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VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation

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During vertebrate neurogenesis, multiple extracellular signals influence progenitor cell fate choices. The process by which uncommitted progenitor cells interpret and integrate signals is not well understood. We demonstrate here that in the avascular chicken retina, vascular endothelial growth factor (VEGF) secreted by postmitotic neurons acts through the FLK1 receptor present on progenitor cells to influence cell proliferation and commitment. Augmenting VEGF signals increases progenitor cell proliferation and decreases retinal ganglion cell genesis. Conversely, absorbing endogenous VEGF ligand or disrupting FLK1 activity attenuates cell proliferation and enhances retinal ganglion cell production. In addition, we provide evidence that VEGF signals transmitted by the FLK1 receptor activate divergent intracellular signaling components, which regulate different responses of progenitor cells. VEGF-induced proliferation is influenced by the MEK-ERK pathway, as well as by the basic helix-loop-helix factor HES1. By contrast, VEGF-dependent ganglion cell suppression does not require MEK-ERK activation, but instead relies on VEGF-stimulated HES1 activity, which is independent of NOTCH signaling. Moreover, elevated HES1 expression promotes progenitor cell proliferation and prevents overproduction of retinal ganglion cells owing to the loss of VEGF or sonic hedgehog (SHH), another signal that suppresses ganglion cell development. Based on previous and current findings, we propose that HES1 serves as a convergent signaling node within early retinal progenitor cells to integrate various cell-extrinsic cues, including VEGF and SHH, in order to control cell proliferation and neuronal specification.

KEY WORDS: VEGF, FLK1, Retina, Development, Proliferation, Retinal ganglion cells, HES, ERK, Sonic hedgehog (SHH), Chicken

INTRODUCTION

The vertebrate retina is an excellent model with which to study molecular mechanisms governing development of multipotent neural progenitor cells in the central nervous system. As development proceeds, retinal progenitor cells undergo cell-intrinsic changes, which generally define the competence state for various cell fate decisions (Cayouette et al., 2003; Harris, 1997; Hatakeyama and Kageyama, 2004; Lillien and Wancio, 1998). Progenitor cells are additionally influenced by extracellular signals in the local environment, which is continuously altered by accumulating postmitotic neurons. Existing evidence supports that both cell-intrinsic molecules and cell-extrinsic cues are important contributing factors to retinal progenitor behaviors (Cepko et al., 1996; Livesey and Cepko, 2001; Perron and Harris, 2000; Yang, 2004).

During vertebrate retinogenesis, the retinal ganglion cell (RGC) is the first type of neuron to emerge from the proliferative neural epithelium (Prada et al., 1991; Young, 1985). Nascent postmitotic RGC neurons begin differentiating immediately at the ventricular surface and the cell bodies of these RGCs translocate to the inner retina to form the ganglion cell layer (Waid and McLoon, 1995). The accumulation of postmitotic RGCs occurs in a center to periphery direction throughout the retina to generate a neurogenic wave (McCabe et al., 1999). A cell-intrinsic factor, the basic helix-loop-helix transcription factor Ath5, crucially dictates the competence of early retinal progenitors to give rise to RGCs (Brown et al., 2001; Kay et al., 2001; Mu and Klein, 2004; Wang et al., 2001; Yang et al., 2003). Among the cell-extrinsic cues that influence RGC development is the secreted molecule sonic hedgehog (SHH), which is produced by differentiated RGCs and regulates two important aspects of RGC genesis. First, SHH signals emanating from postmitotic RGCs are necessary for the propagation of the neurogenic wave towards the peripheral retina (Masai et al., 2005; Neumann and Nuesslein-Volhard, 2000). Second, behind the neurogenic wave front, SHH signals derived from the accumulating RGCs suppress the emergence of additional RGCs from the competent retinal progenitor pool (Wang et al., 2005; Zhang and Yang, 2001a). In addition to diffusible signals, cell contact-mediated DELTA-NOTCH signaling among progenitor cells crucially controls RGC genesis. Either elevated DELTA signal or constitutive NOTCH receptor activity decreases the number of RGCs during the early neurogenic period (Ahmad et al., 1997; Austin et al., 1995; Dorsky et al., 1995; Dorsky et al., 1997).

The secreted protein vascular endothelial growth factor (VEGFA) plays crucial roles during development by signaling through tyrosine kinase receptors (Carmeliet et al., 1996; Millauer et al., 1993). Among these, the cognate high-affinity receptor FLK1 (KDR/VEGFR2) mediates the effects of VEGF in vasculogenesis, angiogenesis and hematopoiesis (Shalaby et al., 1995; Shalaby et al., 1997). Accumulating evidence also suggests that VEGF and FLK1 function in the nervous system (Carmeliet, 2003; Palmer et al., 2000; Weinstein, 2005; Yang and Cepko, 1996). The VEGF family of ligands have been shown to promote cell proliferation and neuronal differentiation in the cortex, enhance sensory and motoneuron survival, and control axonal guidance (Azzouz et al., 2004; Fabel et al., 2003; Jin et al., 2002; Le Bras et al., 2006; Rosenstein et al., 2003;

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Schwarz et al., 2004; Sondell et al., 1999; Sun et al., 2006). Nevertheless, owing to the proximity between the vasculature and neuronal cells, it remains a challenge to understand the precise function of VEGF and FLK1 in the nervous system, as VEGF signals may act directly upon neuronal cells or indirectly upon endothelial cells embedded within the neural tissue to influence neuronal cell populations (Louissaint, Jr et al., 2002; Shen et al., 2004).

We have shown previously that at the onset of retinal differentiation in mouse and chicken, expression of the FLK1 receptor commences in progenitor cells residing in the central retina and subsequently spreads to the peripheral retina, thus suggesting an evolutionarily conserved function of FLK1-mediated signals during vertebrate retinogenesis (Hashimoto et al., 2003; Yang and Cepko, 1996). In this study, we have taken advantage of the chicken retina, which is completely devoid of blood vessels throughout development (De Schaepdrijver et al., 1989), to investigate the potential role of FLK1 receptor-mediated VEGF signaling during the early stages of retinal neurogenesis. By perturbing VEGF signals as well as FLK1 receptor function, we demonstrate that VEGF signals mediated by the FLK1 receptor directly modulate the behavior of uncommitted retinal progenitor cells. We also show that distinct intracellular signaling components mediate the effects of VEGF on progenitor proliferation and neuronal differentiation. Moreover, we reveal a common downstream signal integration mechanism for several extrinsic cues, including VEGF, SHH and NOTCH, all of which participate in the regulation of early progenitor fate decisions.

MATERIALS AND METHODS

Chicken embryos

White Leghorn chicken eggs were purchased from Charles River SPAFAS and incubated at 38°C in a rotating humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH stage) (Hamburger and Hamilton, 1951).

In situ hybridization

Chicken FLK1 cDNA (GenBank AY382882) (Hashimoto et al., 2003) was used as DNA template to generate digoxigenin-labeled RNA probes for in situ hybridization as described (Yang and Cepko, 1996).

Retinal cultures

For explant cultures, the central retina without the peripheral one-pupillary diameter was incubated at 37°C in 5% CO_2 in basal medium consisting of 50% F12/50% DMEM (JRH), 10 mM HEPES, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). Explants were either cultured on polycarbonate filter discs (Costar) floating on the medium, or on hydrophilic polytetrafluoroethylene culture plate inserts (Millipore). Recombinant human $VEGF_{165}$ (R&D Systems) was used at 100 ng/ml in the basal medium containing 1× N2 supplement (Gibco), or 1% fetal calf serum and 0.2% chicken serum (Sigma); except for the dose effect analyses, which were performed in the basal medium containing 1× N2 supplement. To test effects of sFLK1 or control alkaline phosphatase (AP), half of the culture medium containing 1× N2 supplement was replaced with conditioned media. To test effects of inhibitors, 10 μM U0126 (Cell Signaling Technology), 10 μM SU1498 (Calbiochem) or 200 nM cyclopamine (Toronto Research Chemicals) were used.

For collagen gel cultures, dissociated retinal cells were cast at a density of 4000 cells/μl in 1.2 mg/ml rat type 1 collagen (BD Biosciences), 100 mM HEPES in basal medium containing 1× N2 supplement. Explant and collagen gel dissociations were performed as described (Ezzeddine et al., 1997; Zhang and Yang, 2001a).

Expression vectors and cells

The VEGF expression construct (pCMV-VEGF-IRES-GFP) contained the CMV promoter followed by the murine $VEGF_{164}$ cDNA (Yang and Cepko, 1996), the internal ribosomal entry site (IRES), and the enhanced green fluorescent protein (GFP) sequence. The expression construct (pCMV-FLK1Ext-AP) encoding the FLK1 extracellular domain and AP fusion protein (sFLK1) was constructed from a murine FLK1 cDNA clone (Matthews et al., 1991) and the APtag-2 vector (Cheng et al., 1995). Conditioned media containing sFLK1, VEGF or AP were collected between 24 to 72 hours after Lipofectamine transfection of HEK293T cells with pCMV-FLK1Ext-AP, pCMV-VEGF-IRES-GFP, or APtag-4. For cell implantation, 0.5 μl of transfected HEK 293T cells resuspended at 2×10^5 cells/ml in DMEM with 10 mM HEPES was injected intravitreally at HH stage 17 (Zhang and Yang, 2001a).

The mutant HES1 cDNA (pCAG-dnHES1) (Hirata et al., 2002) was cloned downstream of the chicken β-actin promoter with a CMV enhancer (CAG) (Niwa et al., 1991). The wild-type murine HES1 cDNA was expressed from the LTR promoter of the avian retrovirus RCAS (Hughes et al., 1987).

The FLK1 siRNA construct (phU6A-FLK1i) was created by PCR cloning of the human U6 promoter (Fitzgerald et al., 2001) and oligonucleotides containing 29 nucleotides specific to chicken FLK1 (nucleotides 185-213) as a palindrome. A target construct (pCMV-GFP-cFLK1) was made to express from the CMV promoter a chimeric mRNA of GFP and a partial chicken FLK1 fragment (nucleotides 55-232) after the GFP stop codon.

Viral stocks and injection

An avian replication competent retroviral vector (RCAS-FLK1-DN-FLAG) encoding a mutant murine FLK1 with a deletion of the intracellular protein kinase domain (Millauer et al., 1994) was constructed using pMFG-FLK1 and the RCAS(A) viral vector (Hughes et al., 1987). The viral vector expressing siRNA for chicken FLK1 were constructed by inserting the U6-FLK1i cassette and a CMV-GFP expression cassette into the RCAS viral vector. An RCAS vector containing the CMV-GFP cassette and U6 promoter without the FLK1 sequence was used as control. Viral stocks were produced as described (Yang, 2002). Concentrated viral stocks were injected into the optic vesicle at HH stage 10 or the subretinal space at HH stage 17 as described (Zhang and Yang, 2001b).

Electroporation

Retinal explants were electroporated in 1 μg/μl of a cDNA expression plasmid and 0.1 μg/μl of a plasmid with the CAG promoter driving GFP (pAS-CAG-G1) in PBS using an ECM 830 Square Wave Electroporation System (BTX) at 10 V/mm for three 50 mseconds pulses with 950 mseconds intervals. Electroporated explants were further cultured as described.

RT-PCR

Reverse transcriptions were carried out using total RNAs from E6 retinal explants treated with or without 100 ng/ml VEGF for 24 hours and SuperScript II Reverse Transcriptase (Invitrogen) (Hashimoto et al., 1997). Primers used were: for cyclin D1, XJY456 (5′-gccaagcaaacccattagaa-gaagtcctc) and XJY457 (5′-cctgctcgccctcggtgtc); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), XJY361 (5′-ccatcaagtccacaacacggtt-gctgta) and XJY362 (5′-gtcttatgaccactgtccatgccatcac). PCRs were carried out using AmpliTaq polymerase (Roche) at 2.5 mM final $MgCl_2$ at 94°C for 5 minutes, followed by 25 cycles of 94°C for 10 seconds, 60°C for 5 seconds and 72°C for 60 seconds.

Immunoblotting

Western blots were performed using standard protocols. Cells or retinal tissues were lysed in 1% IGEPAL CA-630 (Sigma), 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-Cl (pH 8.0). Blots were incubated with antibodies against AP (Zymed), VEGF (SantaCruz), FLAG (Sigma), GFP (Molecular Probe), α-tubulin (Sigma) or phospho-ERK1/2 (Cell Signaling), followed by secondary antibodies conjugated with horseradish peroxidase (HRP) and detected by enhanced chemiluminescence (ECL plus, Amersham).

Immunostaining

To label proliferating cells, 100 μg of 5-bromo-2′-deoxyuridine (BrdU) was applied in ovo for 3 hours or retinal cultures were incubated with 25 μM BrdU for the last 3 or 6 hours of the culture period. Immunostaining of cryosections was performed as described (Zhang and Yang, 2001a) using

antibodies against BrdU (Amersham), VEGF (SantaCruz), proliferating cell nuclear antigen (PCNA; Sigma), neurofilament 145 (NF 145; Chemicon), NF 200 (Sigma), GFP (Molecular Probes), Islet1/2 [clone 39.4D5; Developmental Studies Hybridoma Bank (DSHB)], AP-2α (clone 3B5; DSHB) or Brn3a (Chemicon). Sections were then incubated with biotinylated secondary antibodies with HRP detection (Vector Laboratories) using 3,3′-diaminobenzidine (Sigma), or binding to Alexa 488-, Alexa 594- (Molecular Probes) or Texas Red- (Jackson ImmunoResearch Laboratories) conjugated antibodies. For nuclear staining, 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI; Roche) was used.

Photomicrographs were captured using a Nikon E800 microscope equipped with a SPOTII digital camera. Confocal images of 1 μm optical sections were obtained using a Zeiss LSM 410 confocal laser-scanning system attached to a Zeiss Axiovert 135M microscope. Quantification of dissociated retinal cells stained for various cell markers was performed using ImagePro PLUS software (Media Cybernetics).

Statistical analysis

In general, 300-1000 cells were counted per experimental condition that was repeated five to nine times ($n \geq 5$). The quantified data are expressed as mean±s.e.m. For pairwise analyses (Figs 2-7 and Fig. 8D,E), the Wilcoxon signed-rank test was used. For comparison of multiple groups (Fig. 8A-C), the ANOVA analysis was performed followed by the Fisher's Protected Least Significance Difference (PLSD) test. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

The avascular chicken retina expresses VEGF and FLK1 in complementary patterns

In the chicken retina, RGC differentiation initiates at embryonic day 2 (E2) and peaks at E6 (Prada et al., 1991). At E5, VEGF immunostaining signals were mainly detected in the inner retina (Fig. 1A), partially co-localized with cell nuclei expressing the LIM homeodomain proteins Islet1 and 2 (Islet1/2), markers of RGCs at early stages of retinogenesis (Zhang and Yang, 2001a) (Fig. 1B,C). By E6, VEGF expression appeared as a protein gradient with the most intense staining signals in the ganglion cell layer (Fig. 1D-F). At E6, FLK1 mRNA was detected in the majority of cells in the ventricular zone (Fig. 1G), overlapping with progenitor cells labeled by the proliferating cell nuclear antigen (PCNA) (Fig. 1H) and BrdU (Fig. 1I). Therefore, during the peak period of RGC production, VEGF is produced by the first emerging postmitotic neurons opposing the proliferative zone occupied by FLK1-expressing progenitor cells. This complementary expression pattern suggests that RGC-derived VEGF may act in a paracrine fashion to signal progenitor cells through the FLK1 receptor.

VEGF promotes proliferation of retinal progenitor cells

To unravel potential roles of VEGF-FLK1 signaling during retinogenesis, we examined whether VEGF influences retinal progenitor cell proliferation. Addition of recombinant VEGF to E5 retinal explants cultured for 48 hours in vitro enhanced BrdU incorporation in a dose-dependent manner (Fig. 2A). Exogenous VEGF at 10 ng/ml and 100 ng/ml resulted in 38.7% and 67.4% increases in BrdU labeling, respectively, within the last 6 hours of culture compared with controls. To examine whether endogenous VEGF plays a mitogenic role, we used a diffusible form of the FLK1 receptor consisting of the extracellular domain fused to alkaline phosphatase (sFLK1), which has been shown to bind and block VEGF signals (Fig. 2B) (Tessler et al., 1994). In contrast to exogenous VEGF stimulation, treatment with sFLK1 resulted in a decrease in BrdU incorporation in retinal explants cultured in vitro from E5 to E7 (Fig. 2E-G).

We next examined effects of VEGF levels on cell proliferation in vivo by performing intravitreal implantation of transfected HEK cells that produce secreted AP, sFLK1 or VEGF (Fig. 2B,C). Compared with the contralateral non-implanted eyes at E5,

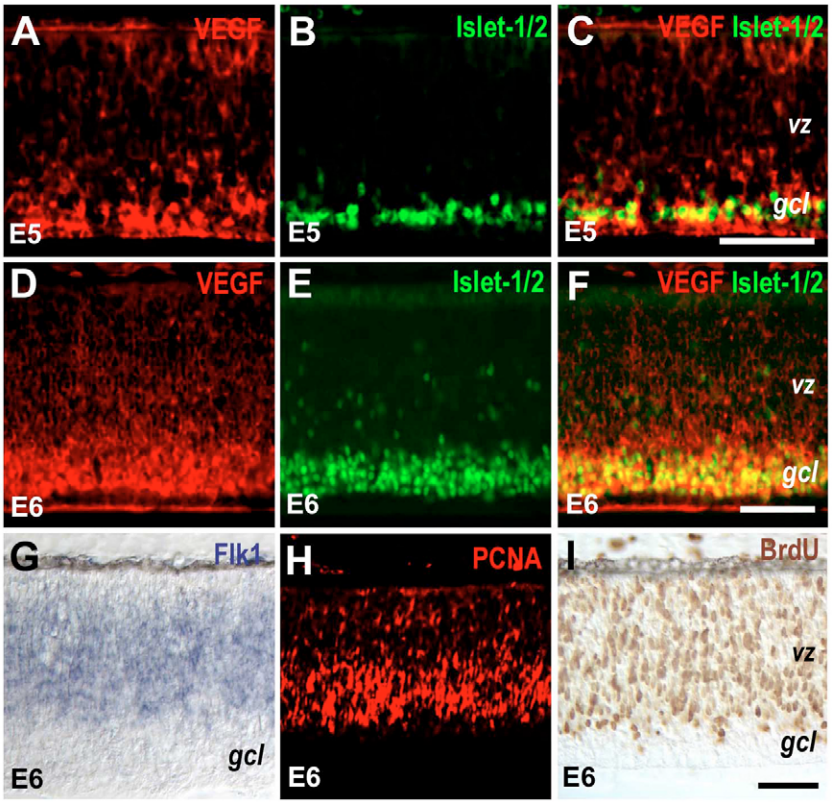

Fig. 1. Expression of VEGF and FLK1 during chicken retinogenesis. (**A-F**) Images of E5 and E6 retinal sections show double immunostaining against VEGF (A,D), Islet1/2 (B,E) and the respective merged images (C,F). (**G-I**) Images of E6 sections show in situ hybridization for FLK1 (G), and immunostaining for PCNA (H) and BrdU (I, in ovo labeling for 3 hours). Scale bars: 50 μm. gcl, ganglion cell layer; vz, ventricular zone.

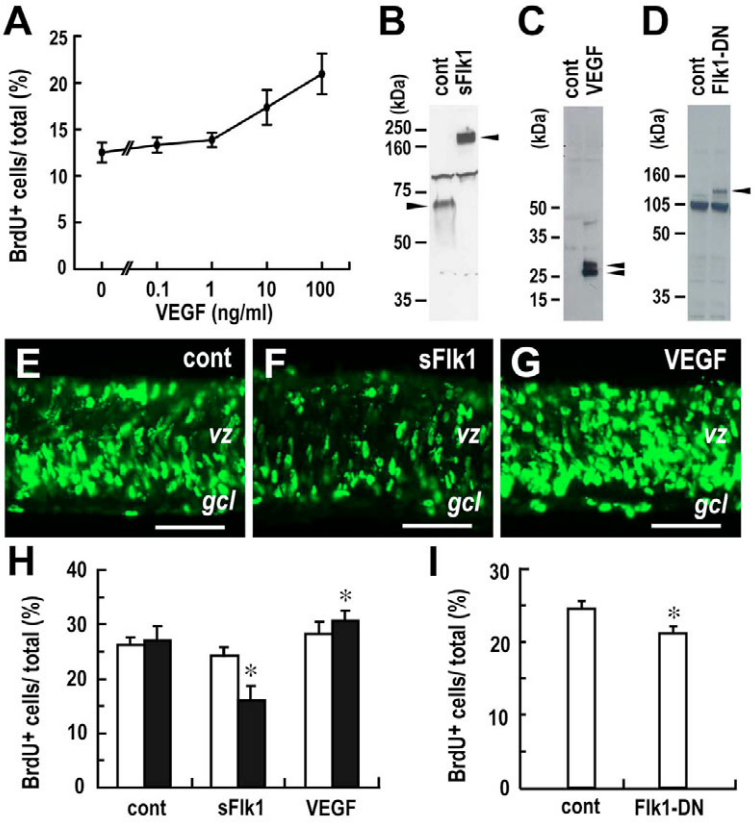

Fig. 2. Influence of VEGF on retinal cell proliferation. (**A**) Effects of VEGF concentrations on BrdU incorporation in vitro. E5-E7 explants were labeled with BrdU for the last 6 hours (n=4 or 5). (**B-D**) Western blots show sFLK1 (B), VEGF (C) or FLK1-DN (D) expression. Culture media of transfected HEK cells (B,C) or infected DF-1 cell extracts (D) were probed with antibodies against AP (B), VEGF (C) or FLAG tag (D). Controls used were CMV-AP (B,C) or RCAS-AP virus (D). Arrowheads indicate bands with expected molecular weights. (**E-G**) Immunostaining show effects of AP (E), sFLK1 (F) or VEGF (G) in E5-E7 explants on BrdU labeling for the last 3 hours. Scale bars: 50 μm. (**H**) Effects of sFLK1- and VEGF-producing cells on BrdU incorporation (3 hours) at E5 in vivo. Black and white bars represent implanted and the contralateral non-implanted eyes, respectively (n=5 or 6; *P<0.03). (**I**) Effect of FLK1-DN expression on BrdU incorporation in vivo. Retinas infected at HH stage 10 with RCAS-AP (control) or RCAS-FLK1-DN virus were harvested at E5 and labeled with BrdU for 3 hours (n=5; *P<0.04).

implanting AP-secreting cells did not alter BrdU incorporation, whereas implanting sFLK1-producing cells decreased BrdU$^+$ cells from 24.2 to 16.0% (P<0.03) (Fig. 2H). Conversely, implanting VEGF-producing cells resulted in an increase of BrdU incorporation from 28.1 to 30.6% (P<0.04) compared with the contralateral eyes (Fig. 2H).

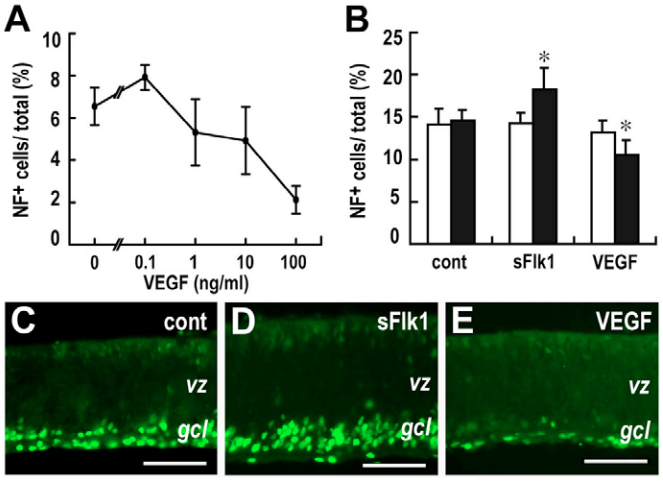

Fig. 3. Influence of VEGF on retinal ganglion cell differentiation. (**A**) Effects of VEGF concentrations on development of NF$^+$ cells in E5-E7 explants in vitro (n=5). (**B**) Effects of sFLK1- and VEGF-producing cells on percentages of NF$^+$ cells in vivo at E5. Black and white bars represent implanted and the contralateral non-implanted eyes, respectively (n=6; *P<0.03). (**C-E**) Immunostaining show effects of AP (C), sFLK1 (D) or VEGF (E) on Islet1/2$^+$ cells in E5-E7 explants. Scale bars: 50 μm.

To test if the proliferative effect of VEGF was mediated by the FLK1 receptor in vivo, we infected the chicken optic vesicle at E1.5 (HH stage 10) with an avian retrovirus (RCAS) that expressed a dominant-negative FLK1 mutant (FLK1-DN) lacking the intracellular tyrosine kinase domain (Fig. 2D) (Millauer et al., 1994). At E5, compared with control RCAS-AP virus (Fekete and Cepko, 1993) infected retinas, the FLK1-DN virus infection reduced BrdU$^+$ cells from 24.3 to 21.1% (P<0.04; average infection rate: 85.6%) (Fig. 2I).

Together, these results indicate that exogenous VEGF promotes early retinal cell proliferation and that endogenous VEGF serves as a mitogen during early retinogenesis. Moreover, the data indicate that the mitogenic effect of VEGF in the retina is mediated by the FLK1 receptor in vivo.

VEGF suppresses differentiation of retinal ganglion cells

We also analyzed if VEGF plays a role in neurogenesis, as maximal FLK1 expression in the chicken retina coincides with the peak period of RGC production (Hashimoto et al., 2003; Yang and Cepko, 1996). In E5 retinal explants cultured for 48 hours in vitro, exogenous VEGF suppressed expression of the RGC marker neurofilament (NF) in a dose-dependent manner (Fig. 3A). VEGF at 100 ng/ml resulted in 67.5% decrease of NF$^+$ cells compared with the control (from 6.5 to 2.1%). Furthermore, sFLK1 treatment caused an increased number of Islet1/2$^+$ cells, whereas supplement of VEGF suppressed differentiation of Islet1/2$^+$ neurons in vitro (Fig. 3C-E).

To examine whether altered VEGF levels in vivo affected RGC development, we implanted intravitreally VEGF- or sFLK1-producing HEK cells at E2.5 (HH stage 17) and performed RGC marker analyses at E5. Compared with contralateral non-implanted

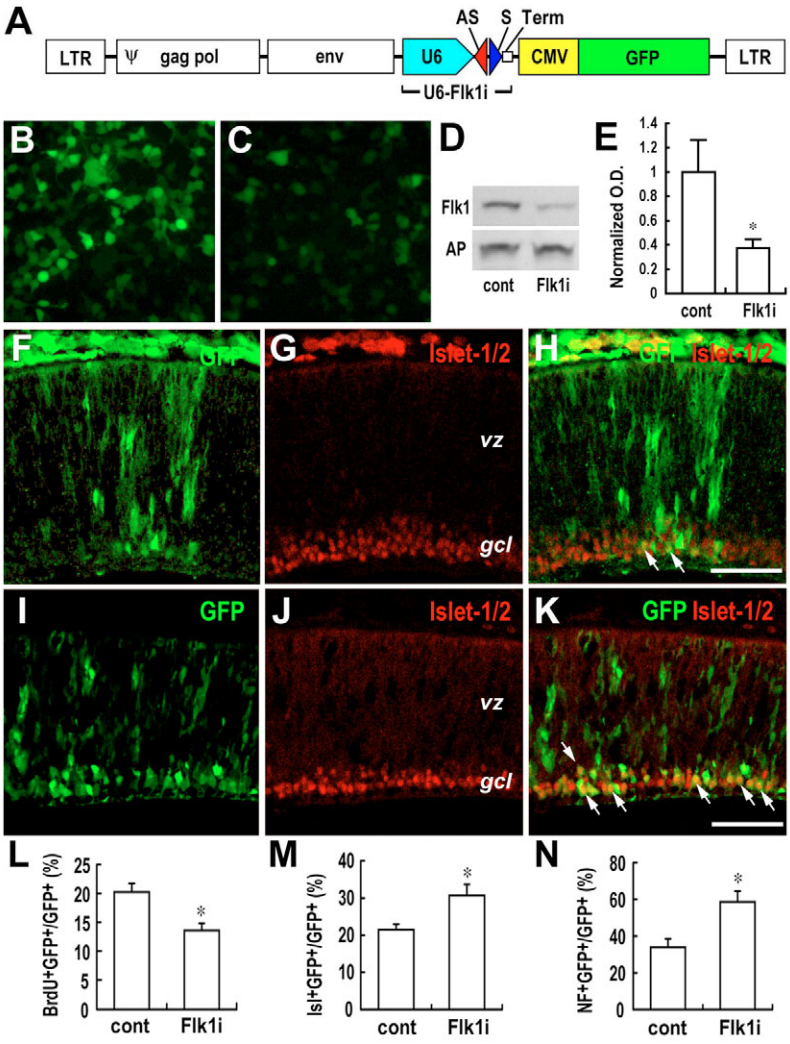

Fig. 4. Effects of RNAi-mediated FLK1 knockdown. (**A**) A schematic represents the RCAS viral vector (white) encoding the U6 promoter and the CMV-GFP cassette. The shFLK1 RNA (FLK1i) contains a 29 nucleotide antisense (AS, red) and a complementary sense (S, blue) sequence of chicken FLK1 followed by transcription termination sequence (Term). (**B-E**) Efficiency of FLK1i knockdown in vitro. Fluorescence images show HEK cells co-transfected with the GFP-FLK1 chimeric target and the control U6 (B) or U6-FLK1i (C) plasmid. Western blots (D) show GFP levels in HEK cells co-transfected with the GFP-FLK1 chimeric target and control U6 or U6-FLK1i plasmid. Blots were probed against GFP (top) and AP (bottom), which serves as a transfection efficiency control. Optical densities of GFP signals (E) were normalized against AP signals (n=6; *P<0.03). (**F-K**) Effects of FLK1i knockdown on RGC development in vivo. Confocal images show retinas infected with the control RCAS (F-H) or RCAS-FLK1i (I-K) viruses at HH stage 17 and immunostained at E6.5 for GFP (F,I), Islet1/2 (G,J) and the respective merged images (H,K). White arrows indicate co-stained cells. Scale bars: 50 μm. (**L-N**) Quantification of FLK1i knockdown effects in vitro. Explants were electroporated at E5 with the control RCAS or the RCAS-FLK1i construct, and labeled with BrdU for 3 hours before dissociation at 48 hours. Percentages of BrdU$^+$ (L), Islet1/2$^+$ (M) or NF$^+$ (N) cells among transected GFP$^+$ cells are shown (n=6; *P<0.03).

eyes, VEGF-secreting cell implantation decreased NF$^+$ cells from 13.3 to 10.6% (P<0.03) (Fig. 3B). By contrast, absorption of endogenous VEGF by sFLK1 increased NF$^+$ cells from 14.3 to 18.3% in vivo (P<0.03) (Fig. 3B). These results show that VEGF signals negatively regulate RGC genesis in vitro and in vivo.

VEGF acts through FLK1 to regulate progenitor proliferation and ganglion cell genesis

To confirm that the effects of VEGF in the developing retina are mediated by the FLK1 receptor, we performed small interfering RNA (siRNA) 'knockdown' of the chicken FLK1. An RCAS retroviral vector encoding the human U6 promoter and a small hairpin RNA specifically targeting the chicken FLK1 (FLK1i) was produced (RCAS-FLK1i, Fig. 4A). The efficiency of siRNA knockdown was determined by co-transfection of the FLK1i construct and an artificial target mRNA that encoded GFP with its stop codon, followed by a 178 nucleotide untranslated sequence of chicken FLK1 mRNA, including the FLK1i target sequence. Analyses of GFP fluorescence and western blots showed that the FLK1i construct, but not the control U6 promoter construct, caused a 60-70% reduction of the target GFP-FLK1 in transfected HEK cells (Fig. 4B-E).

To inhibit FLK1 expression in vivo, E2.5 (HH stage 17) chicken retinas were infected with RCAS-FLK1i or a control virus encoding only the U6 promoter and the CMV-GFP cassette. Double immunostaining showed that at E6.5, a higher proportion of RCAS-FLK1i virus infected cells were labeled positive for the Islet1/2 marker in the RGC layer compared with the control virus-infected retinas (Fig. 4F-K). As RCAS viruses infected only proliferating cells, these results suggest that inhibiting FLK1 receptor expression with siRNA in retinal progenitor cells bias them towards the RGC fate.

We also quantified the effects of FLK1i on retinal cell proliferation and RGC differentiation using retinal explants electroporated with the RCAS-FLK1i or the control viral construct. Compared with controls, FLK1i-expressing GFP$^+$ cells showed reduced BrdU labeling from 20.2 to 13.6% (P<0.03) (Fig. 4L). In addition, FLK1i expression caused increases of Islet1/2$^+$ cells from 21.5 to 30.7%, and of NF$^+$ cells from 33.9 to 58.7% (P<0.03), respectively (Fig. 4M,N). By contrast, expression of FLK1i did not significantly affect amacrine cell differentiation during the E5 to E7 culture period as indicated by staining of the AP-2α marker (West-Mays et al., 1999) (data not shown). These results further support that the effects of VEGF on retinal proliferation and RGC differentiation are both mediated by the FLK1 receptor.

Divergent intracellular transduction machineries mediate VEGF-FLK1 signaling

As VEGF may trigger multiple signaling events in neuronal cells (Zhu et al., 2003), we next examined the intracellular mechanisms responsible for the observed VEGF effects. We first tested if the

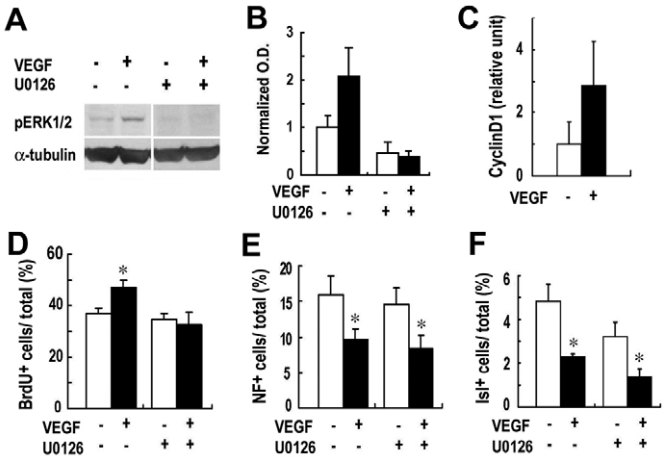

Fig. 5. Effects of MEK-ERK inhibition on VEGF-dependent proliferation and differentiation. (**A**) Western blots of VEGF-induced phospho-ERK. E6 retinas were cultured with or without U0126 for 60 minutes before VEGF was added for 10 minutes. Blots were probed with phospho-ERK1/2 (top) and α-tubulin (bottom) antibodies. (**B**) Quantification of optical densities of phospho-ERK signals were normalized against α-tubulin signals (n=4). (**C**) RT-PCR detection of cyclin D1 transcripts in E6 retinas cultured for 24 hours in the presence or absence of VEGF. Ratios of cyclin D1 and GAPDH products are shown (n=8). (**D-F**) Quantifications of U0126 effects on VEGF-dependent cell proliferation (D) or differentiation (E,F). E5 explants were cultured for 24 hours and BrdU labeled for the last 3 hours (n=6; *P<0.03).

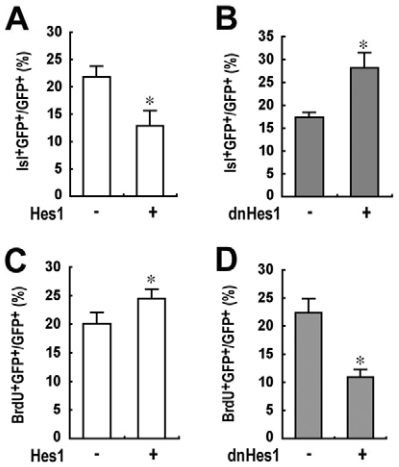

Fig. 6. Influence of HES1 activity on retinal proliferation and differentiation. E5 retinas were co-electroporated with HES1- or dnHES1-expressing construct and a GFP-expressing construct, and cultured as explants for 48 hours with BrdU present for the last 3 hours. Effects of HES1 (**A**,**C**) or dnHES1 (**B**,**D**) on Islet1/2$^+$ (A,B) or BrdU$^+$ (C,D) cells among transfected GFP$^+$ cells are shown (n=6; *P<0.03).

MEK-ERK signaling cascade was involved. In the E5 retina, an endogenous low level of phospho-ERK was detectable by western blot (Fig. 5A). Treatment with 100 ng/ml VEGF for 10 minutes resulted in a twofold increase of ERK phosphorylation (Fig. 5A,B), indicating that VEGF signaling further activated the MEK-ERK cascade. Inclusion of the protein kinase inhibitor U0126, which specifically blocks activities of the MEK1 and MEK2 kinases that act upstream of the ERK1/2 (Favata et al., 1998), significantly reduced both endogenous and VEGF-induced ERK phosphorylation (Fig. 5A,B). Consistent with the observed effect of VEGF on proliferation, RT-PCR assays also detected enhanced cyclin D1 expression in the retinas treated with VEGF (Fig. 5C).

In the E5 to E7 cultures of retinal explants, exposure to VEGF increased BrdU incorporation from 37.0 to 47.0% (P<0.03). Addition of U0126 completely blocked VEGF-enhanced cell proliferation, but did not affect the endogenous level of BrdU incorporation (Fig. 5D). By contrast, U0126 treatment had no effect on VEGF-dependent RGC suppression, as shown by the average 38% decrease of NF$^+$ cells (Fig. 5E) and 45% decrease of Islet1/2$^+$ cells (Fig. 5F). Therefore, the effect of VEGF on cell proliferation requires activation of the MEK-ERK pathway, whereas VEGF-mediated RGC inhibition does not rely on MEK-ERK signaling.

HES1 activity is involved in both VEGF-dependent RGC suppression and progenitor cell proliferation

As DELTA-NOTCH signaling plays a crucial role in controlling RGC specification, we tested if perturbing the activity of the known NOTCH signal effector HES1 (Hatakeyama and Kageyama, 2004; Tomita et al., 1996) had cell-autonomous effects on RGC differentiation. The wild-type or a dominant-negative mutant HES1 (dnHES1) (Hirata et al., 2002; Ström et al., 1997) was co-transfected with a GFP-expressing construct into E5 retinal explants by electroporation. Among transfected E7 cells, forced expression of HES1 resulted in a reduction of Islet1/2$^+$ cells from 21.0 to 12.9% (P<0.03), whereas misexpression of dnHES1 caused an increase of Islet1/2$^+$ cells from 17.3 to 28.1% (P<0.03) (Fig. 6A,B). Analyses of transfected cells also revealed effects of perturbing HES1 activity on cell proliferation, with misexpression of HES1 resulting in a moderate but significant increase of BrdU-labeled cells from 20.1 to 24.5% (P<0.03) and dnHES1 causing a dramatic reduction of BrdU incorporation in retinal explants from 22.3 to 10.9% (P<0.03) (Fig. 6C,D). These results demonstrate that HES1 plays a dual role in cell proliferation and cell fate specification during early retinogenesis.

In order to delineate whether HES1 activity is involved in VEGF-dependent progenitor cell proliferation and RGC fate suppression, we introduced the dnHES1-expressing construct into E5 retinas, and then cultured the explants in the presence or absence of VEGF. Forced expression of dnHES1 resulted in an increase of RGCs at E8, as detected by Brn3a, a marker for more mature RGCs (Liu et al., 2000; Xiang et al., 1995), with or without exogenous VEGF (Fig. 7A). Furthermore, unlike control cells that showed elevated BrdU incorporation because of VEGF stimulation from 23.6 to 29.7% (P<0.03), misexpression of dnHES1 abolished VEGF-induced cell proliferation (Fig. 7B).

To evaluate if HES1 activity involved in VEGF signaling is independent of DELTA-NOTCH interactions, we used a collagen gel culture system in which cell-cell contacts among dissociated retinal cells were minimized. Consistent with previous observations (Austin et al., 1995), the proportion of progenitor cells adopting the RGC fate is greatly increased in gel cultures owing to the elimination of NOTCH signaling (Fig. 7C). Addition of VEGF still resulted in a 22% reduction of RGCs from 45.2 to 35.3% (P<0.02), suggesting that the effect of VEGF on neuronal specification did not depend on cell-cell contacts (Fig. 7C). Among dnHES1-expressing cells, however, the suppression of RGCs by VEGF was eradicated, indicating the direct involvement of HES1 in VEGF-dependent RGC fate regulation (Fig. 7C). In the gel culture, the overall cell proliferation rate was lower than in retinal explant cultures;

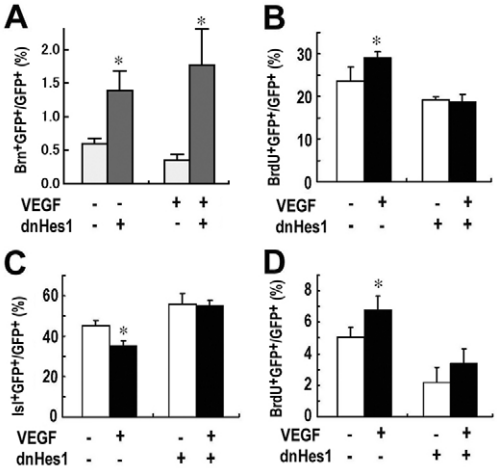

Fig. 7. Requirements of HES1 in VEGF-dependent retinal proliferation and differentiation. E5 retinas were co-electroporated with dnHES1- and GFP-expressing constructs, and then cultured as explants (**A**,**B**) or dissociated cells in collagen gels (**C**,**D**). VEGF was added at 24 hours post transfection and BrdU was added for the last 3 hours. (A,B) Effects of dnHES1 on Brn3a$^+$ cells at E8 (A) or BrdU$^+$ cells at E7 (B) among transfected GFP$^+$ cells (n=6; *P<0.03). (C,D) Effects of dnHES1 in collagen gels on Islet1/2$^+$ cells (C, n=7; *P<0.02) or BrdU$^+$ cells (D, n=5; *P<0.05) among transfected GFP$^+$ cells.

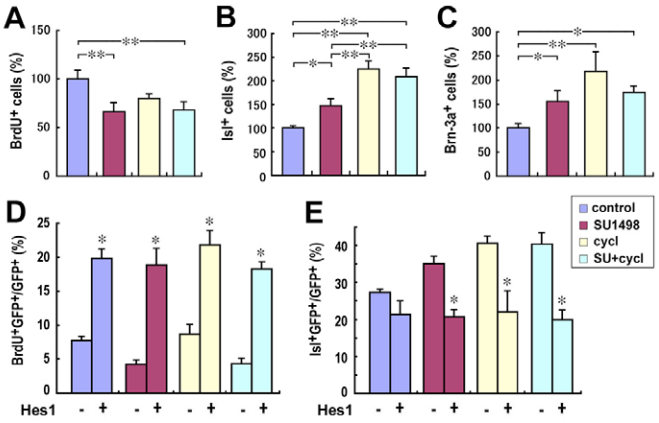

Fig. 8. Effects of signaling inhibitors on cell proliferation and differentiation. (**A-C**) E5 explants were cultured without inhibitors (dark blue), with SU1498 (purple), with cyclopamine (yellow), or with SU1498 and cyclopamine (light blue) for 48 hours. Marker-positive cells per unit length retinal section were quantified and shown as ratios of BrdU$^+$ (A), Islet1/2$^+$ (B) or Brn3a$^+$ (C) cells to the no inhibitor controls (n=9; *P<0.05, **P<0.01). (**D**,**E**) E5 retinal cells transfected with HES1-expressing construct (+) or the control plasmid (–) were cultured in collagen gels for 24 hours before treatment with SU1498, cyclopamine or both for another 24 hours. Bar graphs show BrdU$^+$ (D) and Islet1/2$^+$ (E) cells among transfected GFP$^+$ cells (n=6; *P<0.05).

nonetheless, exogenous VEGF still promoted cell proliferation from 5.2 to 6.8% (P<0.05), and expression of dnHES1 reduced VEGF-enhanced BrdU incorporation (Fig. 7D).

Taken together, these results indicate that HES1 activity is required for VEGF-dependent RGC suppression independent of NOTCH signaling. Furthermore, HES1 is involved in cell proliferation in the absence of NOTCH signaling, and may participate in VEGF-stimulated mitogenic activity.

HES1 participates in both VEGF and SHH signaling in the retina

We have previously demonstrated that behind the neurogenic wave front, SHH secreted by differentiated RGCs negatively regulates RGC production (Zhang and Yang, 2001a). As SHH and VEGF both suppress ganglion cell genesis, we tested whether these two factors act synergistically. To interfere with SHH and/or VEGF signaling, the hedgehog signal inhibitor cyclopamine (Cooper et al., 1998) and an FLK1-specific inhibitor SU1498 (Strawn et al., 1996) were included in E5 retinal explant cultures. Treatment with cyclopamine (200 nM) did not significantly affect BrdU incorporation (Fig. 8A), but resulted in more than 100% increase of both Islet1/2$^+$ and Brn3a$^+$ cells compared with the control (Fig. 8B,C). Addition of SU1498 significantly decreased BrdU incorporation (>30%) (Fig. 8A) and led to a 50% increase of RGC marker-positive cells (Fig. 8B,C). However, the presence of both cyclopamine and SU1498 did not result in additive or synergistic effects on cell proliferation or RGC suppression (Fig. 8A-C), suggesting that VEGF and SHH may share common downstream signaling molecules.

To further investigate the contribution of distinct signaling pathways on proliferation and RGC fate specification, we examined the effects of SHH and FLK1 signaling inhibitors in the absence of DELTA-NOTCH input by using the collagen gel cultures. Similar to results obtained in explant cultures, blocking FLK1 signaling by SU1498 resulted in 50% reduction of BrdU$^+$ cells; however, no effect of cyclopamine on cell proliferation was observed (Fig. 8D). Under the dissociated culture condition, addition of either SU1498 or cyclopamine caused a further increase of Islet1/2$^+$ cells above the already elevated RGC level (Fig. 8E). However, there were no additive or synergistic effects on RGC by blocking both VEGF and SHH signaling pathways (Fig. 8E). To examine if HES1 was involved in mediating VEGF and SHH signals independent of NOTCH, we tested the effects of elevated HES1 expression in dissociated retinal collagen gel cultures. Overexpression of HES1 in dissociated retinal cells yielded a two- to threefold increase of BrdU labeling in the controls and in the presence of either one or both signaling inhibitors (Fig. 8D). Conversely, HES1 overexpression abolished RGC increases caused by SU1498 and cyclopamine (Fig. 8E). Collectively, these results suggest that both VEGF and SHH signals are active in the absence of NOTCH signaling, and that HES1 may mediate the cellular responses of progenitor cells to both VEGF and SHH.

DISCUSSION

This study demonstrates that VEGF signaling directly participates in development of the central nervous system within the neuronal cell lineage. We provide evidence that VEGF secreted by postmitotic retinal neurons signals through the FLK1 receptor to activate divergent intracellular signaling machinery. The VEGF-activated MEK-ERK pathway is required for the proliferative response, whereas HES1 is involved in both neuronal differentiation, as well as cell cycle regulation. Moreover, our study provides insight into the underlying mechanisms responsible for integrating multiple extrinsic signals by uncommitted neural progenitor cells during early retinogenesis.

Results from both gain- and loss-of-function approaches support that VEGF acts as a mitogen during early retinogenesis. We have previously shown that VEGF can bind to postnatal mouse retinal progenitor cells and induce tyrosine phosphorylation of the FLK1 receptor (Yang and Cepko, 1996). Our current data further demonstrate that VEGF induces ERK phosphorylation in the

chicken retina, and that the stimulatory effect of VEGF on proliferation involves the MEK-ERK signaling pathway. Even though the MEK1/2 inhibitor U0126 effectively blocked both endogenous and VEGF-induced ERK phosphorylation, U0126 was unable to eliminate all cell proliferation. This result suggests that robust proliferation during early retinogenesis is dependent on multiple cell-extrinsic cues, which may trigger signaling events other than MEK-ERK activation to regulate the cell cycle (Anchan et al., 1991; Dyer and Cepko, 2001; Jensen and Wallace, 1997; Lillien and Cepko, 1992; Ohnuma and Harris, 2003). Other known signals that regulate cell proliferation in the nervous system include Wnt and hedgehog molecules, which are not thought to depend upon MEK activation (Chesnutt et al., 2004; Kenney et al., 2004). We did not detect altered retinal cell death using TUNEL under conditions that either elevate or diminish VEGF signaling (data not shown). Therefore, the observed effects of VEGF are unlikely to be due to enhanced cell survival or reduced apoptosis.

Intriguingly, interfering with the activity of HES1, one of the effectors of NOTCH signaling (Louvi and Artavanis-Tsakonas, 2006), also affected VEGF-induced cell proliferation. Elegant molecular genetic studies have established that HES1 acts as a transcription repressor to maintain neural progenitor cell potential and regulate differentiation (Hatakeyama et al., 2004; Kageyama et al., 2005). In the mouse retina, loss of HES1 causes premature cell cycle exit and neuronal differentiation, whereas misexpression of HES1 results in the formation of undifferentiated precursor-like cells and Müller glia (Furukawa et al., 2000; Lee et al., 2005; Takatsuka et al., 2004; Tomita et al., 1996). These results are consistent with our observations that manipulating HES1 activity affects proliferation in the chicken retina. The most likely target of the dnHES1 in the chicken retina is the chicken Hairy2 protein, which shares sequence homologies and functional similarities with the mammalian HES1 (Jouve et al., 2000). The dnHES1 may additionally inhibit chicken Hairy1, which also acts as a NOTCH signaling effector (Jouve et al., 2000). Although the precise role of HES1 in cell cycle regulation remains to be determined, recent evidence suggests that HES1 may directly repress transcription of the cell cycle inhibitor $p27^{kip1}$ (Murata et al., 2005). In the developing *Drosophila* eye imaginal disc, NOTCH signaling is required for the G1-S phase transition of the cell cycle and is responsible for triggering the onset of proliferation by multiple signaling pathways (Baonza and Freeman, 2005). However, our results from dissociated retinal cultures demonstrate that the involvement of HES1 in cell proliferation can also be independent of NOTCH signaling. Therefore, HES1 emerges as an important effector for other cell-extrinsic cues in addition to the transmembrane DELTA ligands.

It has been reported that VEGF promotes rhodopsin expression in neonatal rat retinal monolayer cultures (Yourey et al., 2000). Here, we reveal a novel function of VEGF in modulating production of the first-born retinal neurons. Our results demonstrate that VEGF acts simultaneously to promote progenitor proliferation and suppress ganglion cell production. Experiments in the *Xenopus* retina have suggested that cells receiving a proliferative signal are unable to effectively execute a differentiation pathway (Ohnuma et al., 2002). We thus examined whether the mitogenic and neurogenic effects of VEGF in the chicken retina are related to each other. Our findings indicate that VEGF-dependent suppression of RGC does not involve activation of the MEK-ERK cascade, but instead is dependent on HES1 activity. Because HES1 activity is involved in both VEGF effects, at present the mitogenic effect of VEGF can only be partially uncoupled from its effect on neuronal differentiation. The exact mechanism of how VEGF signaling leads to altered HES1 activity is currently unknown. Because VEGF-triggered FLK1 phosphorylation can activate multiple intracellular signaling components (Carmeliet, 2003), it is plausible that FLK1 signaling may influence HES1 activity at both the transcriptional and post-transcriptional levels. The results of MEK1/2 inhibitor U0126 indicate that VEGF-enhanced HES1 function does not require MEK-ERK activation, and thus is probably related to signaling events diverged upstream of MEK.

Current evidence supports that both cell-cell interactions and secreted signals participate in vertebrate RGC fate specification. NOTCH signaling among early retinal progenitor cells plays an important role in controlling RGC formation (Ahmad et al., 1997; Austin et al., 1995; Dorsky et al., 1995; Dorsky et al., 1997), presumably through the downstream effectors HES1 and Hes5 (Hatakeyama and Kageyama, 2004). We have identified SHH as an RGC-derived secreted signal that negatively regulates RGC genesis behind the neurogenic wave front (Zhang and Yang, 2001a). Our model is consistent with a recent study showing that conditional removal of the SHH gene from the peripheral retina results in enhanced local RGC genesis (Wang et al., 2005). Like FLK1, the SHH receptor patched is expressed by mouse and chicken retinal progenitors (Jensen and Wallace, 1997; Zhang and Yang, 2001a). Thus, molecular evidence clearly indicates that early retinal progenitor cells receive multiple extrinsic cues, including DELTA1, SHH and VEGF, which are mediated by distinct cell surface receptors and canonical intracellular signal transduction pathways.

How do uncommitted progenitor cells integrate and respond to multiple environmental signals? Results presented in this study suggest that HES1 protein plays a key role in mediating VEGF effects, independent of NOTCH signaling. Our on-going studies show that SHH signaling enhances HES1 activity in the chicken

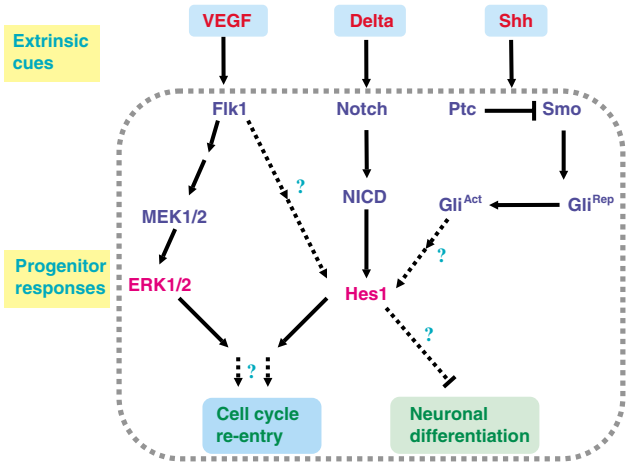

Fig. 9. A model of signal convergence in retinal progenitor cells. DELTA-NOTCH signaling yields the NOTCH intracellular domain (NICD) and activates HES1 transcription. SHH signaling through patched (PTC) and Smoothened (SMO) reduces Gli repressor (GLi^{Rep}) and facilitates the accumulation of the Gli activator (GLi^{Act}). SHH signaling upregulates HES1 by unknown mechanism(s). VEGF stimulation causes the activation of the MEK-ERK cascade and enhances HES1 activity without the involvement of MEK1/2 function. The promotion of cell proliferation may require independent inputs from both MEK-ERK and HES1, whereas suppression of RGC specification mainly involves HES1 activity. Therefore, HES1 serves as a node of signal convergence to integrate inputs of multiple cell-extrinsic cues.

retina (X.-M.Z., T.H. and X.-J.Y., unpublished). Partial reduction of SHH in the mouse retina also leads to a reduction of HES1 mRNA (Wang et al., 2005). Moreover, *Hes1* knockout mice exhibit precocious ATH5 expression and RGC defects (Lee et al., 2005). The lack of synergistic or additive effects of VEGF and SHH on cell proliferation and RGC suppression may reflect that these two pathways share common downstream signaling components that are limiting or tightly regulated. Based on these lines of evidence, we propose a model in which HES1 not only acts as an effector for NOTCH signals, but also serves as a signaling node for the convergence of VEGF and SHH signals during early retinogenesis (Fig. 9). Our model suggests that the negative regulation of RGC genesis by NOTCH, SHH and VEGF signaling all involves HES1 activity. However, the precise function of HES1 in cell cycle progression remains to be further defined. To date, our perturbation results indicate that VEGF but not SHH influences proliferation in the early developing chicken retina. As SHH signaling alone provides enhanced HES1 but not ERK activation, it is possible that cell cycle re-entry may require simultaneous inputs from both activated ERK and HES1. Alternatively, HES1 may play an essential role in regulating cell cycle progression independently of ERK activation. Our model proposes that levels of HES1 activity in early retinal progenitor cells reflect the integrated inputs of multiple extrinsic cues, and critically control progenitor cell fate decisions. Because HES1 protein has been shown to have a short half-life and HES1 gene transcription is auto-repressed by HES1 protein itself (Hirata et al., 2002), it is expected that HES1 protein levels are highly dynamic (Masamizu et al., 2006; Ohtsuka et al., 2006). Future studies on mechanisms that transduce or relay divergent signals to HES1 within the neural progenitor cells and how HES1 interfaces with multiple signals to control the cell cycle will be crucial to further our understanding of neuronal cell fate determination.

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