG vectors were mixed at a 1:1 ratio and electroporated as described above. To study cell proliferation, 1 μl of BrdU (50 mM) was injected intravenously 12 hours after the electroporation and embryos were fixed after a further 12 hours. The vectors used were, RCASBP (A) Wnt2b (a kind gift from J. C. Izpisua-Belmonte), RCASBP (B) Wnt2b, RCASBP (A) dnDelta1 (a kind gift from D. Henrique), RCASBP (A) sonic hedgehog, pCAG-Wnt2b, pCAG-Cath5, and pCAG-mouse Ink4d.

Explant culture of neural retina

The culture medium used was a 1:1 mixture of Dulbecco's Modified Eagle's medium and Ham F12 (Nissui, Japan) supplemented with 10% fetal calf serum and penicillin/streptomycin (DH10). Neural retina of 1 mm^2 was taken from the equatorial region temporal to the optic stalk, and further cut into pieces of 250 μm^2 surface area with fine scissors. In most cases, some region of the Wnt2b-expressing neural retina formed folds. The tissue explants were prepared from unfolded regions in that case. The retinal explants were then placed in the wells of non-coated 24-well dishes (Iwaki, Japan) with 1 ml of DH10, and the dishes were rotated at a speed of 60 r.p.m. on a rotary shaker. Half of the culture medium was changed every day. In some experiments, recombinant EGF and bFGF (Gibco) were added at a concentration of 20 ng/ml each. For labeling proliferating cells, BrdU (Sigma) was added to the medium at a concentration of 2 μM, and the retinal explants were then incubated for 30 minutes at 37°C. To block the signaling pathway downstream of Notch, we used a γ-secretase inhibitor DAPT {N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester; Calbiochem}, which was diluted in DMSO and added at a concentration of 2 μM. To obtain Frizzled 8-CRD, we transfected COS7 cells with pJCH100, which encodes mouse frizzled 8 CRD domain fused to human immunoglobulin heavy chain (Hsieh et al., 1999). The fusion protein was purified on a protein A column, and added to the retinal cultures at a concentration of 50 μg/ml.

Immunohistochemistry and in situ hybridization

The antibodies used were as follows: mouse anti-Hu (clone 16A11; Molecular Probe); mouse anti-middle-molecular-weight neurofilament (clone RMO270; Zymed); mouse anti-Syntaxin (HPC1; Sigma); mouse anti-Glutamine Synthetase (clone 6; Transduction Laboratory); mouse anti-Pax6 (DSHB); mouse anti-islet1 (clone 40.2D6; DSHB); mouse monoclonal antibody 115A10 (a kind gift from Dr Fujita); mouse anti-BrdU (clone BU33; Sigma); mouse anti-radial glia-specific antibody (clone 3CB2; DSHB); mouse anti-Vimentin (clone H5, DSHB), sheep anti-Chx10 (Exalpha); rabbit anti-Visinin (a kind gift from Dr Miki); rabbit anti-phosphorylated Histone H3 (Upstate Biology); Cy3-conjugated anti-rabbit IgG (Chemicon); Alexa 488-conjugated anti-sheep IgG (Molecular Probes) and Alexa 488-conjugated anti-mouse IgG (Molecular Probes).

The tissue explants or embryos were fixed for 1 hour at room temperature in 4% paraformaldehyde in PBS, permeabilized in 100% methanol for 5 minutes at –20°C, and processed using a standard immunostaining protocol.

For BrdU staining, the sections were treated with 2 N HCl for 2 hours at room temperature. Confocal images were collected using a LSM510 microscope (Zeiss).

For in situ hybridization studies, embryos were fixed in 4% paraformaldehyde overnight at 4°C and processed using standard protocols. The cRNA probes for *cNotch1* were kindly provided by Dr. Wakamatsu (Tohoku University, Japan). For *Cath5* and *NeuroM*, the cDNA fragments corresponding to the coding sequences of the proteins were amplified by PCR and subcloned into pCRII (Invitrogen) to make the probes.

RT-PCR analysis of proneural gene expression

Total RNAs were extracted using RNAzol (Tel-Test, Inc., Friendswood, TX, USA) from E5.5 retinas that had been electroporated with either RCASBP(A) Wnt2b or control RCASBP(A) provirus. cDNAs were synthesized from 1 μg of total RNAs using oligo(dT) primer and RevetraAce (TOYOBO) according to the manufacturer's instructions. One-nanogram of the resultant first-strand cDNAs were subsequently used for PCR analysis with the following primer pairs: *Notch1*, 5′-tggagccactgacgttttgt-3′ and 5′-agggatgtcaccataaccat-3′; *Delta1*, 5′-gaacagccagtttccctgga-3′ and 5′-gacgtacaccgactggtact-3′; *Cath5*, 5′-cgtgctggtgtcttacacta-3′ and 5′-ttcacacaagcgtctcattc-3′; *NeuroM*, 5′-gaccttattcctggagaagc-3′ and 5′-cgcttcgctactcgttgaag-3′; *Cash1*, 5′-gtatcctccctgaccagttt-3′ and 5′-cgctcttctgcgtttggaca-3′; *Neurogenin1*, 5′-ccatacgacttggaaaaccc-3′ and 5′-agtggctcaataccccgtgt-3′; *Neurogenin2*, 5′-aggggtccaggttagaagtc-3′ and 5′-ccctttgctcttcagccgta-3′; *Lef1*, 5′-ctcacacaactggaatccct-3′ and 5′-tggtgtcgccgcctttgctt-3′; *Gapdh*, 5′-acatcatcccagcgtccact-3′ and 5′-gatgtaaggtggtacaca-3′. PCR was carried out under cycle conditions of 94°C for 30 seconds, 50°C 30 seconds, and 72°C for 3 minutes. The number of the cycles was carefully determined for each gene so that the resultant products were amplified within a linear range: 25 cycles for *Notch1*, *Delta1*, *Cath5, Cash1* and *Gapdh*; 30 cycles for *NeuroM*, *Ngn2*, and *Lef1*; and 35 cycles for *Ngn1*. The sequences of the PCR products were checked by direct sequencing using each of the primers. For semi-quantitative analysis, the PCR products were run on a 2% agarose gel, and intensities of corresponding bands were measured by ImageMaster TotalLab (Amersham biosciences) after staining with ethidium bromide.

Results

Wnt-2b sustains prolonged proliferation of retinal progenitor cells

In order to compare the effect of Wnt2b and Delta1 on the proliferation of retinal progenitor cells, we sought to stably overexpress these proteins in retinas. To do this, we electroporated optic vesicles of embryonic day 1.5 (E1.5) chick embryos with RCASBP provirus plasmid vectors that produce replication-competent retroviruses in ovo. We first examined the time course of the exogenous expression obtained by this method using an EGFP-expressing provirus. A subpopulation of retinal cells expressed EGFP as early as 24 hours after the electroporation (Fig. 1A), and more than 90% of them were GFP positive 72 hours after the electroporation (Fig. 1B). A similar pattern of exogenous expression was also observed when we used Wnt2b- or Delta1-expressing proviruses (data not shown). At E6, electroporated neural retinas were dissected out, and tissue explants of 250 μm^2 surface area were prepared from the equatorial region temporal to the optic stalk (Fig. 1C). The retinal explants were then placed in a non-coated dish and cultured in vitro for 7 days. The retinal explants electroporated with control provirus formed spherical structures with an average radius of 500 μm (n=48; Fig. 1G). Similarly, Delta1-expressing explants formed slightly larger spherical structures (750 μm on average, n=48; Fig. 1I). In contrast, Wnt2b-expressing explants generated large folded, sheets of tissue

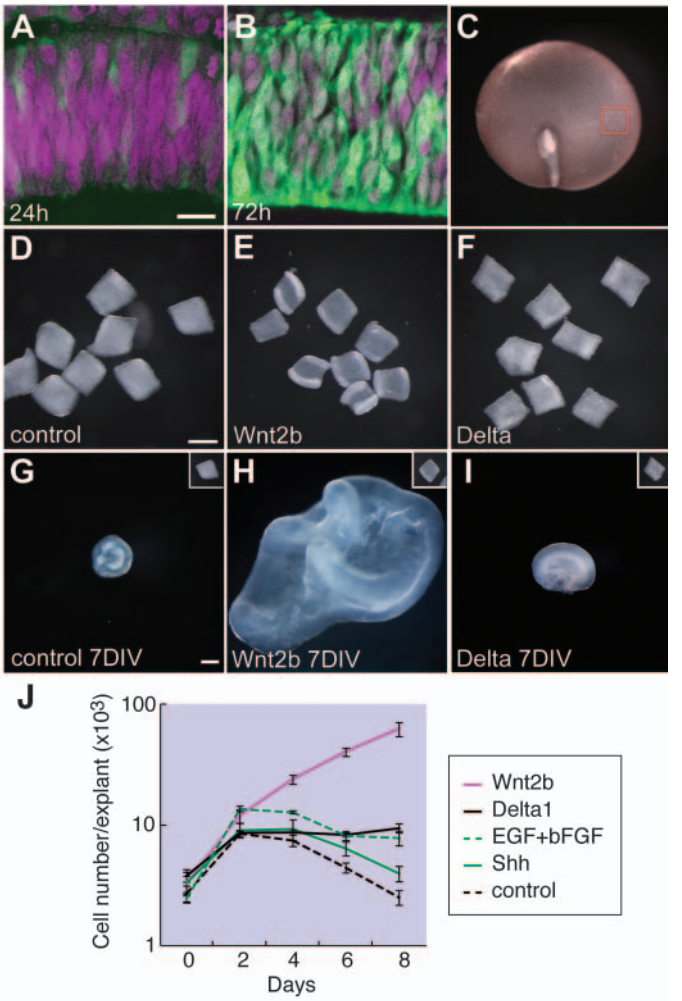

Fig. 1. Wnt2b induces prolonged proliferation of retinal progenitor cells. (A,B) Exogenous gene expression by replication-competent retroviruses. The optic vesicles were electroporated with a GFP-expressing RCASBP provirus vector and fixed after 24 hours (A) or 72 hours (B). Transverse sections of neural retinas were observed using confocal microscopy. Nuclei were counterstained with propidium iodide and pseudocolored in purple. (C) Whole-mount view of E5 retina. The purple box shows the region from which retinal explants were prepared. (D-F) Whole-mount view of the retinal explants taken from control (D,G), Wnt2b-expressing (E,H), and Delta1-expressing (F,I) E6 retina immediately after the dissection (D-F) or after 7 days in culture (G-I). The small insets in G-I show the explants after the dissection at the same magnification. Note that Wnt2b-expressing explants increased in size enormously, making folded sheets (H), whereas control or Delta1-expressing explants made a much smaller sphere (G,I). (J) The change in cell number in the explants during the culture period (shown on the x axis). The cell number is shown in a logarithmic vertical scale. Note the exponential growth in the Wnt2b-expressing explants. Scale bars: 20 μm (A,B); 250 μm (D-I).

(n=48; Fig. 1H). We then prepared secondary tissue explants of 250 μm^2 from those folded sheets, and cultured them for an additional 14 days. In all cases (n=24), the explants formed secondary large sheets just like the primary ones (data not shown). These observations suggest that Wnt2b, but not

Delta1, sustained prolonged proliferation of retinal progenitor cells.

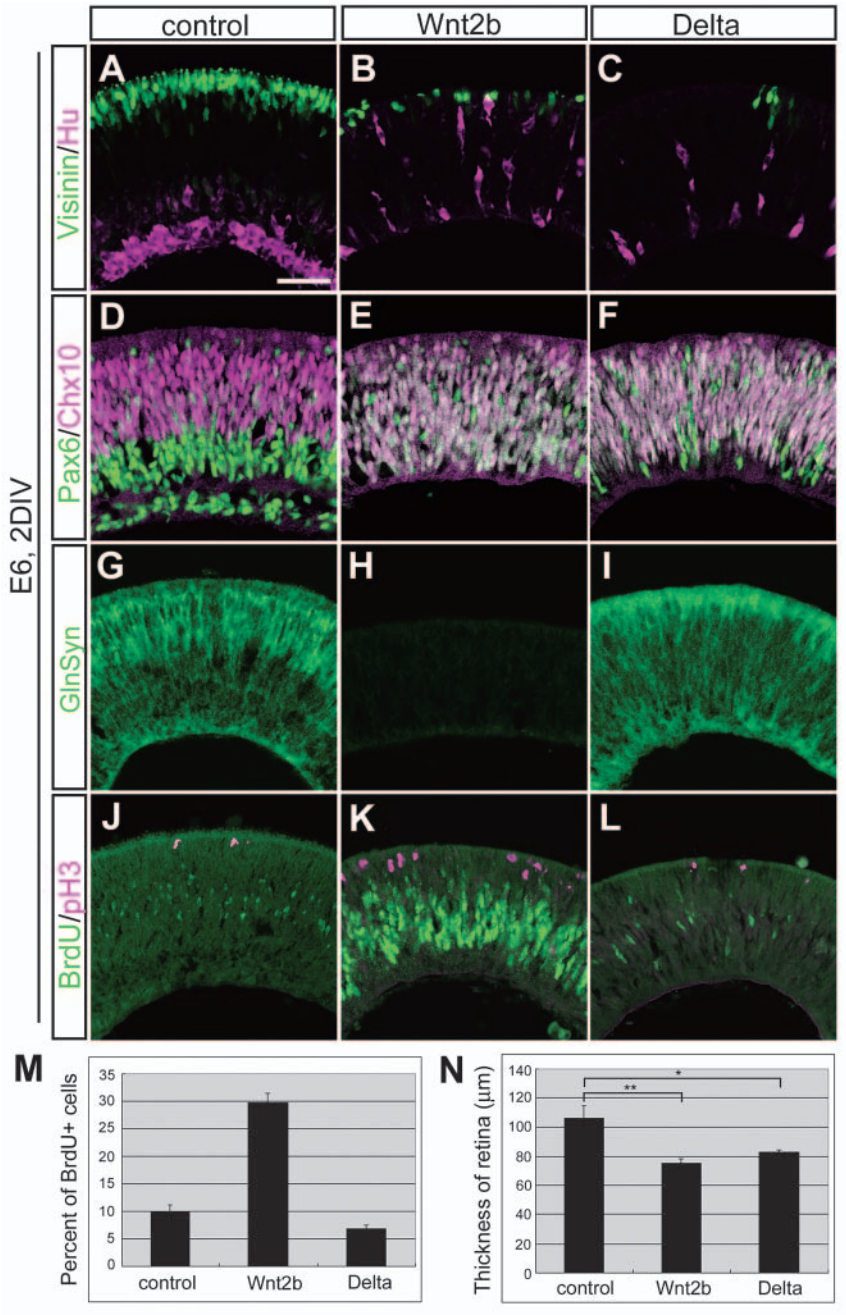

Fig. 2. Wnt2b inhibits the differentiation of retinal progenitor cells. Retinal explants were prepared from E6 retinas electroporated with control (A,D,G,J), Wnt2b-expressing (B,E,H,K), or Delta1-expressing (C,F,I,L) provirus plasmids. They were cultured for 2 days, and sections were prepared and stained for molecular markers shown in the left column: visinin (green) and Hu (purple) in A-C, Pax6 (green) and Chx10 (purple) in D-F, glutamine synthetase (green) in G-I, and BrdU (green) and phosphorylated histone H3 (purple) in J-L. Note the decrease in the number of visinin- or Hu-expressing cells (B,C) and uniform co-expression of progenitor markers Pax6 and Chx10 (E,F) in Wnt2b- or Delta1-expressing retinal explants compared with the marker levels in the control explants. A number of cells incorporated BrdU in Wnt2b-expressing retinal explants (K), whereas few cells were positive for the proliferation markers in control (J) or Delta1-expressing (L) explants. (M) Percentage of BrdU+ cells in the explants expressing each construct (n=4). (N) Thickness of the retinal explants expressing each construct (n=4). $^{**}P<0.01$, $^{*}P<0.05$. Scale bars: 30 μm.

To examine the change in cell number during the culture period, we dissociated each explant into single cells and counted them using a hemocytometer. In the control explants, the cell number reached a plateau during the first 2 days and gradually decreased thereafter (Fig. 1J), presumably reflecting naturally occurring cell death during corresponding embryonic stages (Cook et al., 1998). Similarly, Delta1-expressing explants stopped growing after 2 days, although the cell number remained constant during the following culture periods (Fig. 1J). In contrast, in the Wnt2b-expressing explants the cell number increased exponentially for at least 8 days (Fig. 1J). We also examined the effect of other signaling molecules, such as EGF and bFGF or sonic hedgehog, which have been shown to promote proliferation of retinal progenitor cells (Kelley et al., 1995; Moshiri and Reh, 2004). The continuous growth of the retinal explants was specific to Wnt2b, as it was not observed when the explants were cultured in the presence of EGF and bFGF, or in the explants expressing sonic hedgehog (Fig. 1J).

Both Wnt2b and Delta inhibit neuronal differentiation, but only Wnt2b maintains prolonged proliferation of retinal progenitor cells

To further investigate the effect of Wnt2b and Delta1 on cellular differentiation and proliferation, we examined the expression of various molecular markers in retinal explants cultured for 2 days. We first examined the expression of visinin and Hu, expressed in cone photoreceptor cells and ganglion/amacrine cells, respectively (Hatakenaka et al., 1985; Fischer and Reh, 2000). The number of visinin- or Hu-expressing cells was greatly reduced in the explants expressing Wnt2b or Delta1 compared to the control explants (Fig. 2A-C), suggesting that Wnt2b and Delta1 inhibited neuronal differentiation. Wnt2b-expressing explants and Delta1-expressing explants were thinner than control explants, a situation reminiscent of that for less mature early embryonic neural retina (Fig. 2A-C,N). We then examined the expression of Pax6 and Chx10. These proteins are expressed in distinct subsets of postmitotic neurons, but only retinal progenitor cells co-express both of them (Fischer and Reh, 2000; Belecky-Adams et al., 1997). In the control explants, most of the cells expressed either one of the protein, suggesting they had already differentiated into individual neurons (Fig. 2D). However, a majority of the cells co-expressed the progenitor marker proteins in the explants expressing Wnt2b or Delta1 (Fig. 2E,F), suggesting that both Wnt2b and Delta1 prevented neuronal differentiation. To examine glial cell differentiation in those explants, we

examined the expression of glutamine synthetase, a marker for Müller glia (Norenberg et al., 1980). At this stage, a radial array of Müller glia was already established in the control explants, whereas no glutamine synthetase immunoreactivity was observed in the explants expressing Wnt2b (Fig. 2G,H). In contrast, in the Delta1-expressing explants, the glial marker was uniformly expressed (Fig. 2I), suggesting that cells expressing Delta1 differentiated into Müller glia. Since Müller cells do not normally co-express Pax6 and Chx10, we further characterized the cell type in the Delta-expressing explants by examining other Müller cell markers in a dissociated culture. Most of the cells expressed all the Müller cell markers we examined (data not shown). These observations were consistent with previous reports that activation of Notch signaling instructs retinal progenitor cells to differentiate into Müller cells (Furukawa et al., 2000; Scheer et al., 2001). We then examined the incorporation of BrdU in these explants to study cell proliferation. In the control or Delta1-expressing explants, few cells were positive for the BrdU, suggesting that most of the cells had become postmitotic (Fig. 2J,L,M). However, a number of cells in the intermediate zone incorporated BrdU in the explants expressing Wnt2b (Fig. 2K,M). The number of apoptotic cells in those explants did not change during the 2-day culture period (data not shown). The undifferentiated retinal progenitor cells in the Wnt2b-expressing explants differentiated into retinal neurons and glia upon addition of soluble form of Frizzled 8 cystein-rich domain (CRD) (data not shown), which functions as a Wnt antagonist (Hsieh et al., 1999). Taken together, the data indicated that both Wnt2b and Delta1 inhibited neuronal differentiation but that only Wnt2b supported prolonged proliferation of multipotent retinal progenitor cells.

Wnt2b inhibits progenitor cell differentiation in the absence of Delta-Notch signaling

The observations so far mentioned suggest that Wnt2b inhibited differentiation of retinal progenitor cells through a pathway independent of the inhibitory signals mediated by Delta/Notch. If that was truly the case, it can be predicted that Wnt2b might inhibit progenitor cell differentiation in the absence of the Delta/Notch signaling pathway. To address this issue, we prepared retinal explants from earlier embryos (E5) and treated them for 24 hours with the γ-secretase inhibitor DAPT, which prevents the cleavage of the intracellular portion of Notch and thus functions as a Notch-signaling inhibitor (Dovey et al., 2001; Geling et al., 2002). In the untreated control explants, many cells co-expressed Pax6 and Chx10 and incorporated BrdU (Fig. 3E,I,U), suggesting that the E5 retinal explants had not fully differentiated yet under this culture condition. In the DAPT-treated control explants, characteristic epithelial structures of neural retinas were lost, and spherical aggregates developed that were composed of differentiated neurons (Fig. 3B,F). In the aggregates, almost all the cells expressed either visinin or Hu, and few cells co-expressed the progenitor cell marker Pax6 and Chx10 (Fig. 3B,F). In addition, BrdU-positive cells were rarely observed (Fig. 3J,U), suggesting that progenitor cells had differentiated into postmitotic neurons upon inactivation of Notch signaling as previously reported (Austin et al., 1995; Henrique et al., 1997). Notably, Wnt2b largely suppressed the acute effect of DAPT to promote neuronal differentiation; more than half of the cells still co-expressed Pax6 and Chx10 in the Wnt2b-expressing explants (Fig. 3H), and they incorporated BrdU as well (Fig. 3L,U). The DAPT treatment increased the number of visinin- or Hu-expressing cells in the Wnt2b-expressing explants (Fig. 3C,D), suggesting that a certain population of cells was sensitive to the inhibition of Notch signaling. To further confirm that Wnt2b can suppress the premature neurogenesis caused by an inactivation of Notch signaling, we co-expressed a dominant-negative form of Delta (Henrique et al., 1997) and Wnt2b in the explants. For this experiment, we used two types of provirus vector RCASBP (A) and (B) expressing different envelopes, which enables a double infection of the same cell with two retroviruses (Givol et al., 1994). In the retina co-electroporated with Delta1- and Wnt2b-expressing proviruses, both of the genes were uniformly expressed, as was confirmed by in situ hybridization on adjacent sections (data not shown). As previously reported (Henrique et al., 1997), the dominant-negative form of Delta1 drastically increased the cells expressing the neuronal marker (Fig. 3N). This effect was completely neutralized by co-electroporation of Wnt2b (Fig. 3P). In addition, Wnt2b suppressed the effect of Delta1 to induce Müller glia differentiation when co-expressed in the same explants (Fig. 3Q-T). Those results supported our hypothesis that Wnt2b inhibited progenitor cell differentiation through a pathway independent of Delta/Notch signaling.

Wnt2b downregulates the expression of multiple proneural genes

To explore how Wnt2b could inhibit progenitor cell differentiation in the absence of Notch activity, we studied the expression pattern of proneural genes that are essential for the differentiation of retinal neurons (reviewed by Vetter and Brown, 2001; Perron and Harris, 2000a; Kageyama et al., 1997). Before that, we first examined the expression pattern of *Notch1* mRNA itself in retinas expressing Wnt2b using in situ hybridization. As previously reported (Austin et al., 1995), *Notch1* mRNA was broadly expressed in the undifferentiated cells of the E5 neural retina (Fig. 4A). The expression, however, was significantly reduced in the retina expressing Wnt2b (Fig. 4B), which was consistent with the aforementioned observation that Wnt2b-expressing retinal explants became less sensitive to the treatment that blocks Notch signaling (Fig. 3). We then examined the expression pattern of atonal family proneural genes *Cath5* and *NeuroM* in adjacent sections (Roztocil et al., 1997; Liu et al., 2001). We observed that the expression of both proneural genes was reduced in the Wnt2b-expressing retina compared with that in the control retina (Fig. 4C-F). Futhermore, *Delta1* expression was detected at the same level in both control and Wnt2b-expressing retina (Fig. 4G,H). To further study the effect of Wnt2b on multiple proneural gene expressions, we carried out RT-PCR using mRNAs derived from control or Wnt2b-expressing E5 retina. We found that all the proneural genes we studied, including *Cath5*, *NeuroM*, *Cash1*, *Ngn1* and *Ngn2*, were downregulated in the retina expressing Wnt2b (Fig. 4I,J), while the expression of *Delta1* did not change (Fig. 4I,J). In contrast, the expression of *Lef1* was upregulated in response to the Wnt2b overexpression (Fig. 4I), as previously reported (Kubo et al., 2003).

Exogenous expression of Cath5 suppresses the inhibitory effect of Wnt2b on retinal ganglion cell differentiation

The observations so far described suggested that Wnt2b inhibited progenitor cell differentiation by negatively regulating the expression of proneural genes. To confirm this, we asked if forced expression of a proneural gene using an exogenous promoter could rescue the inhibitory effect of Wnt2b on cellular differentiation. We focused on differentiation of retinal ganglion cells, since molecular mechanisms leading to differentiation of this cell type had been well characterized by a number of previous studies (reviewed by Mu and Klein, 2004). The atonal family bHLH transcription factor Ath5 (Cath5 in chicken, Math5 in mouse, and Xash5 in frog) is the primary factor that specifies the retinal ganglion cell lineage, and it activates transcription of other genes essential for terminal differentiation of the cells (reviewed by Mu and Klein, 2004). We sought to overexpress Cath5 and/or Wnt2b at early stages such as E2 and analyze at E3.5, before the emergence of a huge number of endogenous retinal ganglion cells that might

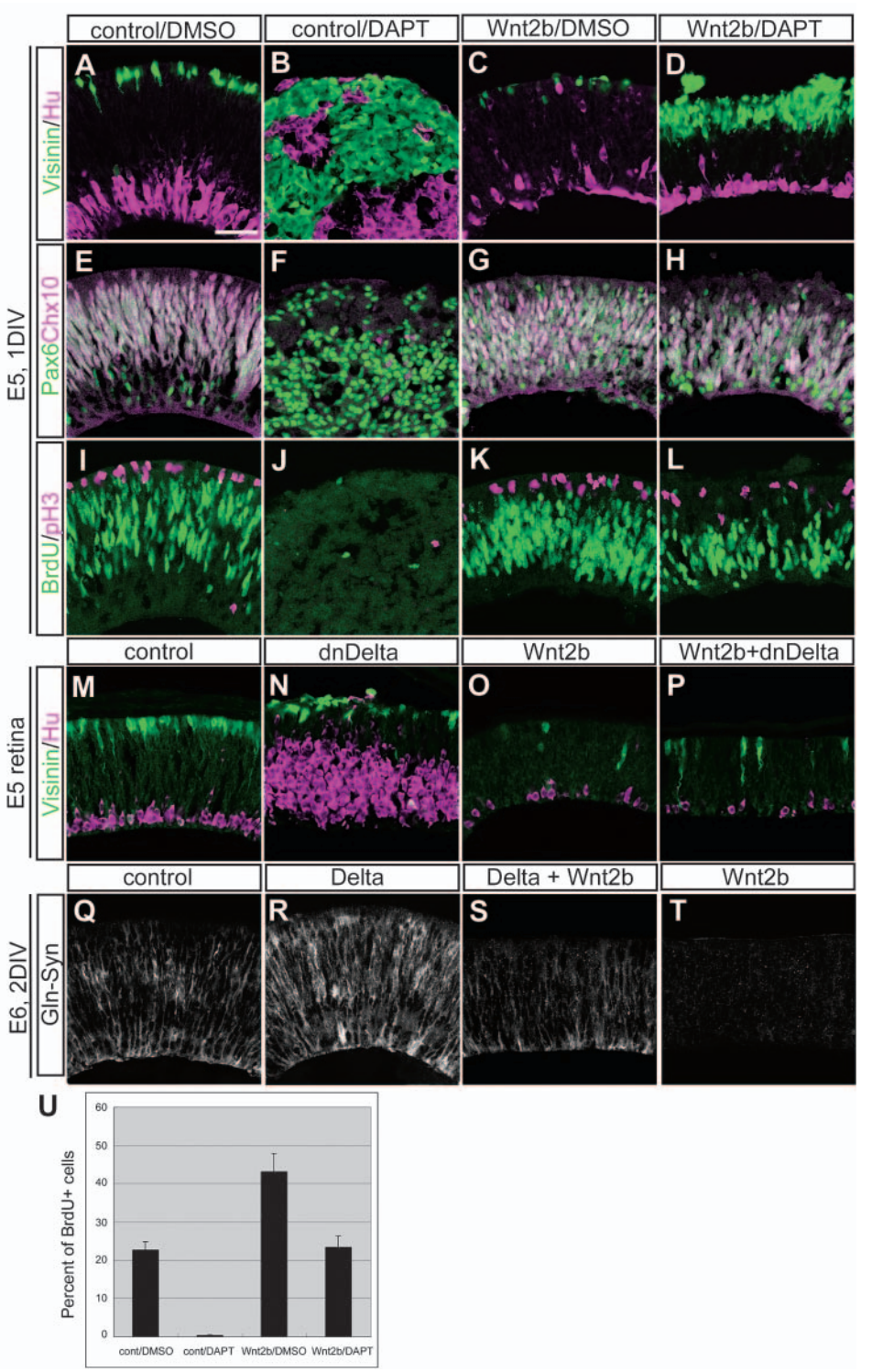

Fig. 3. Wnt2b suppresses premature neurogenesis induced by an inactivation of Notch signaling. (A-L) Effects of DAPT and Wnt2b on progenitor cell differentiation. The retinal explants were prepared from E5 retina electroporated with control (A,B,E,F,I,J) or Wnt2b-expressing (C,D,G,H,K,L) provirus vectors, and were cultured for 2 days in the absence (A,E,I,C,G,K) or presence (B,F,J,D,H,L) of DAPT, a γ-secretase inhibitor that blocks Notch signaling. The sections were stained for visinin (green), and Hu (purple) in A-D, for Pax6 (green) and Chx10 (purple) in E-H, and for BrdU (green) and phosphorylated histone H3 (purple) in I-L. In the control explants, the DAPT treatment induced premature neuronal differentiation, as revealed by the increase of neuronal markers (B) and a decrease of retinal progenitor cell markers (F). In the Wnt2b-expressing retinal explants, however, a large population of the cells still co-expressed the progenitor markers (H) and incorporated BrdU (L). (M-P) Effects of a dominant negative Delta1 and Wnt2b on retinal progenitor cell differentiation. The optic vesicles were electroporated with provirus vectors encoding control RCASBP (A,B) in M, RCASBP (A) encoding a dominant-negative form of Delta1 and RCASBP (B) in N, RCASBP (A) and RCASBP (B) encoding Wnt2b in O, or RCASBP (A) encoding a dominant-negative form of Delta1 and RCASBP (B) encoding Wnt-2b in P. The retinas were fixed at E5, and sections were stained for visinin (green) and Hu (purple). Note that Wnt2b completely suppressed the effect of the dominant-negative Delta in promoting the neurogenesis. (Q-T) Effect of Delta1 and Wnt2b on Müller cell differentiation. Note that Wnt2b suppressed the effect of Delta1 in inducing Müller glia differentiation when co-expressed in the same explants. (U) Percentage of BrdU+ cells in the explants expressing each construct (n=4). Scale bars: 30 μm.

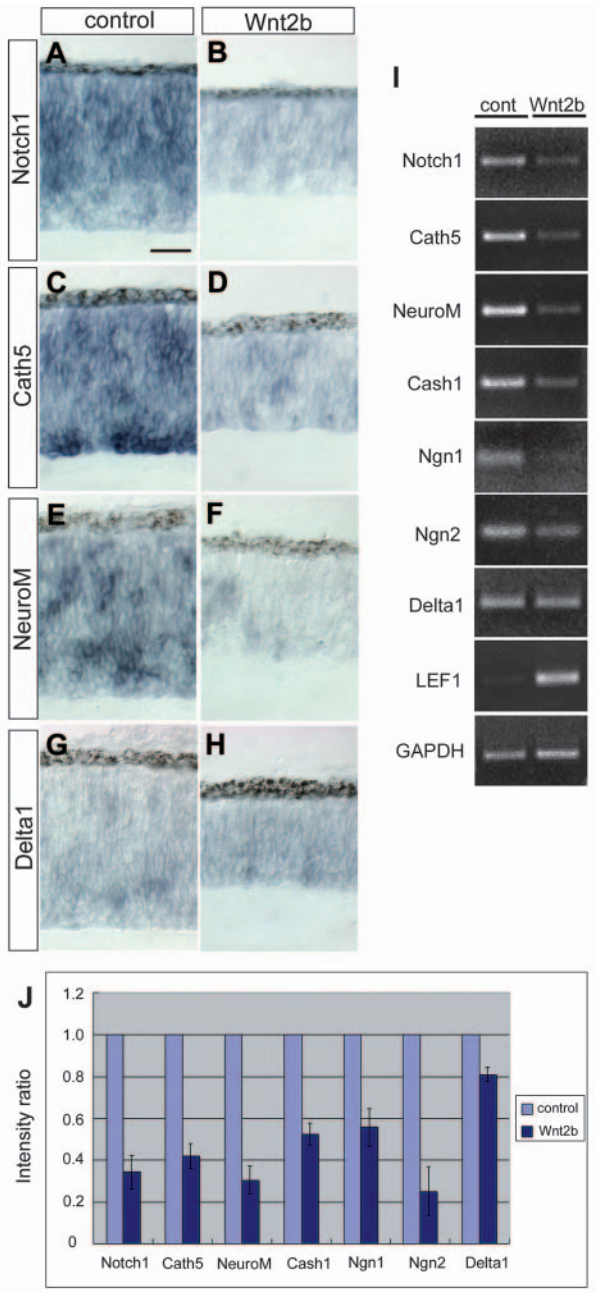

Fig. 4. Wnt2b downregulates the expression of multiple proneural genes. (A-F) mRNA expression pattern of Notch1, Cath5, NeuroM, and Delta1 in the central region of control (A,C,E,G) or Wnt2b-expressing (B,D,F,H) E5.5 retina. These genes were robustly expressed in the control retina, but their expressions were reduced in the Wnt2b-expressing retina, except for Delta1, expression of which was unaffected by exogenous Wnt2b. (I) RT-PCR analysis of proneural gene expressions. The expression of all of the genes, Notch1, Cath5, NeuroM, Cash1, Neurogenin 1(Ngn1), and Neurogenin 2 (Ngn2) was downregulated in the Wnt2b-expressing retina (right lane) compared to the control retina (left lane), whereas the expression level of Delta1 and Gapdh was unchanged. The expression of Lef1 was upregulated in the Wnt2b-expressing retina. (J) Percentage expression of each proneural gene in Wnt2b-expressing retina to that in the control retina. Intensity of PCR products corresponding to each proneural gene was measured and normalized to that of Gapdh. Relative signal intensity of each gene in the control experiment was represented as 1. Scale bar: 30 μm.

obscure the exogenous ganglion cells induced by Cath5 overexpression. For this purpose, we overexpressed exogenous genes under the control of the strong ubiquitous promoter CAG (Niwa et al., 1991), the expression of which starts earlier than that of the retrovirus promoter we used in the prior experiments mentioned above. As previously reported (Liu et al., 2001; Matter-Sadzinski et al., 2001; Kanekar et al., 1997), overexpression of Cath5 increased the number of retinal ganglion cells compared with their number in the control embryo (Fig. 5B,F,I). On the contrary, Wnt2b overexpression completely inhibited differentiation of retinal ganglion cells (Fig. 5C,G,I). When Wnt2b and Cath5 were co-introduced, differentiation of the retinal ganglion cells was rescued in the cells expressing co-electroporated GFP (Fig. 5D,H,I). These results indicated that downregulation of proneural genes was necessary for Wnt2b to inhibit progenitor cell differentiation.

Inhibitory effect of Wnt2b on retinal ganglion cell differentiation is not dependent on its mitogenic activity

Previously, regulation of the cell cycle exit has been shown to be important for proper retinal cell differentiation (Ohnuma et al., 2002). As activation of Wnt signaling has a mitogenic effect in other regions of the central nervous system (Megason and McMahon, 2002; Chenn and Walsh, 2002), it seemed possible that continuous progression of the cell cycle was the primary effect of Wnt2b, resulting in the inhibition of neuronal differentiation. To address this issue, we tested whether blocking of the cell cycle progression would suppress the inhibitory effect of Wnt2b on ganglion cell differentiation. Wnt signaling upregulates the transcription of cyclinD1 to promote cell cycle progression in cancer cells or mouse neural tube (Tetsu and McCormick, 1999; Megason and McMahon, 2002). We therefore overexpressed Ink4D (p19), which specifically inhibits cyclinD/Cdk4 complex during the early G1 phase in the retina (Cunningham et al., 2002) (reviewed by Dyer and Cepko, 2001), to arrest the progression of the cell cycle. We initially examined cellular proliferation 12 hours after the electroporation when the first exogenous gene expression was observed. In the retina electroporated with a mock or Wnt2b-expressing plasmid, most of the cells expressing co-electroporated GFP (96.3±0.4% for mock transfected and 96.1±2.1% for Wnt2b transfected, n=4) incorporated BrdU (Fig. 6A,C,M). In contrast, in the Ink4D-electroporated retina, only about one-third of the cells (29.1±6.4%) incorporated BrdU (Fig. 6B,M), indicating that the cell cycle progression was blocked by the overexpression of Ink4D. A significantly smaller number (38.4±5.8%) of the cells co-electroporated with Wnt2b and Ink4D incorporated BrdU (Fig. 6D,M) compared to cells electroporated with Wnt2b alone, suggesting that cell cycle progression was also inhibited by the overexpression of Ink4D even in the presence of Wnt2b. We subsequently examined the differentiation of retinal ganglion cells 48 hours after electroporation. In mock-electroporated retina, a subpopulation (3.8±0.4%) of the cells expressing co-electroporated GFP differentiated into ganglion cells, as revealed by the expression of neurofilament or islet 1 (Fig. 6E,I,N). The number of ganglion cells was not significantly altered by the overexpression of Ink4D (Fig. 6F,J,N, 3.1±0.6%), suggesting that overexpression of Ink4D itself did not affect ganglion cell differentiation. As mentioned above,

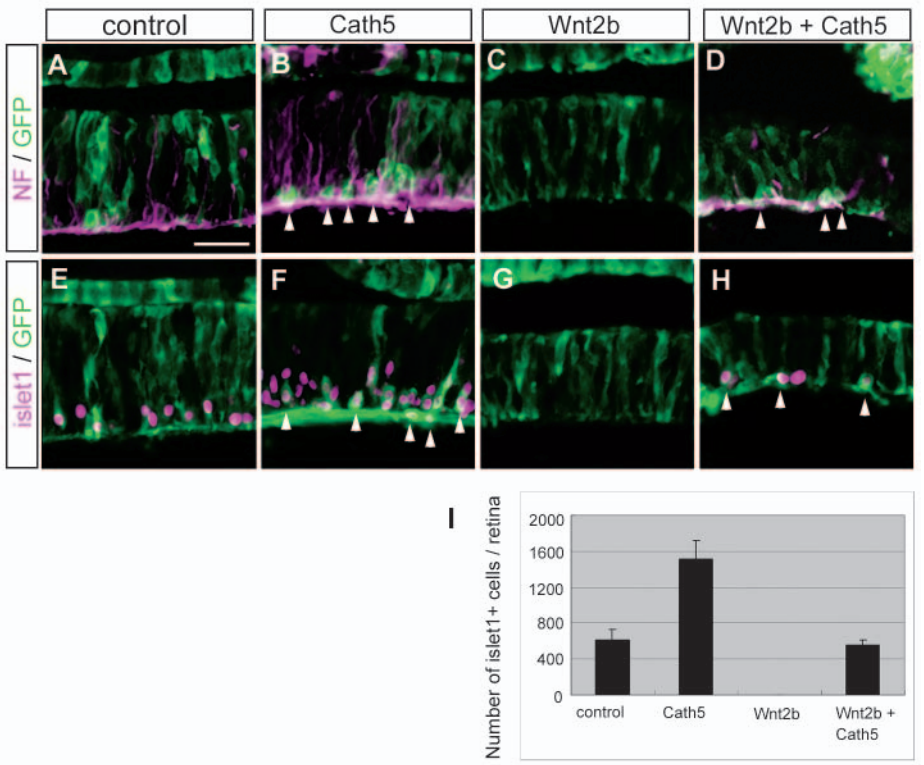

Fig. 5. Introduction of exogenous Cath5 suppresses the inhibitory effect of Wnt2b on the differentiation of retinal ganglion cells. Expression pattern of neurofilament (A-D) and islet1 (E-H) in the retina electroporated with plasmids expressing control (A,E), Cath5 (B,F), Wnt2b (C,G), and Cath5 and Wnt2b (D,H). The marker expressions are shown in purple, and co-electroporated GFP signals, in green. Arrowheads indicate the cells expressing co-electroporated GFP and the retinal ganglion cell markers. (I) The number of islet1-expressing cells per retina (n=4). The cells were counted on serial sections of the retina electroporated with each construct as indicated. Note that the exogenous expression of Cath5 increased the number of islet1-positive cells (B,F,I), whereas no islet1-positive cells were observed in the Wnt2b-expressing retina (C,G,I). When Cath5 was introduced together with Wnt2b, inhibition of the ganglion cell differentiation by Wnt2b was suppressed (D,H,I). Scale bar: 30 μm.

Wnt2b overexpression completely inhibited the differentiation of ganglion cells (Fig. 6G,K,N). Significantly, this effect of Wnt2b was not suppressed by co-expression of Ink4D (Fig. 6H,L,N), suggesting that the inhibitory effect of Wnt2b on neuronal differentiation did not require continuous cell-cycle progression.

Discussion

We have demonstrated that Wnt2b promotes prolonged proliferation of retinal progenitor cells and inhibits their differentiation by downregulating proneural gene expressions independently of Notch activity (Fig. 7). The following observations support this conclusion. First, Wnt2b-overexpression induced continuous growth of retinal explants whereas that of Delta did not. Second, Wnt2b downregulated the expression of Notch1 itself, thus making the retinal progenitor cells less sensitive to signals mediated by the receptor. Third, Wnt2b inhibited progenitor cell differentiation even under the condition where Notch signaling was blocked, either by a pharmacological reagent DAPT or by a dominant negative form of Delta. Finally, Wnt2b reduced the expression of multiple proneural bHLH genes, and restoring Cath5 expression by an exogenous promoter rescued ganglion cell differentiation in the Wnt2b-expressing retina. Although the downregulation of proneural genes was necessary for Wnt2b to inhibit progenitor cell differentiation, we currently do not know if it is sufficient or not. To test this hypothesis, it might be essential to knock down several proneural genes simultaneously as well as the Wnt downstream signaling pathway, which is technically not feasible at the moment.

Wnt activates diverse signaling pathways depending on the cell type (reviewed by Wodarz and Nusse, 1998). Since Wnt2b stabilizes a cytosolic fraction of β-catenin in embryonic retinal cells (Kubo et al., 2003), it is likely that Wnt2b stimulates the canonical Wnt pathway in the retina, which activates target gene transcriptions through a downstream effector β-catenin/Lef (Tcf) complex (reviewed by Wodarz and Nusse, 1998). Nevertheless, we observed downregulation of expression of multiple proneural gene as well as Notch in the Wnt2b-overexpressed retina. Recently, the β-catenin/Lef (Tcf) complex has been shown to bind an upstream regulatory region of Ngn1, a proneural bHLH gene essential for neuronal differentiation, and to activate its transcription (Hirabayashi et al., 2004). Interestingly, the activation of the canonical Wnt pathway promotes the differentiation of cultured neural precursor cells derived from the E13.5 mouse cortex or E11.5 telencephalon, but inhibits their differentiation when the precursor cells are prepared from E10.5 embryo (Hirabayashi et al., 2004; Muroyama et al., 2004). Therefore, the β-catenin/Lef (Tcf) complex may function both as a repressor and an activator of the same target genes such as Ngn1 to exert opposite effects, depending on the cellular context. Alternatively, Wnt2b may activate the expression of certain transcriptional repressor(s), which in turn downregulates the expression of target proneural genes in the retina (Fig. 7A). Since an activation of Notch signaling leads to glial cell differentiation (Furukawa et al., 2000; Satow et al., 2001; Scheer et al., 2001), it is unlikely that Wnt2b stimulates Notch-downstream effector genes such as Hes to inhibit proneural gene functions. Several studies have indicated the importance of the exit from the cell cycle to the proper control of differentiation (reviewed by Ohnuma and Harris, 2003), suggesting that Wnt2b might inhibit cellular differentiation through its mitogenic activity, which promotes continuous cell cycle progression. However, we have demonstrated that Wnt2b

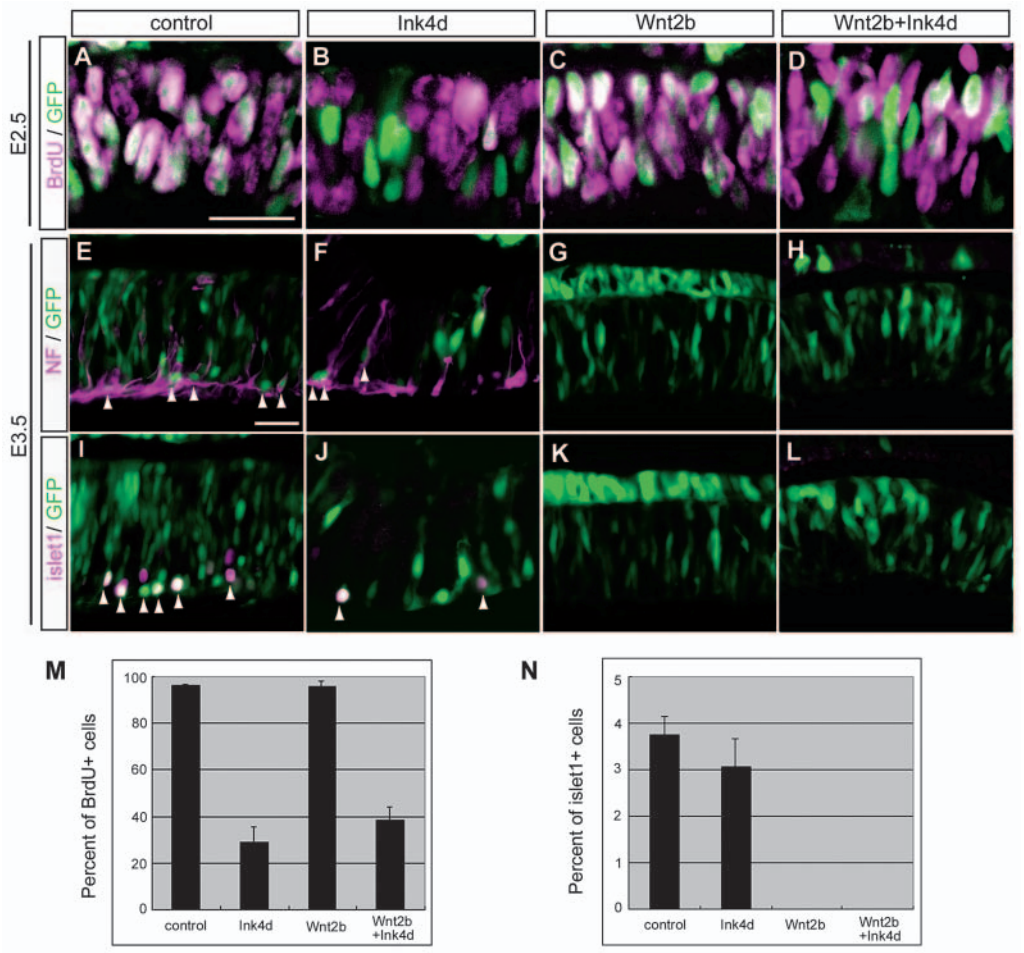

Fig. 6. Blockade of cell-cycle progression by a CDK inhibitor, Ink4D (p19), does not affect the inhibitory effect of Wnt2b on ganglion cell differentiation. (A-D) Effect of Ink4D and Wnt2b on cellular proliferation. Optic vesicles of E1.5 embryos were electroporated with vectors, as indicated above. After 12 hours, the sections were stained for BrdU (purple) and for co-electroporated nlsGFP (green). Note that Ink4D-electroporated cells did not incorporate BrdU in B and D. (E-L) Expression patterns of neurofilament (purple in E-H) and islet1 (purple in I-L) in the retina 48 hours after the electroporation. Arrowheads indicate the cells expressing co-electroporated GFP and the retinal ganglion cell markers. Wnt2b inhibited ganglion cell differentiation in both the presence and absence of co-electroporated Ink4D (G,H,K,L). (M) Percentage of BrdU+ cells in retina (n=4) expressing each construct. (N) Ratio of islet1+ cells/co-electroporated nlsGFP in the retina (n=4). Scale bars: 20 μm (A-D); 30 μm (E-L).

can inhibit ganglion cell differentiation even when the cell cycle progression is perturbed by Ink4D overexpression. We thus favor a model in which Wnt2b exerts its negative effect on differentiation independently of its mitogenic activity, presumably by negatively regulating proneural genes.

In the marginal region of the retina, progenitor cells continue to proliferate for a long period, showing stem cell-like properties (reviewed by Reh and Levine, 1998; Perron and Harris, 2000b). bFGF and EGF sustain proliferation of progenitor cells derived from human fetal retina for up to 300 days in monolayer cultures (Kelley et al., 1995), and the administration of these factors to the eyes of post-hatched chickens increases the number of proliferating cells in the marginal retina (Fischer and Reh, 2000). In the heterozygous patched mutant mouse, where the hedgehog signal is supposed to be hyper-activated, the marginal progenitor cells continue to proliferate even after the stage when they normally stop proliferating in this species (Moshiri and Reh, 2004). These studies imply that bFGF/EGF or hedgehog family protein may control the progenitor cell

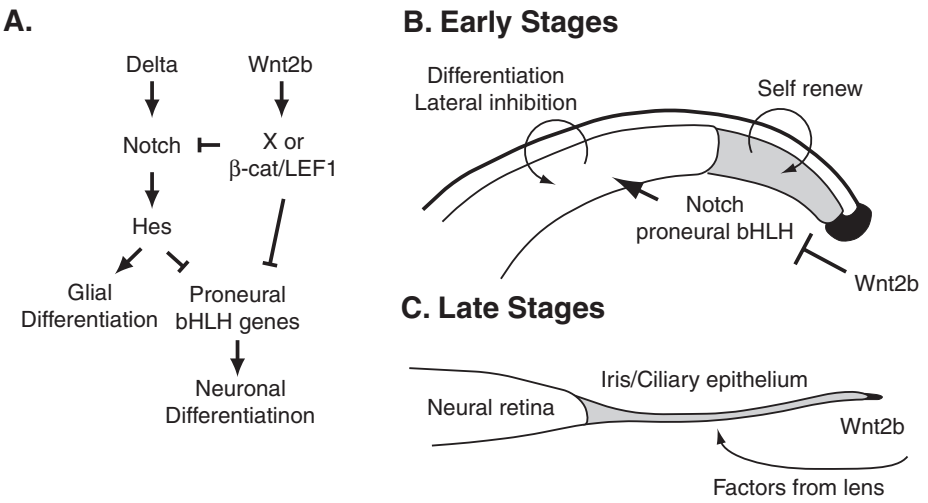

Fig. 7. Proposed model for the action of Wnt2b in the marginal retina. (A) Gene cascade leading to the inhibition of cellular differentiation by Wnt signaling. See the text for details. (B) During early retinal development, Wnt2b sends a signal to the peripheral region of the optic vesicles to keep the progenitor cells undifferentiated. Centrally located progenitor cells do not receive the Wnt signals and express proneural genes as well as Notch, which triggers the differentiation cascades. (C) At later stages, the marginal retina differentiates into iris and ciliary epithelium, induced by factors coming from surrounding tissues such as the lens or cornea.

proliferation in the marginal retina. However, the retinal explants cultured in the presence of bFGF and EGF or those overexpressing sonic hedgehog stopped growing after 2-4 days under our culture conditions; and among the factors we have tested, only Wnt2b sustained prolonged proliferation of the retinal explants. This discrepancy might be partially explained by different culture systems. In particular, culturing progenitor cells in monolayer conditions should provide different cell-cell or cell-substrate interaction, resulting in an activation of divergent signaling pathways that act together with EGF/FGF signaling to sustain prolonged proliferation. Importantly, the progenitor cells maintained by Wnt2b in the retinal explants could differentiate into neurons and glia, making a correctly laminated retina, upon the addition of a Wnt antagonist, suggesting that those cells also retained multipotency. The effect of the canonical Wnt signaling to promote progenitor cell proliferation has also been demonstrated in another region of the central nervous system: the overexpression of an activated form of β-catenin in mice delays the cell-cycle exit of progenitor cells, leading to the formation of a folded cerebral cortex resembling that of primates (Chenn and Walsh, 2002). In the spinal cord, Wnt1 and Wnt3a cooperate to stimulate cyclinD1 expression, which results in the formation of a growth gradient from dorsal to ventral (Megason and McMahon, 2002). Taken together, our data allow us to speculate that Wnt2b is responsible for the stem-cell-like properties of marginal progenitor cells (Fig. 7): in the marginal retina, the progenitor cells receive Wnt2b signals from the anterior rim, which leads to downregulation of proneural bHLH genes. These cells thus remain undifferentiated and therefore self-renew, adding new cells in the periphery. When the Wnt signal is absent, these cells prematually differentiate into neurons, as we have previously reported (Kubo et al., 2003). In contrast, centrally located cells do not receive the Wnt signals, and progress into the differentiation cascades by expressing the proneural genes as well as Notch. This hypothesis is consistent with a previous study showing that the marginal-most part of the ciliary marginal zone in *Xenopus*, where true retinal stem cells reside, does not express any of the known proneural genes and neurogenic genes (Perron et al., 1998).

The marginal optic vesicles neighboring the Wnt2b-expressing regions finally give rise to non-neural anterior eye structures including the iris and ciliary epithelium (Bard and Ross, 1982). Since Wnt2b-expressing retinal explants started to differentiate into neural retina upon being deprived of the ligand, Wnt2b alone was not sufficient to induce iris/ciliary epithelium, at least in vitro. Considering that the lens induces the expression of iris/ciliary epithelium-specific markers in cultured retinas (Thut et al., 2001), Wnt2b possibly prevents the peripheral optic vesicles from becoming neural retina by downregulating proneural and neurogenic genes, so they differentiate into the anterior eye structures through interaction with surrounding tissues at later stages (Fig. 7C). Recently, retinal stem cells have been isolated from adult ciliary epithelium or iris under appropriate culture conditions (Tropepe et al., 2000; Ahmad et al., 2000; Haruta et al., 2001). In the neurosphere cultures, prepared from pigmented ciliary margin, stem cell-containing spheres are always composed of a mixture of darkly pigmented cells and non-pigmented cells (Tropepe et al., 2000). Since Wnt2b is also expressed in a certain population of pigmented cells (Fuhrmann et al., 2000; Jasoni et al., 1999), it will be intriguing to test whether Wnt2b is responsible for maintenance of those retinal stem cells in the neurosphere cultures.

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