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Wnt2b controls retinal cell differentiation at the ciliary marginal zone

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SUMMARY

The ciliary marginal zone of the vertebrate retina contains undifferentiated progenitor cells that continue to proliferate and add new neurons and glia peripherally during the embryonic stages – even after the formation of a functional retina. To understand the molecular mechanism that controls the prolonged progenitor cell proliferation in the ciliary marginal zone, we employed a candidate molecule approach, focusing on Wnt2b (formerly know as Wnt13), which is expressed in the marginal most tip of the retina. Frizzled 4 and 5, seven-pass transmembrane Wnt receptors, were expressed in the peripheral and central part of the retina, respectively. LEF1, a downstream Wnt signaling component, was expressed at high levels in the ciliary marginal zone with expression gradually decreasing towards the central retina. The LEF1-expressing region, which is where Wnt signaling is supposedly activated, expressed a set of molecular markers that are characteristic of the progenitor cells in the ciliary marginal zone. Overexpression of Wnt2b by use of in ovo electroporation in the central retina inhibited neuronal differentiation and induced the progenitor cell markers. Blocking of the Wnt downstream signaling pathway by a dominant-negative LEF1 inhibited proliferation of the cells in the marginal area, which resulted in their premature neuronal differentiation. The progenitor cells in the ciliary marginal zone differentiated into all the neuronal and glial cell types when cultured in vitro, and they proliferated for a longer period than did centrally located progenitor cells that underwent a limited number of cell divisions. In addition, the proliferation of these progenitor cells was promoted in the presence of Wnt2b. These results suggest that Wnt2b functions to maintain undifferentiated progenitor cells in the ciliary marginal zone, and thus serves as a putative stem cell factor in the retina.

Key words: Wnt, Frizzled, Canonical pathway, Chicken, Retina, Stem cell, Differentiation, Electroporation

INTRODUCTION

The marginal part of the vertebrate retina is distinct from the other parts, showing unique differentiation and proliferation properties. The marginal-most region neighboring the embryonic lens becomes non-neural anterior eye structures such as the iris and ciliary epithelium (Coulombre, 1965). The ciliary marginal zone (CMZ) is a region between the neural retina and ciliary epithelium, and contains retinal progenitor cells that proliferate for a long period to add neurons and glia peripherally even after the functional retina has formed (Perron and Harris, 2000; Reh and Levine, 1998). In lower vertebrates, such as fish and frog, the CMZ progenitor cells continue to divide throughout life, displacing the 'old' neural retina centrally as the eyes increase in size (Beach and Jacobson, 1979; Hollyfield, 1968; Johns, 1977; Meyer, 1978; Straznicky and Gaze, 1971). In the post-hatched chick, the CMZ progenitor cells give rise to multiple types of neurons and glia, suggesting the progenitor cells are also maintained in that region in the higher vertebrates (Fischer and Reh, 2000). In adult rodents, cells located in the pigmented ciliary margin, which topologically correspond to the CMZ, show stem cell characteristics when they are cultured in vitro (Ahmad et al., 2000; Tropepe et al., 2000).

The CMZ progenitor cells are distinct from other progenitor cells in the central part of the developing retina, although both of them produce all the retinal neurons and glia (Layer et al., 2001; Perron and Harris, 2000; Reh and Levine, 1998). For example, in *Xenopus*, the CMZ progenitor cells produce a large number of progenitor clones forming wide columns, whereas the retinal progenitor cells in the central retina undergo a limited number of cell divisions to generate progenies forming narrow radial columns (Holt et al., 1988; Wetts and Fraser, 1988; Wetts et al., 1989). In addition, the embryonic retinal margin, but not the central retina, possesses the capacity to regenerate a correctly laminated neural retina after retinoectomy, despite the fact that both of the retinal regions contain multipotential retinal progenitor cells (Coulombre and Coulombre, 1965; Coulombre and Coulombre, 1970). The two progenitor populations can also be distinguished by molecular marker expression. Both of the retinal progenitor cells are identified by co-expression of Pax6 and Chx10 (Belecky-Adams et al., 1997; Fischer and Reh, 2000). The CMZ progenitor cells, however, do not express neural precursor

marker Notch1 (Dorsky et al., 1995; Perron et al., 1998), which is strongly expressed in the proliferating progenitor cells in the central retina (Austin et al., 1995; Henrique et al., 1997). It is generally accepted that the continuous proliferation of the CMZ progenitor cells add cells peripherally during the embryonic period, accounting at least in part for the centro-peripheral gradient of neural differentiation (Coulombre, 1965; Layer and Willbold, 1993).

To address the molecular mechanism that controls the prolonged proliferation of the CMZ progenitor cells, we employed a candidate molecule approach. Wnt genes were originally identified as oncogenes, and had been shown to regulate a variety of developmental processes (reviewed by Wodarz and Nusse, 1998). We noted that many of the Wnt family molecules were expressed in the region where multipotential progenitor cells are maintained, such as the neural crest cell-producing dorsal neural tube, primitive streak, tail bud and proliferating region of the hair follicles (Wodarz and Nusse, 1998). In the present study, we analyzed the function of chicken Wnt2b expressed in the marginal-most tip of the embryonic chicken retina (Jasoni et al., 1999). When ectopically overexpressed in ovo, Wnt2b induced expression of molecular markers characteristics of CMZ progenitor cell. On the contrary, blocking of Wnt2b function by a dominant-negative LEF1 inhibited proliferation of the marginal cells, leading to their premature differentiation. Furthermore, the CMZ progenitor cells proliferated for a longer period than the centrally located ones, and their proliferation was further promoted by chicken Wnt2b in vitro. Our results suggest that Wnt2b regulates the maintenance of a retinal progenitor population in the CMZ, and thus function as a putative stem cell factor in the retina.

MATERIALS AND METHODS

Animals

Fertilized eggs were obtained from a local farm (Yamagishi, Japan) and incubated at 38.5°C until they reached the appropriate embryonic stages (Hamburger and Hamilton, 1951).

Molecular cloning and construction of the vectors

Degenerate primers were designed for conserved sequences of Frizzled family proteins, YPERPII and YFHLAAW, and RT-PCR was carried out by using cDNA from E5 chicken embryonic retina as a template. The amplified fragments were subcloned into pCR2.1 (Invitrogen), sequenced, and subsequently used for making cRNA probes for in situ hybridization. For amplifying Wnt family genes, the same set of degenerated primers were used as previously described (Gavin et al., 1990). To obtain a cDNA fragment of chicken Ptmb4, we carried out RT-PCR by using the following the following set of primers: 5′-atgtctgacaaaccagatat-3′ and 5′-ttcttacaaagttaaacagc-3′. To obtain a full-length clone of the mouse Wnt2b gene, we designed primers according to the published sequences (Katoh et al., 1996) and amplified the entire coding region by RT-PCR using cDNA from E14 retinas. The construction of pcDNA3.1-mFzd4-CRD-myc and hFzd5-CRD-myc was described elsewhere (S. N., S. Takada, R. Takada and M. T., unpublished). For in ovo overexpression studies, cDNA fragments were subcloned into pCA-pA (Niwa et al., 1991), which enabled strong exogenous expression in chicken embryos (Momose et al., 1999). The cDNA clone of chicken Wnt2b was a kind gift from Dr Izpisua Belmonte (Kawakami et al., 2001). The entire coding region of chicken Wnt2b was subcloned into pEF-Fc (Suda and Nagata, 1994) to generate a fusion protein of chicken Wnt2b and the Fc region of human IgG. RCAS-ΔLEF1 was a kind gift from Dr Kengaku, and the coding region was subcloned into pCA-pA for the in ovo electroporation study. The GenBank Accession Number for chicken Frizzled 5 is AF463494.

Fzd-binding study using immunoprecipitation

As both Wnt2b-Fc and the Fzds-CRD-myc were poorly secreted into the culture medium, we analyzed the binding of those proteins by co-transfecting cells with both proteins, followed by immunoprecipitation of Wnt2b-Fc. COS7 cells were cultured in DH10, which is 1:1 mixture of Dulbecco's MEM (DMEM, Nissui, Japan) and Ham's F12 (F12, Nissui, Japan) supplemented with 10% FCS, 0.3% glucose and antibiotics. The cells (3×10^5) were co-transfected with pcDNA3.1-Fzds-CRD-myc and pEF-Wnt2b-Fc by using the Effectene reagent (Qiagen) according to the manufacturer's instructions. As a control experiment, pEF-c-cad7-Fc (Nakagawa and Takeichi, 1998) was used instead of pEF-Wnt2b-Fc. After 48 hours, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in 1 ml of binding buffer (1% Triton-X-100, 1% NP-40, 1 mM EDTA in PBS) for 30 minutes at 4°C. The cell lysates were then centrifuged at 17,400 ***g*** for 5 minutes to remove the cell debris and incubated with 50 μl of Protein-A Sepharose-4B beads (Zymed) for 30 minutes at 4°C. After extensive washing with the binding buffer, the immunoprecipitated complexes were lysed in SDS-PAGE sample buffer, run on a 10% acrylamide gel, and then analyzed by using a standard western blotting technique. For detecting the myc and the Fc antigen, we used a mouse monoclonal antibody 9E10 (Sigma) and rabbit anti-human IgG-Fc (Jackson Laboratory), respectively, followed by HRP-conjugated goat anti-mouse IgG (Amersham) and HRP-conjugated goat anti-rabbit IgG (Amersham), respectively.

Preparation of conditioned medium of chicken Wnt2b producing cultures and the lysate of mFzd4-CRD producing cells

To obtain functional chicken Wnt2b proteins, we collected conditioned medium (CM) prepared from 293 cells stably transfected with pCA-Wnt2b. The transfectants were grown to confluence, and the medium was replaced with fresh DH10, and the cells were further incubated for 48 hours. The supernatants were collected and centrifuged at 48,384 ***g*** to remove insoluble materials, filtered, aliquoted and stored at –80°C until used. For collecting soluble mFzd4-CRD, 5×10^5 COS-7 were plated on 5 cm dish and transfected with pcDNA3.1-mFzd4-CRD-myc. On the next day, the medium was replaced with fresh DH10, and the cells were cultured for 48 hours. The cells were then washed with PBS, and incubated for 30 minutes in 0.5 ml of 1% CHAPS in PBS containing 1 mM EDTA on ice. After the centrifugation, the cell lysates were extensively dialyzed against PBS, and then against DH, and were thereafter kept frozen until used. As a control, GFP-expressing COS7 cells were uses as the source of the cell lysates.

Retinal monolayer culture

The cultures were prepared according to the methods previously described (Willbold et al., 2000) with minor modifications. Briefly, the marginal part of E5 neural retinas was dissected in F12 and treated for 10 minutes at room temperature with 0.1% crude trypsin (1:50; DIFCO) in saline buffered with HEPES (10 mM, pH 7.4) and supplemented with 1 mM EDTA. After 2 washes in DH10, DNAseI (Sigma) was added at a final concentration of 0.001% in DH10, and the retinal fragments were dissociated into single cells by gentle pipetting with a fire-polished Pasteur pipette. After low-speed centrifuge at 700 ***g*** for 5 minutes, 2×10^6 cells were resuspended in 2 ml of culture medium and plated into each well of six-well dishes (Falcon). A 300 μl volume of CM (prepared from chicken Wnt2b-expressing or parental 293 cells) and 700 μl of cell lysates (prepared from mFz4-CRD or GFP-expressing cells) were then added to the

medium, and the cells were cultured for 6 hours. For preparing cytoplasmic and membrane-associated β-catenin, fractionation was performed according to the procedures described previously (Shibamoto et al., 1998). To detect the β-catenin on a western blot, we used a rabbit polyclonal antibody against β-catenin (Shibamoto et al., 1998) and HRP-conjugated goat anti rabbit Ig (Amersham). After visualization of the signal by using ECL (Amersham), the intensity of the signals was analyzed with an Image Master (Amersham Pharmacia Biotech). For RT-PCR to study the gene expression, the following primers were used: 5′-catgtgaagcctcagcac-3′ and 5′-cctggataaagctgcatg-3′ for LEF1 and 5′-aagcccattgactttgag-3′ and 5′-tggactctcattcacatc-3′ for N-cadherin. The PCR conditions used were 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 3 minutes, for a total of 25 cycles. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and analyzed with the Image Master. The amplified fragments were also subcloned into pCR2.1 and sequenced to confirm specific amplification of the desired genes.

Reaggregation culture for clonal analysis

The reaggregation culture was prepared according to the methods previously described (Belliveau and Cepko, 1999) with minor modifications. We employed this culture system because retinal cells plated on a culture dish firmly adhered to the substrate within 12 hours of the addition of Wnt2b CM, which hampered the analysis because cell-substrate interaction promoted nonspecific cell differentiation. Small tissue explants were prepared from the CMZ or the central part of the neural retina of E4.5 quail retina, treated with 0.1% trypsin and dissociated into single cells as described above. They were then mixed with an excess of dissociated cells (4×10^3-fold) prepared from the central part of E5 chicken retina, and centrifuged at 700 *g* for 1 minute. The cell pellet was then suspended in culture medium at a concentration of 2×10^5 cells/μl. Cell suspension (1 μl) was placed on a Millicell CM filter, 0.4 μm pore size (Millipore) and cultured for 7 days. The culture medium used was DH10 supplemented with control or chick Wnt2b CM, which was added to the culture medium at a dilution of 1:5. The half volume of the culture medium was exchanged every 2 days. To examine the secondary clone formation, we cultured the primary clones for 4 days either in the presence or absence of chicken Wnt2b CM. The pellets were dissociated into single cells and then pelleted to be cultured for a further 7 days as described above.

In ovo electroporation

The purified DNA for each plasmid was diluted to a final concentration of 5 μg/μl and mixed with 1.5 μg/μl of the GFP-expressing plasmid to identify regions of gene introduction. pCA-mWnt2b-pA was used at a concentration of 1 μg/μl. Fast Green (1%) was added to facilitate visualization of the injected DNA solution. DNA solution (~30 nl) was injected into the optic vesicles of stage 10-11 embryos. After the injection, the anode was placed beside the optic vesicle, and a tungsten microelectrode was inserted into the vesicle through the anterior neuropore. An electric pulse was applied for 25 ms, three times at 7 V. Electroporated embryos were incubated at 38.5°C until they reached the appropriate stages. Strong exogenous expression began approximately 12 hours after electroporation (12 hours AE), corresponding to stage 16-18 (Momose et al., 1999).

Immunostaining and in situ hybridization

Mouse anti collagen type IX (clone 2C2, Developmental Studies of Hybridoma Bank; DSHB), mouse anti-Hu (clone 16A11, Molecular Probe), mouse anti-Islet1 (clone 40.2D6, DSHB), mouse anti-glutamine synthetase (clone 6, Transduction), mouse anti-middle molecular weight neurofilament (clone RMO270, Zymed), mouse anti-quail nucleus (clone QCPN, DSHB), mouse anti-BrdU (clone BU33, SIGMA), rabbit anti-visinin (kind gift from Dr Miki) (Hatakenaka et al., 1985), rabbit anti-GFP (Chemicon), Cy3-conjugated anti-mouse IgG (Chemicon), Alexa 488-conjugated anti-rabbit IgG (Molecular Probes) and Alexa 647-conjugated anti-mouse IgG (Molecular Probes) antibodies were used. To study cell proliferation at E3.5, a few microliters of 50 mM BrdU were injected into an allantoic vein ~1 hour before fixation. For immunostaining experiments, embryos were fixed for 1 hour at room temperature in 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose in PBS for 30 minutes, and then embedded in Tissue-Tek (Sakura). Sections at a thickness of 10 μm were collected on silane-coated slide glasses, rehydrated in PBS, and permeabilized in 100% methanol for 5 minutes at –20ºC. Following re-hydration in PBS, nonspecific binding on these samples was blocked with 4% skim milk (Difco) for 5 minutes. For triple-color immunostaining of the reaggregation culture, the pellets were fixed in 4% paraformaldehyde for 1 hour at room temperature, permeabilized in –20ºC methanol for 15 minutes. After dehydration with PBS, nonspecific binding was blocked with PBST (0.2% Triton X-100 in PBS) supplemented with 5% fetal bovine serum (FBS), and the reaggregates were incubated with primary antibodies overnight at 4°C. After extensive washing with PBT, they were incubated with secondary antibodies for 4-5 hours. After extensive washing, they were then incubated with a second set of primary antibodies that had been directly labeled with either ZENON Alexa 555 (Molecular Probe; for labeling QCPN) or Alexa-488 Antibody labeling kit (Molecular Probe; for labeling HuD). Confocal images at a thickness of 1.7 μm optical sections were collected using LSM510 (Zeiss). For in situ hybridization studies, embryos were fixed in 4% paraformaldehyde overnight at 4°C and processed using standard protocols (Perron et al., 1998). The cRNA probes for LEF1 and Notch1 were kindly provided by Dr Kengaku (Kyoto University, Japan) and Dr Wakamatsu (Tohoku University, Japan), respectively, and those for Chx10, Pax6 and Rx1 by Dr Sakagami and Dr Yasuda (Nara Institute for Science and Technology, Japan). For the Wnt, Frizzled and Ptmb4 sequences, the PCR fragments described above, cloned into pCR2.1, were used as templates.

RESULTS

Chicken Wnt2b and its signaling components are expressed in the marginal retina

To identify molecules that regulate the proliferation of the CMZ progenitor cells, we first tried to identify Wnt genes expressed in the retina, which were considered to be good candidate molecules. We first carried out RT-PCR using the same set of degenerated primers as previously reported, designed for the sequences conserved between Wnt family proteins (Gavin et al., 1990). The cDNA from E4 (stage 22) (Hamburger and Hamilton, 1951) chicken retina was used as a template, because the retinal progenitor cells actively proliferate to increase the eye size at this stage. We obtained three independent cDNA fragments, which were found identical to the previously identified chicken Wnt2b (formerly referred to as cWnt-13), chicken Wnt5a and chicken Wnt7a (Jasoni et al., 1999; Kawakami et al., 2000; Kawakami et al., 1999). We focused on Wnt2b, as its mRNA was expressed in the marginal tip of the developing retina (Fig. 1A).

Wnt proteins transmit their signal by binding to a member of the Frizzled family molecules, which are seven-pass transmembrane receptors (Wodarz and Nusse, 1998). To characterize the subtypes of Frizzled proteins that would mediate the chicken Wnt2b signaling from the eye margin, we carried out RT-PCR again, using primers designed for conserved sequences of the Frizzled transmembrane domains. We obtained cDNA fragments that were identical to previously cloned chicken Frizzled 3 and Frizzled 4 (Nohno et al., 1999),

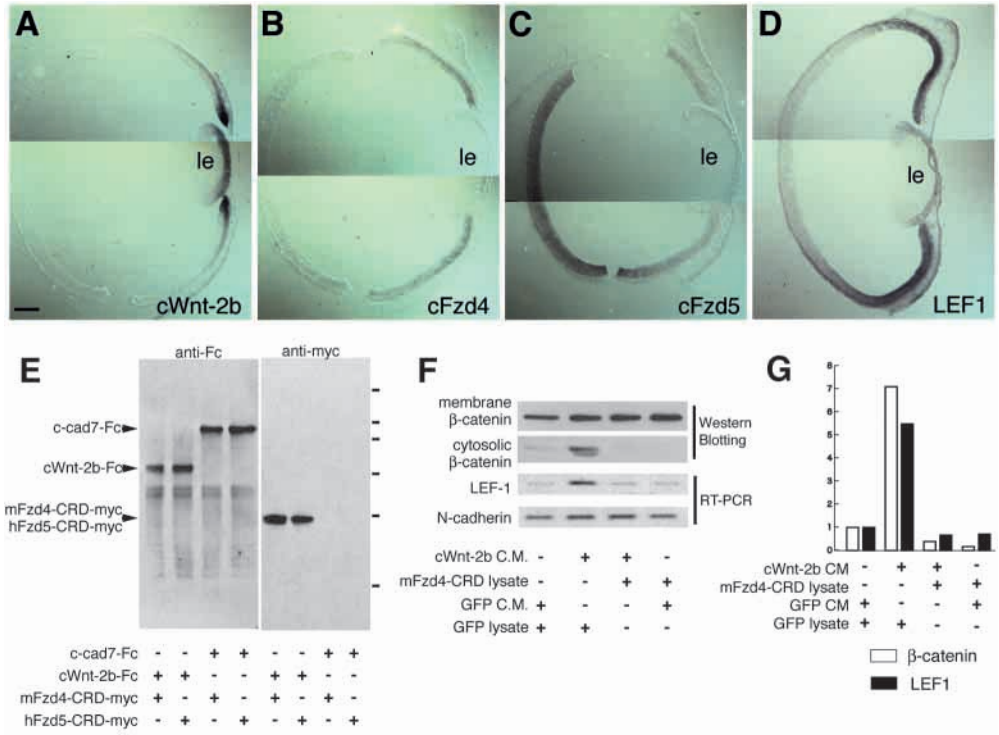


Fig. 1. Wnt signaling components are expressed in the marginal neural retina. (A-D) In situ hybridization of stage 22 (E4) neural retina probed with chicken Wnt2b (A), Fzd4 (B), Fzd5 (C) and LEF1 (D). le, lens. Scale bars: 100 μm. (E) Western blot of the immunoprecipitates from COS7 cells co-transfected with Fzds-CRD and Wnt2b-Fc. The cells were co-transfected with the construct described below, and the cell lysates were immunoprecipitated with protein A. The immunoprecipitates were detected with the antibodies shown at the top. The Wnt2b-Fc binds both mouse Fzd4-CRD and human Fzd5-CRD, whereas the control c-cad7-Fc did not. (F) Stabilization of cytosolic β-catenin and upregulation of LEF1 mRNA by chicken Wnt2b. Retinal cultures were incubated for 6 hours in the absence or presence of chicken Wnt2b-conditioned medium (CM) or soluble mouse Fzd4 lysate described below. The membrane-associated and cytosolic β-catenins were then analyzed by western blotting, and the level of LEF1 mRNA expression was analyzed by RT-PCR. mRNA expression of N-cadherin was used as a control for the RT-PCR analysis. Cytosolic β-catenin and LEF1 mRNA expression were clearly upregulated in the presence of chicken Wnt2b, an effect completely blocked by the addition of mouse Fzd4-CRD. Molecular weight markers are 200 kDa, 116 kDa, 98 kDa, 66 kDa, 45 kDa and 31 kDa. (G) Relative ratio of cytoplasmic β-catenin to membrane-bound β-catenin and of LEF1 mRNA to N-cadherin mRNA. The ratio in the control culture medium was normalized to 1.

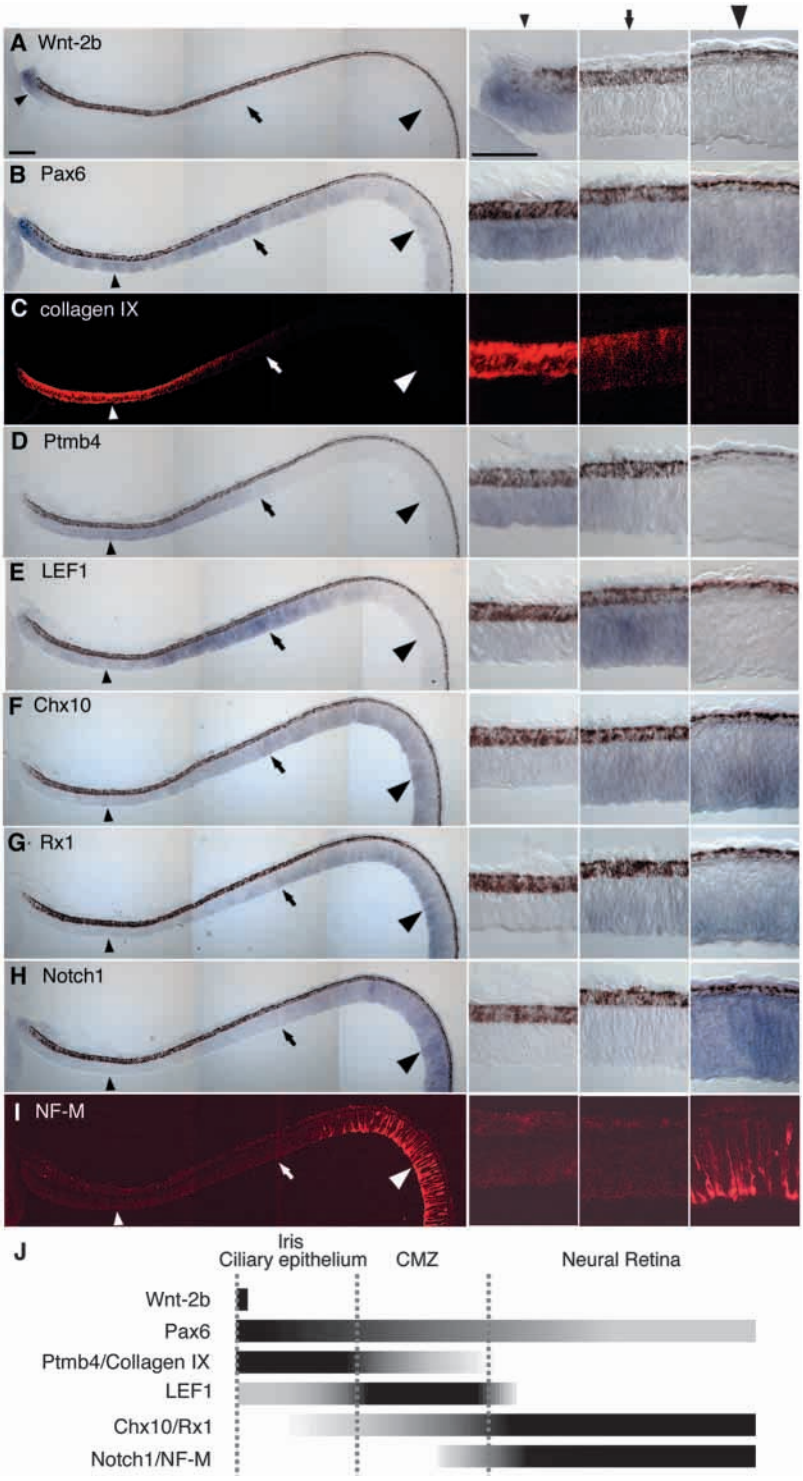
in addition to one fragment that showed its highest sequence similarity to human Frizzled 5 (Wang et al., 1996). We then examined the expression pattern of these chicken Frizzled proteins (Fzd3, 4, 5) at stage 22 by in situ hybridization. The Fzd4 and Fzd5 mRNAs were expressed in the peripheral (Fig. 1B) and the central (Fig. 1C) region of the retina, respectively. We could not detect specific signals for Fzd3 in the retina at the stage tested (data not shown).

We subsequently examined the expression pattern of LEF1, which is an essential component in one of the Wnt signaling pathways called the canonical pathway and forms a transcriptional activator complex with β-catenin (Behrens et al., 1996; Molenaar et al., 1996). In the chicken embryo, LEF1 mRNA is highly expressed where the Wnt-canonical pathway is operating, such as the apical ectodermal ridge of developing limb buds, medial somites, primitive streak and tail buds (Kengaku et al., 1998; Schmidt et al., 2000). In addition, exogenous activation of the Wnt signaling pathway leads to an upregulation of LEF1 mRNA (Kengaku et al., 1998; Schmidt et al., 2000). Accordingly, the strong LEF1 mRNA expression is considered to be a tentative molecular marker for an active Wnt canonical pathway (Kengaku et al., 1998; Schmidt et al., 2000). The expression of LEF1 mRNA was particularly higher in the peripheral part of the retina next to the chicken Wnt2b-expressing region, in which expression decreased gradually towards the central retina (Fig. 1D).

To test a possible interaction between chicken Wnt2b and Fzd4 or Fzd5, we carried out immunoprecipitation experiments using tagged proteins. We co-transfected COS7 cells with myc-tagged Fzd-CRDs (cysteine-rich domain) and chicken Wnt2b fused to the Fc region of human IgG. We then prepared cell lysates and immunoprecipitated the chicken Wnt2b-Fc with Protein-A beads. As a control experiment, we co-transfected the cells with the extracellular domain of cadherin 7 fused to the IgG-Fc (Nakagawa and Takeichi, 1998). For these experiments, we used mouse frizzled 4 and human frizzled 5 (Wang et al., 1996), because the full-length chicken clones were not available at the time of the study. Both mouse Fzd4-CRD-myc and human Fzd-5-CRD-myc were specifically co-immunoprecipitated with chicken Wnt2b-Fc, but not with the control cadherin 7-Fc (Fig. 1E), suggesting that chicken Fzd4 and Fzd5 were able to transmit the chicken Wnt2b signal in the retina.

To confirm the activation of the Wnt canonical pathway by

chicken Wnt2b, we prepared dissociated cells from E5 retina and cultured them in the presence or absence of chicken Wnt2b-conditioned medium. We then studied the level of cytosolic β-catenin by western blotting, as cytosolic β-catenin is stabilized after Wnt canonical pathway activation (Behrens et al., 1996; Molenaar et al., 1996). In control cultures, little or no cytosolic β-catenin was detected; however, the ratio of the cytosolic β-catenin to the membrane-bound form increased sevenfold as early as 6 hours after addition of chicken Wnt2b CM (Fig. 1F,G). We also examined the upregulation of LEF1 mRNA by chickenWnt2b by using RT-PCR. The LEF1 expression level increased more than fivefold by the addition of chicken Wnt2b CM, whereas control N-cadherin expression remained constant (Fig. 1F,G). To confirm that these effects were specific to the Wnt2b in the conditioned medium, we added a soluble form of the extracellular domain of Frizzled CRD domain into the culture. The soluble Frz4-CRD neutralized cytosolic β-catenin stabilization and LEF1 mRNA upregulation, indicating the specificity of the chicken Wnt2b CM for the Wnt pathway (Fig. 1F,G).



LEF1 mRNA is upregulated in a region between the presumptive iris and the neural retina

The marginal retina is largely divided into three parts: the marginal-most area forming iris and ciliary epithelium; the centrally located area determined to become the neural retina; and the intermediate region CMZ, containing retinal progenitor cells (Coulombre, 1965; Perron and Harris, 2000). To further characterize the LEF1 mRNA-expressing region, we examined the expression pattern of various molecular markers using adjacent sections of E5 retina, when the future iris and ciliary epithelium can be morphologically identified (Bard and Ross, 1982). At this stage, chicken Wnt2b remained expressed in the marginal-most tip of the retina (small arrowhead in Fig. 2A). LEF1 mRNA was downregulated in the neighboring region (small arrowhead in Fig. 2E), which strongly expressed collagen type IX and Ptmb4 (small arrowheads in Fig. 2C,D), markers for the presumptive iris or ciliary epithelium (Thut et al., 2001). The marginal region expressed neither Chx10 nor Rx1 (small arrowheads in Fig. 2F,G), markers for the retinal progenitor cells (Belecky-Adams et al., 1997; Fischer and Reh, 2000; Ohuchi et al., 1999). We

Fig. 2. Subdivision of the marginal retina characterized by expression of a combination of markers. The expression patterns of various molecular markers were examined by using adjacent sections of the marginal region of the E5 retina. The S-shape of the retina is the artifact produced during the fixation (see Fig. 1D). In these figures, the marginal region is towards the left and the central region is towards the right. (A,B,D-H) In situ hybridization or (C,I) immunohistochemistry of E5 adjacent sections stained for chicken Wnt2b (A), Pax6 (B), collagen type IX (C), Ptmb4 (D), LEF1 (E), Chx10 (F), Rx1 (G), Notch1 (H) and NF-M (I). The small arrowheads, arrows and large arrowheads indicate the retinal regions that presumably give rise to the iris/ciliary epithelium, the CMZ and the neural retina, respectively (shown at higher magnification in the right-hand panels). For Wnt2b, the marginal tip of the retina is shown at higher magnification instead of the presumptive iris region. Note that the LEF1-expressing CMZ is characterized by weak expression of collagen type IX and Ptmb4; co-expression of Pax6, Chx10 and Rx1; and the absence of Notch1 and NF-M (J). Scale bars: 50 μm.

could not detect the expression of Notch1 or middle molecular weight neurofilament (NF-M) in the marginal region, either (small arrowheads in Fig. 2H,I), which are markers for the neural precursor cells and the retinal ganglion cells, respectively (Austin et al., 1995; Henrique et al., 1997; McCabe et al., 1999). Notch1 was highly expressed in the centrally located neural retina, which also expressed NF-M (large arrowheads in Fig. 2H,I). Strong LEF1 mRNA expression was observed in the CMZ, a region between the presumptive iris/ciliary epithelium and the neural retina (arrow in Fig. 2E). Weak expression of Ptmb4 and collagen type IX were also observed in the LEF1-expressing region; however, their signals were much weaker compared with the signals in the iris and ciliary epithelium (arrows in Fig. 2C,D). The LEF1 mRNA expressing region also expressed moderate level of Pax6, Chx10 and Rx1 (arrows in Fig. 2B,F,G), but did not express Notch1 or NF-M (arrows in Fig. 2H,I). These observations are consistent with the idea that LEF1 mRNA was upregulated in the CMZ progenitor cells, although we could not confirm the marker expression at a single cell level because of the diffuse signals of in situ hybridization.

Wnt2b overexpression inhibits expression of differentiation markers in ovo

To investigate the involvement of chicken Wnt2b in the maintenance of CMZ progenitor cells, we carried out overexpression experiments using the in ovo electroporation technique (Momose et al., 1999). We initially studied the expression pattern of Wnt signaling components at stage 18, as we examined the effects of the chick Wnt2b overexpression around this stage (see below). Chicken Wnt2b was upregulated in the dorsal margin of the invaginating eye bud (Fig. 3A), and Fzd4 and Fzd5 were expressed in the peripheral and the central region of the retina, respectively (Fig. 3B,C). The chicken Wnt2b expression was detected in the ventral margin, as well as dorsal margin at slightly later stages, such as stage 19 (data not shown). LEF1 was expressed at high levels in the marginal region, decreasing centrally (Fig. 3D). We then electroporated stage 10 embryos with mouse Wnt2b and studied the time course of the overexpressed message by using a mouse cRNA probe, which did not crossreact with the endogenous chicken Wnt2b mRNA (Fig. 3E-G). The ectopic expression of mouse Wnt2b was first observed in the entire eye bud in addition to the ventral diencephalon at stage 13 (12 hours after the electroporation, Fig. 3E). The overexpressed message was also detected at stage 16 (24 hours after the electroporation, Fig. 3F), but little or no signal was observed at stage 21 (48 hours after the electroporation, Fig. 3G). We then studied LEF1 mRNA expression using the adjacent sections to examine where the Wnt canonical pathway was activated. We could not detect any LEF1 mRNA at stage 13; however, it was strongly upregulated at stage 16 (Fig. 3I), whereas little or no expression was observed in the control retina at that stage (data not shown). The LEF1 mRNA continued to be expressed ectopically in the central retina at stage 21, even though the exogenous expression of mouse Wnt2b had decreased under the detection level (Fig. 3J). Interestingly, the retina electroporated with mouse Wnt2b became folded at high frequency (Fig. 3G,J). To investigate cellular differentiation in the folded retina, we studied the expression pattern of molecular markers described previously (Fig. 4). In the following experiments, we co-electroporated with a GFP-expressing plasmid to identify the region where the overexpressed gene was introduced. In control embryos injected with GFP only, the central part of the retina started to express neural precursor marker Notch1 and ganglion cell marker NF-M (Fig. 4A,C). In the folded retinas expressing mouse Wnt2b, however, neither Notch1 nor NF-M signal was detected (Fig. 4B,D); no change was observed in the expression of these markers in the ventral diencephalon of the same embryo, despite the expression of the co-electroporated GFP (Fig. 4B,D), suggesting the specific effect in the retina. We then studied the expression pattern of progenitor cell markers in the folded retina (Fig. 4E-J). At this stage, a strong Pax6 signal was observed only in the marginal area (Fig. 4E). However, strong expression of Pax6 was induced in the central part of the mouse Wnt2b-electroporated retina (Fig. 4F). The cells in

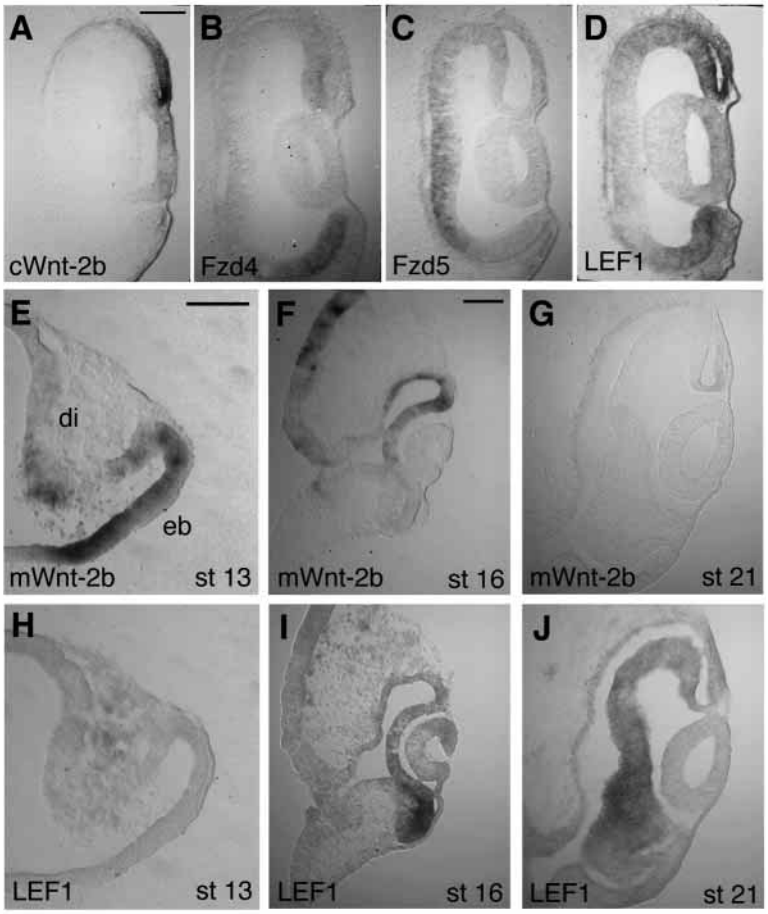
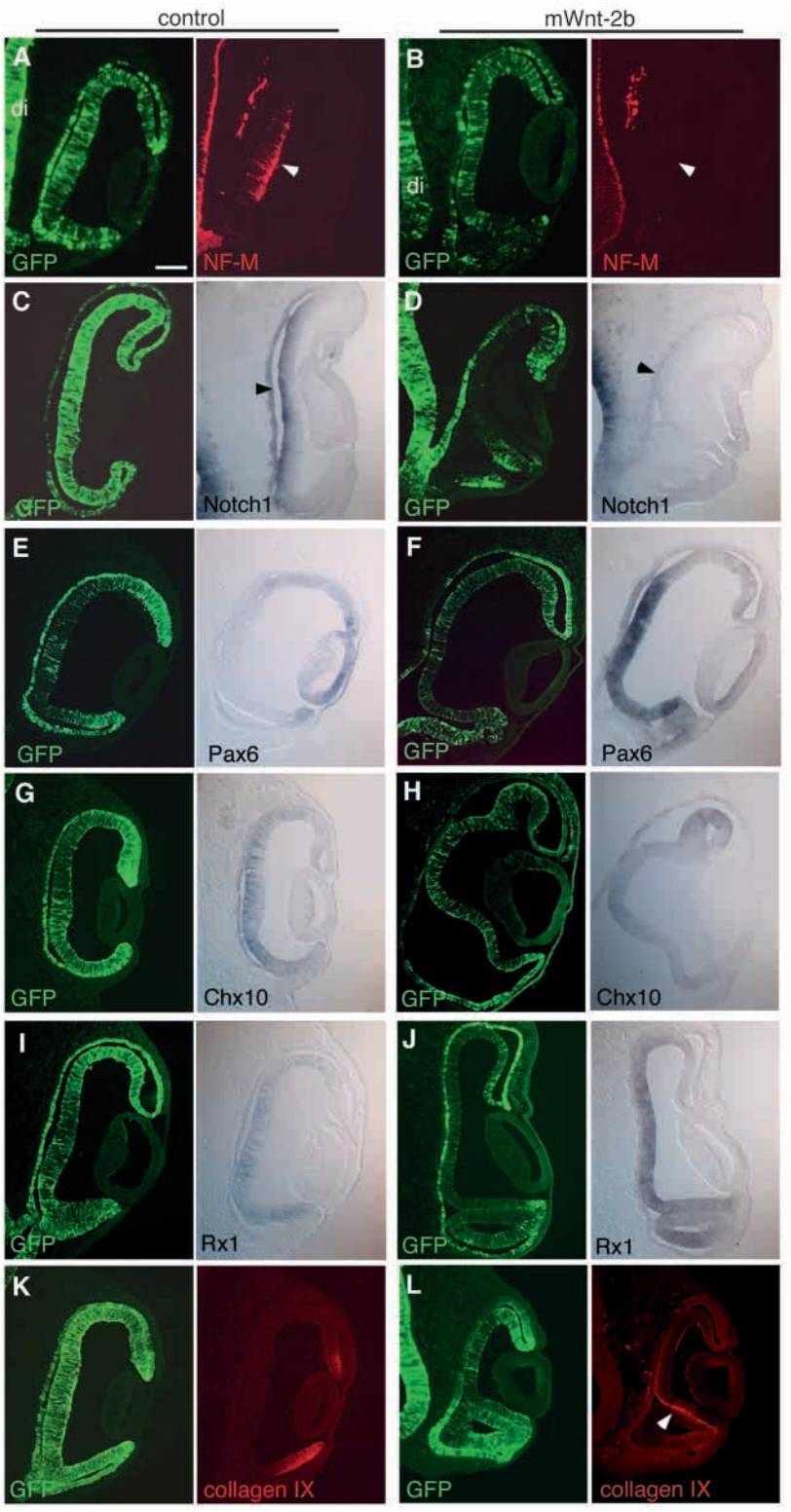


Fig. 3. Time course of Wnt2b overexpression and induction of LEF1 expression by in ovo electroporation. (A-D) In situ hybridization of normal E3 retina probed with chicken Wnt2b (A), Fzd4 (B), Fzd5 (C) and LEF1 (D). (E-J) In situ hybridization of the electroporated embryos probed with the overexpressed mouse Wnt2b (E-G) and LEF1 (H-J). The embryos were fixed 12 hours (E, H), 24 hours (F, I) and 48 hours (G,J) after the electroporation. The upregulation of LEF1 mRNA was detected 12 hours after the initiation of exogenous mouse Wnt2b expression, which continued for at least 24 hours. di, diencephalon; eb, eye bud. Scale bars: 100 μm.

the folded retina also expressed other progenitor markers, i.e. Chx10 and Rx1 (Fig. 4H,J). We also observed weak expression of collagen type IX in the central retina of mouse Wnt2b-electroporated embryo (Fig. 4L). The signals, however, were much weaker compared with those in the E5 iris or ciliary epithelium (Fig. 2C). These finding suggests that the cells in the folded retina were equivalent to the CMZ progenitor cells.



Notably, these effects were not restricted to the cells expressing co-electroporated GFP, and whole retina was equally affected even when chicken Wnt2b was introduced in a mosaic pattern (Fig. 4). We also studied the incorporation of BrdU in the chicken Wnt2b-electroporated embryo to see if cell proliferation is promoted. At this stage, essentially all the cells incorporated BrdU and we could not detect any obvious change in the intensity of the BrdU signals or number of cells positive for it (data not shown).

Functional blocking of Wnt2b leads to premature ganglion cell differentiation

We then carried out complementary experiments to block the chicken Wnt2b downstream signaling by overexpressing a dominant-negative form of LEF1 (ΔLEF1) (Kengaku et al., 1998) by using the same in ovo electroporation technique. At stage 21, ΔLEF1-expressing cells tended to form small cell clumps in the dorsal retinal margin, which expressed stronger co-electroporated GFP compared with the control embryo (Fig. 5B,D). Because the cell division was assumed to dilute the number of plasmids in each cells, the strong GFP signals suggested the cell proliferation had been inhibited by ΔLEF1 overexpression. This decrease in cell proliferation was confirmed by the reduced BrdU incorporation by the ΔLEF1-expressing cells (Fig. 5G,H). The cells in the clumps expressed NF-M (Fig. 5K,L), suggesting that the marginal cells had prematurely differentiated into retinal ganglion cells. We also studied the expression of other post-mitotic neuronal markers such as Islet1 (Fig. 5N,P) and Hu (data not shown) in the ΔLEF1-electroporated embryos. These markers, however, were not expressed in the marginal cells, even when ΔLEF1 had been introduced by electroporation (Fig. 5P).

The multipotential progenitor cells in the CMZ proliferate over a longer period than the centrally located progenitor cells

Finally, we carried out clonal analysis to examine

Fig. 4. Induction of the CMZ markers and inhibition of neural retinal markers by Wnt2b overexpression in ovo. (A-H) The expression pattern of NF-M (A,B), Notch1 (C,D), Pax6 (E,F), Chx10 (G,H), Rx1 (I,J), and collagen type IX (K,L) in the embryos electroporated with control (A,C,E,G,I,K) or chicken Wnt2b (B,D,F,H,J,L)-expressing plasmids. The embryos were fixed at stage 21, and adjacent transverse sections were stained for co-electroporated GFP (shown in left panels) and each molecular marker (shown in right panels). In the Wnt2b-electroporated embryos, the inner layer of the optic cup formed folded retinas that did not express neural retina-specific markers such as NF-M or Notch1 (B,D). The folded retinas expressed progenitor marker genes, including Pax6 (F), Chx10 (H) and Rx1 (J). Note that whole retina was affected by relatively uneven introduction of the electroporated chicken Wnt2b. Arrowheads indicate the central neural retina. di, diencephalon. Scale bar: 100 μm.

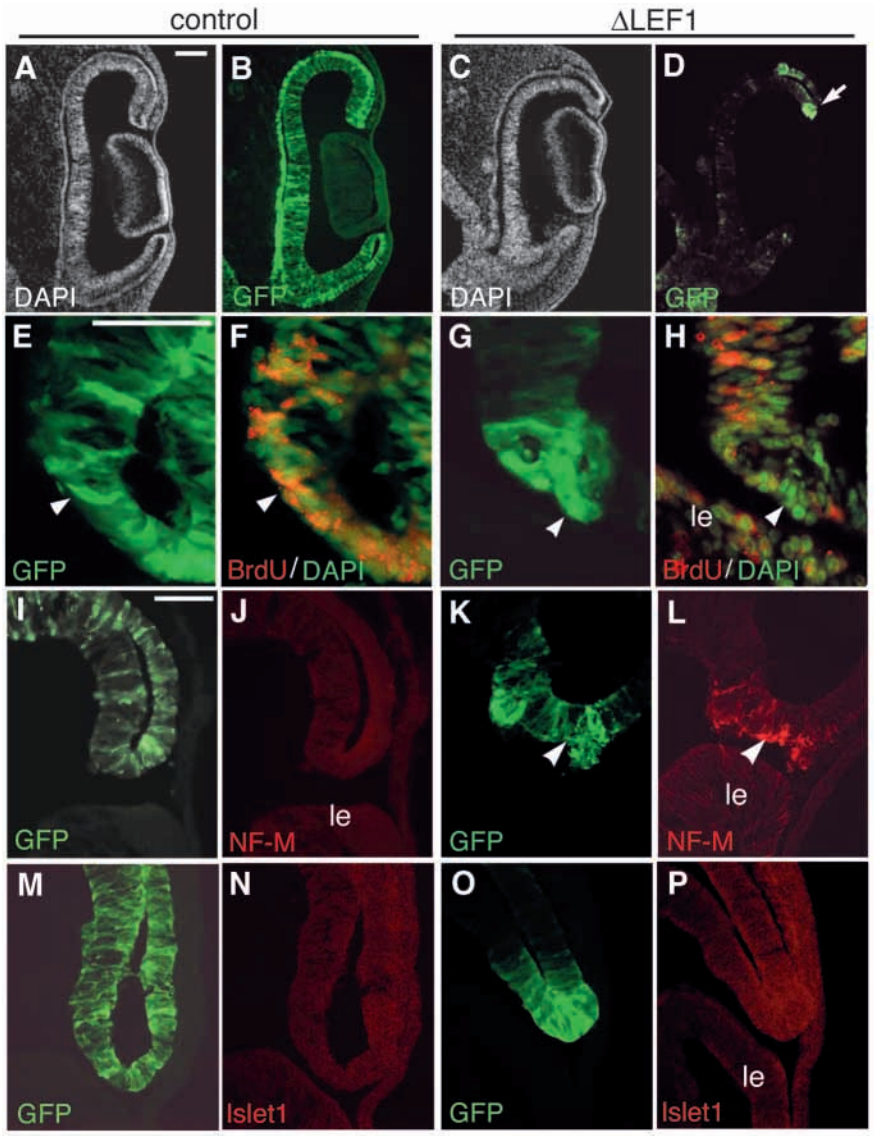


Fig. 5. Precocious differentiation of the retinal stem cells by inhibition of the Wnt canonical pathway with ΔLEF1. (A-D) Transverse sections of stage 21 embryo electroporated with control (A,B) or ΔLEF1 (C,D) stained with DAPI (A,C) and the co-electroporated GFP (B,D). Note that the cell clump in the dorsal retinal margin strongly expressed GFP (arrow). (E-H) The incorporation of BrdU (red) in the control (F) and ΔLEF1-electroporated (H) retina. The co-electroporated GFP is shown in E,G. (F,H) Pseudo-colored DAPI signals are shown in green. (I-P) The expression of co-electroporated GFP (I,K,M,O), NF-M (J,L), Islet1 (N,P) in the cell clump of ΔLEF1-electroporated retina adjacent to the lens (le). Arrowheads indicate the ectopic NF-M expression. Scale bars: 100 μm.

the proliferation and differentiation capacity of the CMZ progenitor cells that expressed a high level of LEF1 mRNA, and compared these cells with progenitor cells located in the central retina, which expressed little or no LEF1 mRNA. We employed the reaggregate culture system previously described (Belliveau and Cepko, 1999) because the progenitor cells did not grow very well at a clonal density in a monolayer culture. We first prepared singly dissociated cells from either the CMZ or central retina of E4.5 quail embryo (equivalent to E5 in chicken embryo). The dissection of the CMZ was carefully carried out to exclude any contaminating cells from the iris or neural retina, which was confirmed by the marker expression in the dissected CMZ explant (data not shown). The dissociated cells were then mixed with an excess amount of feeder cells prepared from E5 chicken retinas to dilute the quail cells at a clonal density, pelleted on a filter and cultured for a further 7 days. In the reaggregation culture, ~70% of the dissociated cells proliferated to make clones regardless of the region from which they had been prepared, suggesting the existence of progenitor cells in each region of the retina (Fig. 6A,B; Fig. 7A,E). The CMZ progenitor cells, however, produced a larger number of progenies (average=18.20; Fig. 6B, Fig. 7E) compared with the progenitor cells in the central neural retina (average=4.58; Fig. 6A, Fig. 7A). We subsequently examined the expression of differentiation markers in these clones to study if the clones were multipotential. The markers we used were visinin (photoreceptor cells), Hu (ganglion cells and amacrine cells) and glutamine synthetase (Müller glia). At least two of these differentiation markers were typically expressed within a clone derived from a progenitor cell taken either from the central retina (Fig. 7B-D) or the CMZ (Fig. 7F-H), suggesting that both progenitor cells were multipotential. To study the effect of chicken Wnt2b on the differentiation and proliferation of the progenitor cells, we cultured them in the presence of chicken Wnt2b CM and examined the number of progenies and the expression of the differentiation markers. The number of progenitor clones was significantly increased by the addition of chicken Wnt2b CM (Fig. 6C,D; Fig. 7I,M), suggesting proliferation of the progenitor cells was promoted in the presence of chicken Wnt2b, regardless of the retinal region from which they were prepared. The CMZ clones, however, generally contained larger numbers of progenies (average=84.04; Fig. 6D, Fig. 7M) than the clones that had originated from the central progenitor cells (average=17.51; Fig. 6B, Fig. 7I). These clones contained both neurons and glia, suggesting that cellular differentiation had occurred normally even in the continuous presence of chicken Wnt2b CM (Fig. 7J-L,N-P). To test if cell proliferation continued for a longer period in the presence of chicken Wnt2b, we dissociated the primary clones into single cells after 4 days in culture, and cultured them once again in the pellets to make secondary clones. All the cells remained as a single cells in the secondary reaggregation culture, when they had been cultured in the control CM to make the primary clones (data not shown), suggesting that all of the progenitor cells had became postmitotic under that culture condition.

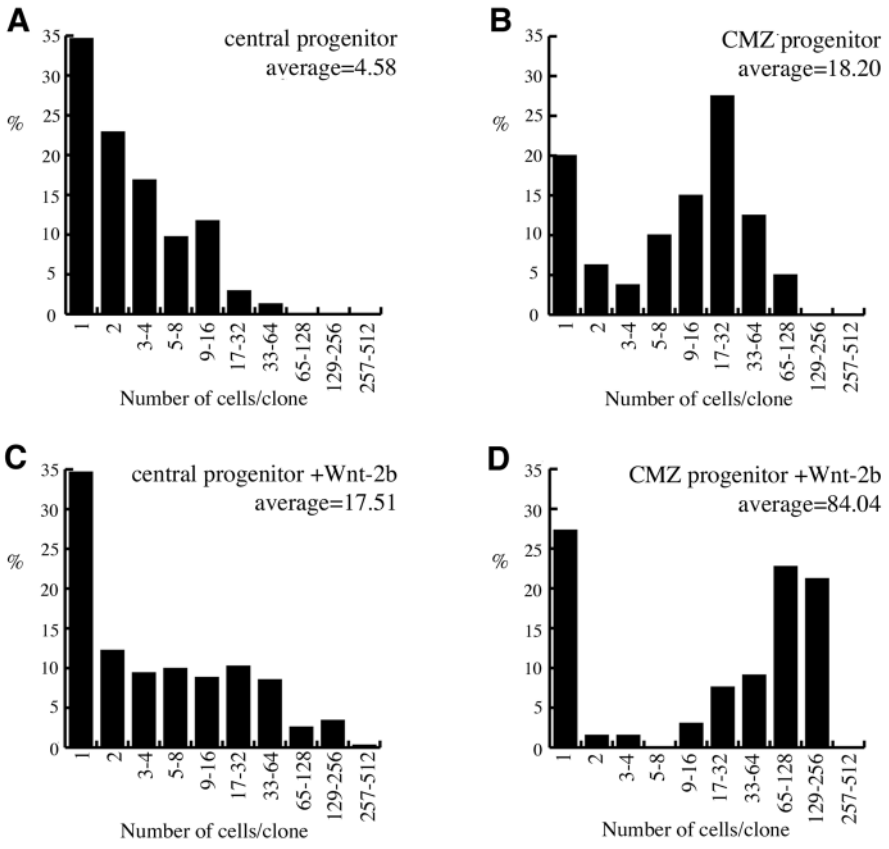


Fig. 6. CMZ progenitor cells produce a larger number of progenies compared with central progenitor cells. Histograms of the number of progenies derived from single progenitor cells prepared from the central retina (A,C) or the CMZ (B,D) either in the absence (A,B) or presence (C,D) of chicken Wnt2b CM. The number of progenies within a clone was counted and placed into the bins indicated below. Each value represents the number of clones in each bin divided by the total number of clones. Note that the CMZ progenitor clones contained a larger number of progenies compared with the central progenitor cells.

However, ~10% of the primary progenies derived from the CMZ progenitor cells made secondary clones if they had been cultured in the presence of chicken Wnt2b CM during the first 4 days (Fig. 7Q). These secondary clones contained cells expressing visinin, Hu and glutamine synthetase, suggesting that they were multipotential (Fig. 7R-T). The progenitor cells prepared from the central retina, however, remained as single cells even if they had been cultured in the presence of chicken Wnt2b (data not shown).

DISCUSSION

The results presented here suggest that chicken Wnt2b controls the prolonged cell proliferation of the CMZ progenitor cells, preventing them from differentiating into neural retina or non-neural anterior eye structures, such as iris and ciliary epithelium. The following observations support this idea: first, LEF1 mRNA, a tentative marker for the active state of Wnt signaling, was upregulated in the CMZ specified by a combination of progenitor and differentiation markers. Second, overexpression of chicken Wnt2b inhibited neuronal differentiation and induced the expression of molecular markers characteristic of CMZ progenitor cells, which lead to the formation of folded retina presumably owing to the overgrowth of the progenitor cells. Third, blocking of the downstream Wnt signaling pathway by a dominant-negative form of LEF1 inhibited progenitor cell proliferation, which resulted in premature neuronal differentiation in the marginal region. Finally, multipotential progenitor cells located in the CMZ proliferated for a longer period than the centrally located ones, and their proliferation was further promoted in the presence of chicken Wnt2b when they were cultured in vitro.

The marginal-most region of the retina differentiates into non-neural anterior eye structures such as the iris and ciliary epithelium (Coulombre, 1965). Because the chicken Wnt2b-expressing region corresponds to the future rim of the iris, the inhibition of neuronal differentiation by Wnt2b overexpression could possibly be explained by transformation of the central neural retina into the non-neural eye structures. This may apparently agree with our observation that the expression of collagen type IX was induced in the central part of the folded retina in the chicken Wnt2b-electroporated embryos. However, the collagen type IX signals in the folded retina were much weaker than those in the presumptive iris and ciliary epithelium of the E5 retina, when the first morphological transformation for their differentiation is observed (Bard and Ross, 1982). In addition, the folded retina expressed retinal progenitor markers such as Chx10 and Rx1, which were not expressed in the retinal region forming the iris and ciliary epithelium. Considering that the weak expression of collagen type IX was also observed in the CMZ of the E5 retina, Wnt2b supposedly inhibits both neuronal and iris/ciliary epithelium differentiation by maintaining the progenitor cell state, rather than by transforming the neural retina into non-neural tissues.

The upregulation of LEF1 mRNA is considered to be a tentative marker for the active state of the Wnt downstream pathway. Indeed, our RT-PCR analysis showed that LEF1 mRNA was upregulated in vitro upon the addition of chicken Wnt2b CM to the retinal cell culture. The upregulation of LEF1 mRNA was also induced when chicken Wnt2b was overexpressed by the electroporation in vivo. In the developing retina, the LEF1-expressing region was not restricted to the cells immediately next to the chicken Wnt2b expressing retinal margin, but was widely distributed making a gradient decreasing centrally. In *Drosophila* imaginal disks, Wingless proteins are transported for a long distance by the argosome (Greco, 2001). It is possible that a similar mechanism also works in the vertebrate neural retina to activate the Wnt signaling pathway at a distance. The long-range effect of Wnt is supported by our observations that the cellular differentiation

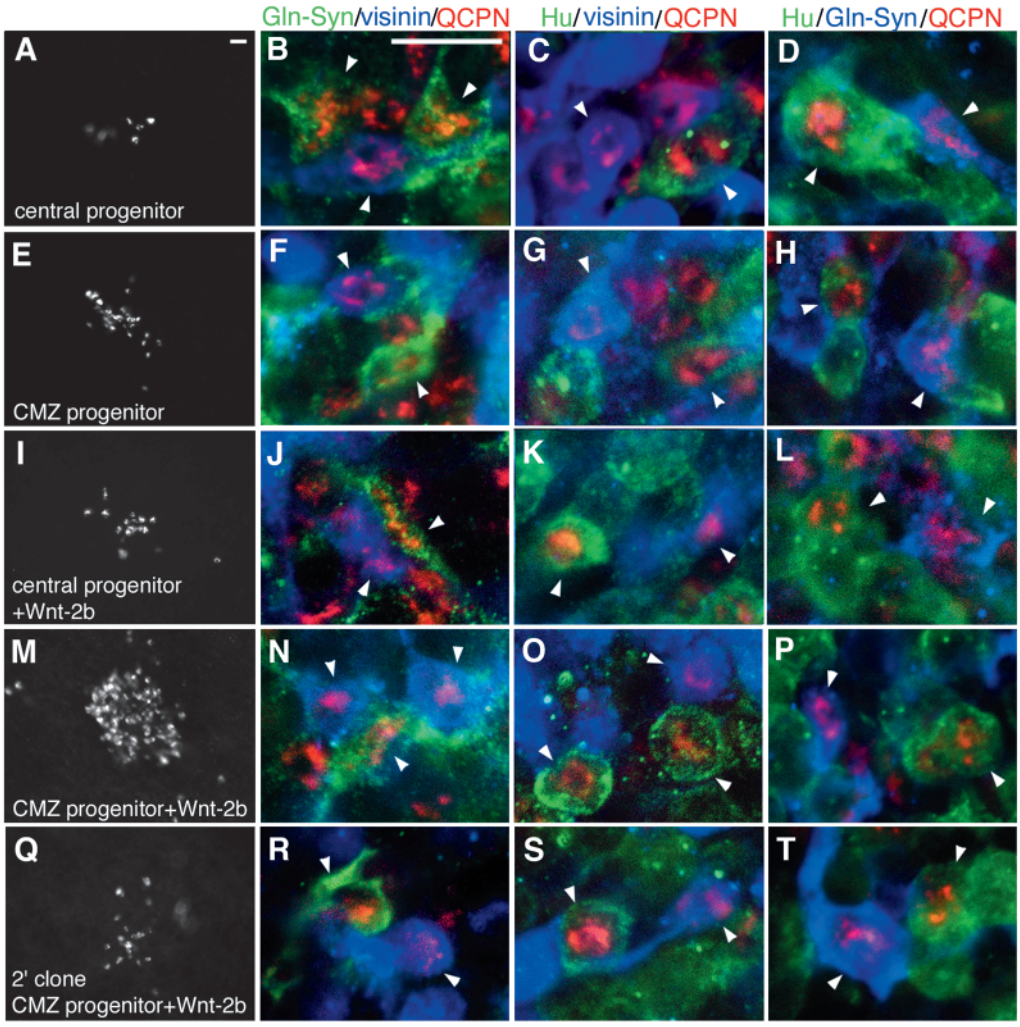


Fig. 7. Clonal analysis of multipotential retinal progenitor cells using reaggregation culture. (A,E,I,M,Q) Low-magnification image of the clones visualized with anti-quail nucleus antibody, QCPN. The singly dissociated retinal progenitor cells, prepared from the central part of the E4.5 quail neural retina (A,I) or CMZ (E,M,Q), were mixed with feeder cells from E5 chicken retina, and cultured on a filter for 7 days either in the control (A,E) or chicken Wnt2b (I,M,Q)-conditioned medium. The CMZ progenitor cells produced larger clones than the central progenitor cells (A,E), and proliferation of both progenitor cells was promoted by the addition of chicken Wnt2b-conditioned medium (I,M). The CMZ progenitor cells produced secondary clones when they had been cultured in the presence of chicken Wnt2b (Q). Confocal images of progenitor clones visualized with QCPN (red), which were triple-labeled for Müller cell marker glutamine synthetase (Gln-Syn) and photoreceptor cell marker visinin (B,F,J,N,R), ganglion/amacrine cell marker Hu and visinin (C,G,K,O,S), or Hu and glutamine synthetase (D,H,L,P,T). Scale bar: 10 μm. Arrowheads indicate quail cells expressing those differentiation markers.

was inhibited by chicken Wnt2b in a region not neighboring the cells expressing co-electroporated GFP and the whole retina was uniformly affected by relatively uneven introduction of the gene. Alternatively, it is equally possible that LEF1 mRNA is kept upregulated for a while, even when the Wnt ligand is no longer available, and the LEF1-expressing cells are simply displaced centrally by addition of new cells at the peripheral region. Direct observation of chicken Wnt2b protein is crucial to discriminate these two possibilities.

At E5, we observed downregulation of LEF1 mRNA in the presumptive iris and ciliary epithelium immediately next to the chicken Wnt2b-expressing marginal tip. It should be noted that the morphological change caused by the iris differentiation begins at E5 (Bard and Ross, 1982), which correlates with the downregulation of LEF1 mRNA in the corresponding regions. The identities of the non-neural marginal retina are determined through interaction between the retina and the neighboring lens, which produces diffusible factors that regulate the formation of the anterior eye structures (Breitman et al., 1989; Stroeva, 1960). Considering that the lens-derived factors were shown to act over a short range (Thut et al., 2001), the factors may inhibit Wnt downstream signaling in a region close to the lens to induce iris and ciliary epithelium differentiation. The induction of iris and ciliary epithelium by the lens factor may also account for the absence of Islet1 and Hu expression in the marginal retina of the ΔLEF1-electroporated embryo, which prematurely expressed NF-M. In the chicken embryonic retina, the expression of NF-M starts much earlier than that of other ganglion cell markers (McCabe et al., 1999). The lens factor may thus inhibit the maturation of the ectopic ganglion cells expressing NF-M into Islet1- or Hu-positive ganglion cells in the vicinity of the lens.

While the expression of the neuronal markers was completely inhibited by in ovo overexpression of chicken Wnt2b, neuronal differentiation occurred normally in the reaggregation cultures, even in the continuous presence of chicken Wnt2b CM. As Wnt proteins are poorly secreted into the medium (Wodarz and Nusse, 1998), the amount of the protein in the conditioned medium may not be enough for complete inhibition of their neuronal differentiation. Alternatively, additional factors are required to keep CMZ progenitor cells undifferentiated, which are not present in the feeder cells prepared from the central part of the E5 neural retina. Considering that the retinal cell differentiation is regulated by both intrinsic and extrinsic factors (Belliveau and Cepko, 1999), progenitor cells may require factors present in the CMZ in addition to chicken Wnt2b to keep them undifferentiated. This idea could be tested by using CMZ cells

as feeder cells to make the reaggregation pellets; however, this is not feasible because of the small size of the tissue for collection of the feeder cells.

Chicken Wnt2b was expressed in the marginal-most tip, and chicken Fzd4 and Fzd5 were expressed in the marginal and the central part of the retina, respectively. Although both Fzd proteins bind to chicken Wnt2b in vitro, chicken Fzd4 would supposedly play a major role for transmitting the endogenous chicken Wnt2b signal from the eye margin considering its spatial distribution; and a smaller amount of chicken Wnt2b would thus be available in the central region expressing a high level of chicken Fzd5. Although we could not detect the expression of other Frizzled genes in the embryonic chicken retina, a previously conducted RNase protection assay showed that many of the mouse frizzled genes (Fzd2-7) were expressed in the adult mouse eye (Wang et al., 1996). In addition, frizzled 3 is expressed in the central nervous system, including the presumptive eye field, in *Xenopus*, and overexpression of this gene led to ectopic eye formation (Rasmussen et al., 2001). It is therefore possible that these frizzled genes are also expressed in the chicken retina and transmit the Wnt signals to regulate multiple aspects of retinal development. It should also be noted that the soluble forms of the frizzled-related proteins Sfrp2 and Sfrp5 are expressed in the neural retina and retinal pigment epithelium, respectively (Chang et al., 1999), suggesting that Wnt signaling is modulated in a region-specific or stage-specific manner. Further studies will definitely be required for the complete understanding of Wnt function during ocular development.

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