Regulation of Retinal Progenitor Cell Differentiation by Bone Morphogenetic Protein 4 Is Mediated by the Smad/Id Cascade

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Purpose. Bone morphogenetic proteins (BMPs) are secreted signaling molecules that are implicated in the control of multiple events during mouse eye development. However, little is known about the mechanisms by which BMP signaling regulates these retinal developmental processes.

Methods. Real-time PCR, Western blot, and immunohistochemistry were used to investigate the expression of components of BMP signaling in the mouse retina. Retinal progenitor cells (RPCs) were used to study the effects of BMP4 on retinal cell differentiation and regulation of Id protein expression.

Results. Results showed that BMP2, -4, and -7; BMP receptor (BMPRIb) mRNAs; and proteins and downstream signaling molecule Smad1/5/8 proteins were all highly expressed in the mouse retina during the embryonic (E13.5–E18.5) and early postnatal (P)1 stage and that the expression was downregulated in the adult. On stimulation with BMP4, cultured mouse RPCs differentiated into neuronal lineage whereas astrocyte cell differentiation was inhibited. BMP4 mainly stimulated production of retinal ganglion cells (RGCs). Results also revealed that BMPs and BMPRIb were co-localized with inhibitors of differentiation (Id) (mainly Id1 and -3) in RGCs in the adult mouse retina. Exposure of RPCs to BMP4 upregulated Id1–3 expression levels, mediated through the phosphorylation of Smad1/5/8 proteins.

Conclusions. These results suggest that Id genes are one of the potential targets of BMP signaling in the differentiation of RPCs. (*Invest Ophthalmol Vis Sci.* 2010;51:3764–3773) DOI: 10.1167/iovs.09-4906

Retinal development involves a complex progression of tissue induction, proliferation of retinal progenitor cell (RPC) populations and terminal differentiation of these cells into specific functional types. Growing evidence indicates that both extrinsic cues and intrinsic properties play critical roles in retinal cell development.[1] Elucidation of the interactions between the extrinsic cues and the intrinsic properties may lead to the identification of the signaling pathways that are involved.

One such extrinsic molecule type, bone morphogenetic protein (BMP), is a member of the transforming growth factor (TGF)-β family of signaling molecules, which are known to regulate a variety of cell functions in the developing nervous system, including neural induction, cell fate determination, apoptosis, and proliferation.[2] BMP2, -4, and -7 and their receptors (BMPRs) are expressed in the eye during embryogenesis and are essential for multiple aspects of retinal development.[3] Although the role of BMPs in early eye patterning and retinal neurogenesis has been established, it is unclear how they mediate such a diversity of responses. BMPs transduce their signals by binding to and activating complexes of specific type I and -II serine/threonine kinase receptors. There are two type I BMP receptors (BMPRIa/ALK3 and -Ib/ALK6) and one type II receptor (BMPRII). Binding of BMP receptors results in the activation of downstream signaling through phosphorylation of the receptor-regulated Smad proteins (R-Smad)1, -5, and -8, which can then heteromerize with Smad4 and translocate into the nucleus to activate transcription of target genes, such as the inhibitors of DNA-binding proteins (Ids).[4–6] BMPs have been shown to regulate Id expression in several cell types, including embryonic stem cells.[7–10]

Id proteins are a family of four proteins (Id1–Id4) that have been implicated in several cellular processes, including control of cell cycle progression and differentiation.[11,12] They lack basic DNA-binding domains, and heterodimers formed between Id and bHLH transcription factors cannot bind DNA; thus, the gene transcription function of these transcription factors is inhibited.[12,13] The expression of Ids has been detected in the developing nervous system[14–18] and also in the developing mouse retina.[19] It is possible that the role of Id proteins in the retina is to bind to and inhibit the function of bHLH transcription factors such as Hes1, Mash1, Math5, and NeuroD1, which are critical for retinal development.

Despite extensive experimental studies, little is known about how extrinsic BMP signaling molecules and intrinsic transcription factors combine to regulate differentiation of RPCs. In this study, we examined the expression patterns of BMPs and BMPRs in the developing mouse retina and the effects of BMP4 on the differentiation of RPCs, which are known to be multipotential and retain their ability to produce different types of retinal cells, even in the late stages of retinogenesis.[20] We then investigated whether the effects of BMP4 on RPC differentiation is mediated through the regulation of Id expression by BMP/Smad signaling.

Materials and Methods

Animals

Timed-pregnant female Institute for Cancer Research (ICR) mice were obtained from the Laboratory Animal Unit at the University of Hong

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TABLE 1. List of Primary Antibodies

Target Protein	Immunogen	Species	Concentration (μg/mL)	Source†
Bone morphogenetic protein 2 (BMP2)	CHO-derived, recombinant human bone morphogenetic protein 2, amino acids 283–296	Mouse, monoclonal antibody (IgG)	2.5	R&D Systems; Cat. no. MAB3551
Bone morphogenetic protein 2 (BMP2)	Peptide mapping at the N-terminus of the mature chain of BMP-2 of human origin	Goat, polyclonal antibody (IgG)	1.0	Santa Cruz; Cat. no. sc-6895
Bone morphogenetic protein 4 (BMP4)	NS0-derived, recombinant human bone morphogenetic protein 4	Mouse, monoclona antibody (IgG)	2.5	R&D Systems; Cat. no. MAB7571
Bone morphogenetic protein 4 (BMP4)	Peptide mapping at the N-terminus of BMP-4 of human origin	Goat, polyclonal antibody (IgG)	1.0	Santa Cruz; Cat. no. sc-6896
Bone morphogenetic protein 7 (BMP7)	CHO-derived, recombinant human bone morphogenetic protein 7	Mouse, monoclonal antibody (IgG)	2.5	R&D Systems; Cat. no. MAB3541
Bone morphogenetic protein 7 (BMP7)	Peptide mapping near the C-terminus of BMP-7 of human origin	Goat, polyclonal antibody (IgG)	1.0	Santa Cruz; Cat. no. sc-34766
Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428)	Synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Ser463/465 of human Smad5	Rabbit, polyclonal antibody (IgG)	NA*	Cell Signaling; Cat. no. 9511
Bone morphogenetic protein receptor types IB	Peptide mapping near the N-terminus of BMPR-IB of human origin	Goat, polyclonal antibody (IgG)	1.0	Santa Cruz; Cat. no. sc-5679
Inhibitor of differentiation/DNA binding proteins 1 (Id1)	Peptide mapping at the C-terminus of Id1 of mouse origin	Rabbit, polyclonal antibody (IgG)	0.2	Santa Cruz; Cat. no. sc-488
Inhibitor of differentiation/DNA binding proteins 3 (Id3)	Peptide mapping at the C-terminus of Id3 of human origin	Rabbit, polyclonal antibody (IgG)	0.4	Santa Cruz; Cat. no. sc-490
β-Tubulin class III	Peptide corresponding to amino acids 436-450 of neuronal specific βIII tubulin	Mouse, monoclonal antibody (IgG)	1.0	Sigma-Aldrich; Cat. no. T8578
Bm3b	Peptide mapping within an internal region of Bm-3b of human origin	Goat, polyclonal antibody (IgG)	1.0	Santa Cruz; Cat. no. sc-31989

* NA concentration is not provided by supplier's product information sheet.

† R&D Systems, Minneapolis, MN; Santa Cruz Biotechnology, Santa Cruz, CA; Cell Signaling Technology, Danvers, MA; Sigma-Aldrich, St. Louis, MO.

Kong and were used according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Timed-pregnancy of the mice was determined by checking the vaginal plug, with midday of plug observation designated as embryonic day (E) 0.5. To ensure consistency, we used the retinas from littermates, and all the experimental steps were performed according to identical protocols.

Immunofluorescence Staining

The eyeballs of E13.5, E16.5, E18.5, postnatal day (P) 1, and adult ICR mice were fixed in 4% paraformaldehyde for 2 hours at 4°C and then changed into 30% sucrose in phosphate-buffered saline (PBS) overnight at 4°C. The samples were cut into 10-μm-thick cryosections. Antigen retrieval was performed by incubating sections in protein kinase K (5 μg/mL; Sigma-Aldrich, Inc., St. Louis, MO) in PBS for 10 minutes followed by blocking in 10% normal donkey serum, 1% bovine serum albumin, and 0.3% Triton X-100 in 0.01 M PBS for 1 hour at room temperature. The sections were then incubated with different primary antibodies (Table 1) in the blocking buffer overnight at 4°C. The slides were then washed in PBS (three times, 15 minutes each time) and incubated with secondary antibody (Table 2) diluted in 0.01 M PBS for 1 hour at room temperature in the dark. Nuclei were visualized by counterstaining with either a fluorescent DNA probe (1:10,000; Sytox; Molecular Probes) or DAPI (1:10,000; Sigma-Aldrich) nuclear dye. The sections were mounted in fluorescence mounting medium (DakoCytomation, Fort Collins, CO). Images were acquired with a confocal microscope (LSM510 Meta; Carl Zeiss Microimaging, Hamburg, Germany). The specificity of the immunostaining with the antibodies was evaluated in various ways. First, the antibody was preabsorbed with 2 μg of the corresponding peptide antigen per 100 μL incubation mixture for 4 to 6 hours at room temperature before they were added to the tissue sections. In a separate test of the antibody specificity, non- or preimmune purified IgG (mouse, goat or rabbit; depending on the primary antibody) was used as the isotype control at the same concentration as the specific primary antibody. Mouse preimmune IgG and nonimmune goat IgG was used as isotype control for anti-BMP2, -4, and -7. Rabbit preimmune IgG was used as the isotype control for anti-phospho-Smad1/5/8, anti-Id1, and anti-Id3; mouse nonimmune IgG for anti-β-tubulin III; and goat preimmune IgG for anti-Brn3b. The negative control samples did not show any labeling.

Real-Time PCR Analysis

Total RNA was extracted from retinas, RPCs, and differentiated retinal cells (Trizol reagent; Invitrogen-Gibco, Grand Island, NY); cDNA was prepared (Superscript III RT Reaction Mix; Invitrogen, Carlsbad, CA); and real-time PCR was performed (iCycle iQ Multi-color Real Time PCR Detection System; Bio-Rad, Hercules, CA). Gene-specific primers are listed in Table 3. Reactions were performed with qPCR master mix

TABLE 2. List of Secondary Antibodies

Antibody	Catalog No.*
Donkey anti-mouse Alexa Fluor 488	A21202
Donkey anti-rabbit Alexa Fluor 488	Cat. no. A21206
Donkey anti-rabbit Alexa Fluor 568	Cat. no. A10042
Donkey anti-goat Alexa Fluor 488	Cat. no. A11055
Donkey anti-goat Alexa Fluor 568	Cat. no. A11057

All concentrations were 5.0 μg/mL.

* All antibodies were from Invitrogen, Carlsbad, CA.

TABLE 3. Primer Sequences for Mouse Retina

Target	Forward Primer	Reverse Primer
Id-1	ccagtgggtagagggtttga	agaaatccgagaagcacgaa
Id-2	acccgatgagtctgctctac	ctggttctgtccaggtctct
Id-3	aggtgtctcttttcctccct	atgtcgtccaagaggctaag
BMP2	ccagggttagtgaatcagaacac	tcatctttggtgcaaagacctgc
BMP4	attggctcccaagaatcatgg	cgtgatggaaactcctcacagt
BMP7	cgatttcagcctggacaacg	cctgggtactgaagacgg
BMPRIa	cttctccagctgcttttgct	atagcggcctttaccaacct
BMPRIb	agctggttccgagagactga	cagcatggactttgcgtcta
β-tubulin-III	gctgtccgcctgccttttt	gacctcccagaacttggcc
GFAP	gaagaaaaccgcatcaccat	tcacatcaccacgtccttgt
Opsin	gtaccacctcaccagcacct	gggtgtcccagaacgaagta
Rhodopsin	tcaccaccaccctctacaca	tgatccaggtgaagaccaca
Thy1.1	actgccgccatgagaataac	atccttggtggtgaagttgg
Brn3b	cggagagcttgtcttccaac	gatgggcacagaagaagagg
Syntaxin	gaacaaagttcgctccaagc	attcctcactggtcgtggtc
Calbindin	gacggaaagctggaactgac	agcaaagcatccagctcatt
PKC	gaaggtgatgcttgctgaca	cgttgacgtattccatgacg
Hes-1	ctaccccagccagtgtcaac	atgccgggagctatctttct
Mash-1	gttggtcaacctgggttttg	gaacccgccatagagttcaa
Ngn-2	gatgccaagctcacgaagat	acgtggagttggaggatgac
NeuroD1	gctccagggttatgagatcg	ggcttttgatcctcctcctc
Chx10	tccgattccgaagatgttcc	gacttgaggatagactctggcagg

(SYBR Green qPCR SuperMix; Invitrogen). Relative gene expression was calculated by using the comparative threshold cycle ($^{\Delta\Delta}C_t$) method. PCR products were separated by 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Western Blot Analysis

Retinas and RPCs were homogenized in RIPA lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors (PMSF 1 mM, leupeptin 1 mg/mL, and aprotinin 1 mg/mL). Equal amounts of protein were separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 10% skim milk in TBST (Tris-buffered saline [pH 7.4], 0.1% Tween-20) for 1 hour and probed with the antibodies BMP2, -4, and -7; phosphorylated Smad (pSmad)1/5/8; total Smad1/5/8 (Santa Cruz Biotechnology, Santa Cruz, CA); BMPRIb; Id1, -2, and -3 (Santa Cruz); and β-actin (Sigma-Aldrich) overnight at 4°C. After the membranes are washed three times in TBST, they were incubated in HRP-conjugated goat anti-rabbit secondary antibody and HRP-conjugated goat anti-mouse secondary antibody (1:2000; Cell Signaling Technology, Danvers, MA) for 1 hour at room temperature and visualized with enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL).

RPC Culture

Eyes from E14.5 ICR mouse pups were isolated in cold Hanks' balanced salt solution (HBSS; Sigma-Aldrich) containing 3% D-glucose (Sigma-Aldrich) and 0.01 M HEPES (Sigma-Aldrich). The retinas were separated from sclera and pigmented epithelium by dissection, cut into small pieces, and digested in 0.05% trypsin (Invitrogen) for 5 minutes at 37°C. Fetal bovine serum (10% FBS; Invitrogen) was added to stop the trypsin treatment. The cells were pelleted by gentle centrifugation for 5 minutes at 1000 rpm. They were then resuspended in progenitor cell culture medium (F12/DMEM [Invitrogen-Gibco] containing 20 ng/mL EGF and 20 ng/mL bFGF [both from Sigma-Aldrich]), and triturated into a single-cell suspension by pipetting up and down. Cell suspension ($1.5–2 \times 10^6$ cells) was seeded into 25-cm^2 tissue culture flasks (IWAKI, Japan) and cultured in progenitor cell culture medium for 6 to 7 days at 37°C with 95% air and 5% CO_2, to form retinal neurospheres.

Differentiation of RPCs

To induce RPC differentiation, retinal neurospheres were digested with 0.05% trypsin for 5 minutes at 37°C and neutralized with 10% FBS. Neurospheres were resuspended and triturated into a single-cell suspension. The viable cell count in an aliquot was assessed by using the trypan blue (Invitrogen) exclusion assay. Thereafter, the cells (1×10^5 cells/coverslip) were plated on coverslips coated with poly-D-lysine (250 μg/mL; Sigma-Aldrich) and laminin (5 μg/mL; Sigma-Aldrich) in four-well culture plates (NUNC) and were grown in the differentiation medium (progenitor cell culture medium without EGF and FGF, with 1% FBS added) for 6 days. For BMP4 treatment, recombinant BMP4 (20 ng/mL; R&D Systems) was added to the medium. For some of the experiments, the RPCs were pretreated with Noggin (80 ng/mL; R&D systems) 1 hour before the addition of BMP4.

Analyses of Cell Proliferation and Cell Death

The effects of BMP4 on cell proliferation were determined by adding 10 nM bromodeoxyuridine (BrdU; Sigma-Aldrich) to the culture medium 2 hours before collecting the cells. The BrdU-positive cells were stained in mouse monoclonal anti-BrdU antibody (Roche Diagnostics, Indianapolis, IN) and counted in randomly selected fields from different groups. The effect of BMP4 on cell death was determined with a cytotoxicity assay (LDH-Cytotoxicity Assay kit; Roche Diagnostics), a colorimetric method for the measurement of the amount of lactate dehydrogenase (LDH) released by dead cells in culture. The reaction products were analyzed by absorbance at 495 nm on a microplate reader. The amount of LDH in the culture is directly proportional to the number of dead or damaged cells.

Immunocytochemistry

After the medium was removed, and the cells were washed three times in PBS, the cells were fixed in cold methanol for 1 hour at −20°C and blocked in 10% normal donkey serum, 1% bovine serum albumin, and 0.3% Triton X-100 in 0.01 M PBS for 1 hour at room temperature. The cells were then incubated with BMPRIb and BMPRIa antibodies (Santa Cruz Biotechnology), β-tubulin III antibody (Sigma-Aldrich), GFAP antibody (Chemicon International, Inc., Temecula, CA), Brn3b (Santa Cruz Biotechnology), and anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology) in the blocking buffer overnight at 4°C. The slides were washed three times in PBS and incubated with secondary antibodies (Table 2) in PBS for 1 hour at room temperature. The cell nuclei were visualized by staining with DAPI (Sigma-Aldrich).

Id1-Promoter Luciferase Assay

RPCs plated on precoated 12-well plates were transfected with 0.5 μg Id1 promoter plasmid together with 0.01 μg pRL-luc plasmid as an

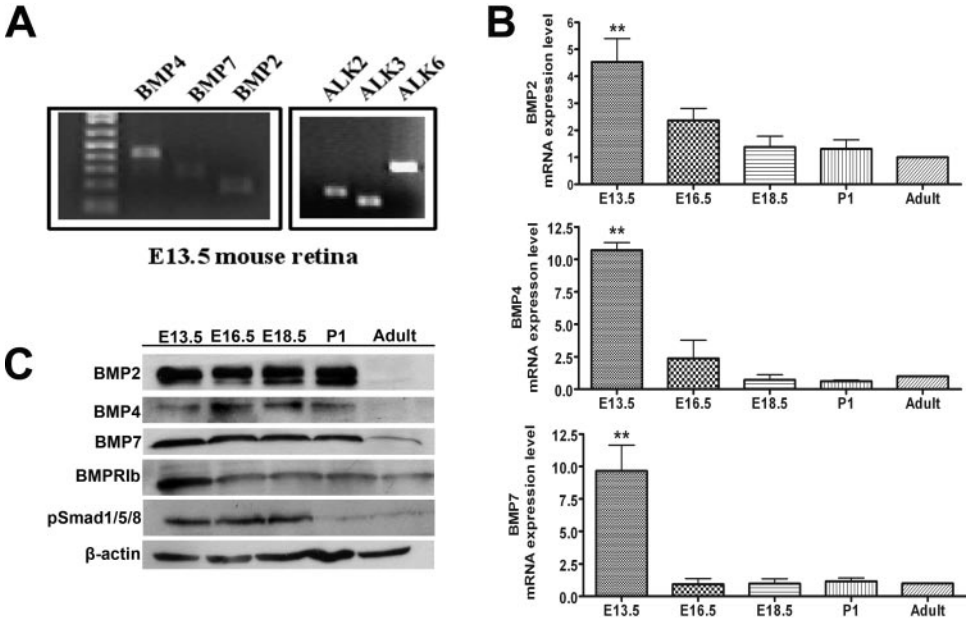

FIGURE 1. Expression patterns of BMP and BMPR mRNAs and proteins during retinal development. (**A**) mRNAs of BMP2, -4, and -7 and the corresponding BMP receptors ALK2, -3, and -6 were detected in the E13.5 mouse retina by RT-PCR analysis. (**B**) Quantitative analysis of BMP mRNA expression levels in the developing and adult retina. Real-time PCR was performed and BMP mRNA expression levels were normalized relative to the adult retina, with GAPDH as the internal control. (**C**) Western blot analysis of BMP2, -4, and -7; BMPRIb; and pSmad1/5/8 proteins, with β-actin as the endogenous control (*$P < 0.05$; **$P < 0.01$).

internal control for normalizing transfection efficiency (Lipofectamine 2000; Invitrogen). PGLbasic3 plasmid was used as an external control. The pID1SB-Luc construct, containing the core promoter of Id1, was kindly provided by Judith Campisi (University of California, Berkeley, CA). On the following day, the cells were stimulated with 20 ng/mL of BMP4 for 24 hours. They were harvested in passive lysis buffer, and luciferase activity in the cell lysates was measured with a luciferase assay kit (Promega Corp., Madison, WI).

Statistical Analysis

All experiments were repeated three times. One-way ANOVA and Tukey multiple-comparisons test or Student's *t*-test was used to determine the statistical significance of differences (Prism 4 software; GraphPad Software, Inc., San Diego, CA). Data are presented as the mean ± SE, and differences are considered significant at $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

Expression Patterns of BMPs and Their Receptors during Retinal Development

Expression of BMP2, -4, and -7 and the corresponding mRNA of the BMP receptors ALK2 (BMP7 receptor), -3 (BMP2 receptor), and -6 (BMP4 receptor) was observed by RT-PCR in the E13.5 mouse retina (Fig. 1A). BMP2, -4, and -7 mRNAs exhibited similar expression patterns, showing the highest expression levels at E13.5 and then gradually decreasing from E16.5 to the adult stage (Fig. 1B). Western blot results demonstrated the dynamic changes in protein expression levels of BMP2, -4, and -7; BMPRIb; and pSmad1/5/8 during retinal development. They were all highly expressed from the early embryonic stage (E13.5) to the early postnatal stage (P1) and then decreased in the adult stage (Fig. 1C).

BMPs and pSmad1/5/8 Protein–Expressing Cell Types in the Adult Mouse Retina

Expression of BMP2 and -7 (Figs. 2A, 2E, arrows) was localized in Brn3b$^+$ cells (Figs. 2B, 2F) in the GCL (Figs. 2D, 2H). BMP4 (Fig. 2I, arrows) and pSmad1/5/8 proteins (Fig. 2M, arrows) were expressed in β-tubulin III$^+$ cells (Figs. 2J, 2N, arrows) in the GCL (Figs. 2L, 2P, arrows) of the adult mouse retina. Using a fluorescent DNA probe (Sytox; Molecular Probes) as a nuclear marker (Figs. 2C, 2G, 2K, 2O), we found that BMP2, -4, and -7 proteins were expressed in the cytoplasm of RGCs (Figs. 2D, 2H, 2L), whereas pSmad1/5/8 were expressed in the nuclei of RGCs (Fig. 2P). Preabsorbing the BMP2 and pSmad1/5/8 antibodies with the corresponding antigen peptides resulted in the elimination of specific BMP2 and pSmad1/5/8 immunostaining (Figs. 2Q, 2S). Furthermore, positive staining, as demonstrated by BMP2 and pSmad1/5/8 primary antibodies (Figs. 2A, 2M), was not seen when the sections were incubated with the matching isotype control (Figs. 2R, 2T). Blocking and isotype controls for BMP4 and -7 (not shown) revealed similar results. Together, these results confirmed the specificity of the antibody reaction.

BMP Signaling Molecules in RPCs

The sphere-forming RPCs expressed nestin, a common neural stem/progenitor marker (Fig. 3A). BMP2, -4, and -7; BMPRIa and -b (Fig. 3B); and Id1, -2, and -3 (Fig. 3C) transcripts were detected in the RPCs. We also identified expression of mRNAs of RPC markers Pax6 and Chx10 and retinal bHLH transcription factors Hes1, Mash1, NeuroD1 and Ngn2 in the RPCs (Fig. 3C).

Effect of BMP4 on RPC Proliferation and RPC Differentiation into Neuronal Lineage

On treatment of RPCs with BMP4, the total cell number was decreased in the BMP4-treated group (Fig. 4Ab), compared with the control group (Fig. 4Aa) after 6 days in culture. Also, BMP4 increased β-tubulin III$^+$ cell immunoreactivity and decreased GFAP$^+$ astrocytes in the BMP4-treated group (Fig. 4Ab), compared with the control group (Fig. 4Aa). Real-time PCR analysis revealed that BMP4 treatment increased β-tubulin III mRNA transcript and decreased GFAP mRNA transcript (Fig. 4B). The BMP4-treated culture showed a 3.5-fold decrease in BrdU incorporation relative to control (Fig. 4C). The LDH assay showed that BMP4 did not increase RPC death at 20 ng/mL (Fig. 4D).

BMP4 and RGC Differentiation

Real-time PCR analysis showed that after 3 days in culture, BMP4 significantly increased Math5 mRNA transcript (Fig. 5A). Consistent with the increase in Math5 expression, expression of Brn3b, a target gene of Math5, was also increased in the BMP4-treated culture (Fig. 5B). Real-time PCR analysis showed that of all the

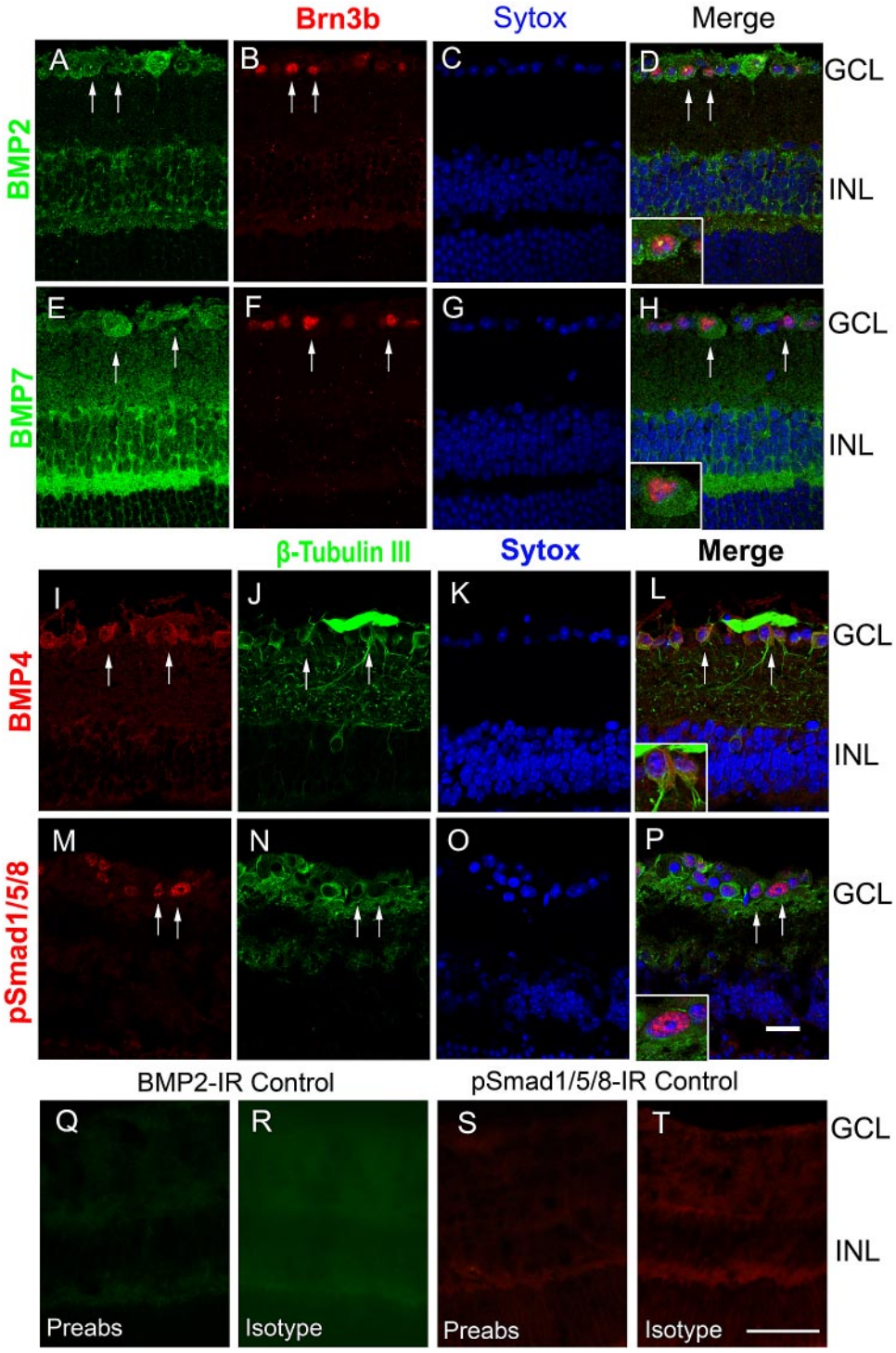

FIGURE 2. Confocal micrographs of BMP and pSmad1/5/8 protein-expressing cell types in the adult mouse retina. BMP2 and -7 (**A**, **E**, *arrows*; *green*) were co-labeled with the RGC marker Brn3b (**B**, **F**, *arrows*) stained in *red* in the GCL (**D**, **H**). Immunofluorescently labeled BMP4 (**I**, *arrows*) and pSmad1/5/8 proteins (**M**, *arrows*; *red*) co-localized with the RGC marker β-tubulin III (**J**, **N**, *arrows*; *green*) in the GCL (**L**, **P**, *arrows*). The fluorescent DNA probe (Sytox; Molecular Probes) (**C**, **G**, **K**, **O**, *blue*) showed expression of BMP2, -4, and -7 proteins in the cytoplasm of retinal cells (**D**, **H**, **L**), whereas pSmad1/5/8 proteins were expressed in the nucleus (**P**). (**Q**, **S**) Preabsorption controls with the antigen and the same antibody resulted in the complete loss of BMP2- and pSmad1/5/8 immunoreactivity, and (**R**, **T**) incubation with the concentration-matched IgG isotype control revealed no specific BMP2 and pSmad1/5/8 staining. Blocking and isotype controls for BMP4 and -7 (not shown) also revealed that pretreatment of the antibodies with the peptide antigens from which they were generated abolished immunostaining in the mouse retina, and incubation with the isotype control reveal no specific staining. Scale bar: (**A**–**P**) 20 μm; (**Q**–**T**) 50 μm.

genes that we examined (Thy1.1 for RGCs, syntaxin for amacrine cells, calbindin for horizontal cells, and PKC for bipolar cells), only the Thy1.1 mRNA expression level was significantly increased by BMP4 stimulation. By contrast, the mRNA levels in inner nuclear neurons, such as amacrine, horizontal, and bipolar cells, did not show significant increases (Fig. 5B).

BMP Signaling Molecules and Id1 and -3 Proteins in the Adult Mouse Retina

In immunolocalization experiments, Id1-expressing cells (Figs. 6B, 6F, 6J, arrows) colocalized with BMP2, -4, and -7 (Figs. 6A, 6E, 6I, arrows), mainly in the GCL and some in the inner nuclear layer (INL; Figs. 6D, 6H, 6L, arrows). Similarly, BMP2, -4 and -7 (Figs. 6M, 6Q, 6U, arrows) were co-localized with Id3 (Figs. 6N, 6R, 6V, arrows), mainly in the GCL and some in the INL (Figs. 6P, 6T, 6X, arrows). BMPRIb expression (Figs. 7B, 7F, arrows) was found in $Id1^+$ and -3^+ cells (Figs. 7A, 7E, arrows) in the GCL (Figs. 7D, 7H, arrows). The fluorescent DNA probe (Sytox; Molecular Probes) revealed nuclear staining. No specific staining was observed in sections when the Id1 and Id3 antibodies were preabsorbed with the corresponding peptides (Figs. 7I, 7K) or incubated with isotype control IgG (Figs. 7J, 7L).

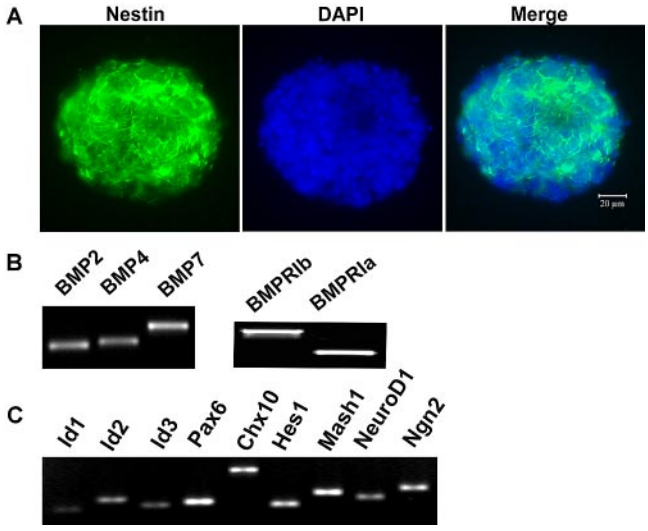

FIGURE 3. RPCs expressed BMPs and BMPRs. (**A**) The stem/progenitor cell marker nestin demonstrated that neurospheres were mainly composed of neural progenitor cells. Scale bar, 20 μm. (**B**, **C**) RT-PCR results showed BMP2, -4, and -7; BMPRIa; and BMPRIb mRNA expression in RPCs. Expression of Id1–3 mRNA, the RPC markers Pax6 and Chx10, and the retinal bHLH transcription factors Hes1, Mash1, NeuroD1, and Ngn2 were also detected.

BMP4 Activation of Smad1/5/8 in RPCs

Western blot analysis showed that BMP4 induced the phosphorylation of Smad1/5/8 in RPCs 30 minutes after BMP4 treatment, whereas the total Smad1/5/8 protein level did not change (Fig. 8A). Immunocytochemistry showed that after BMP4 treatment, pSmad1/5/8 proteins were detected in the nucleus (Figs. 8Bb, 8Bc, arrows), whereas pSmad1/5/8 proteins were not detected in the control culture (Figs. 8Be, 8Bf).

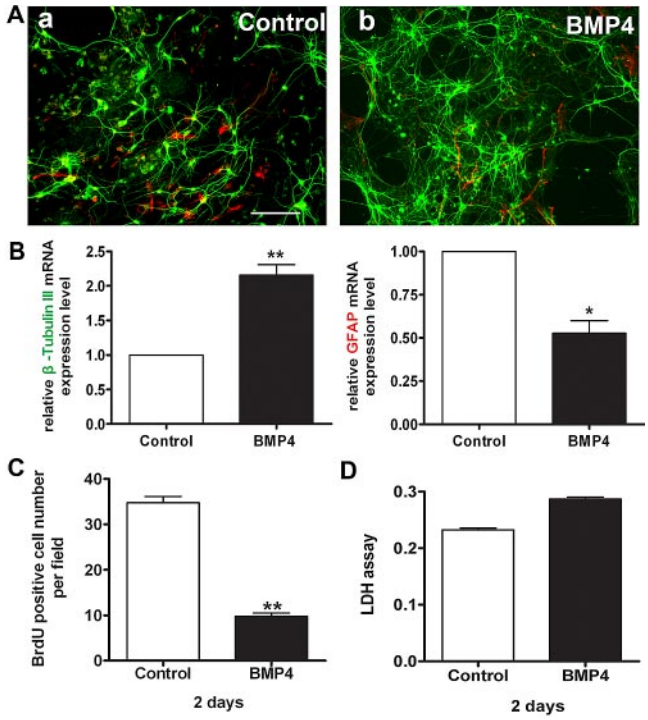

FIGURE 4. BMP4 promoted RPC differentiation. (**A**) β-Tubulin III$^+$ immunoreactivity was increased in neuronal cells (*green*), whereas GFAP$^+$ astrocyte (*red*) immunoreactivity was decreased in the BMP4-treated group compared with the control group. Scale bar, 100 μm. (**B**) Real-time PCR results showed that BMP4 promoted β-tubulin III mRNA expression, but inhibited GFAP mRNA expression. (**C**) BrdU staining results showed that BMP4 decreased the number of proliferating cells. (**D**) The LDH assay results revealed that BMP4 did not increase cell death ($^*P < 0.05$; $^{**}P < 0.01$).

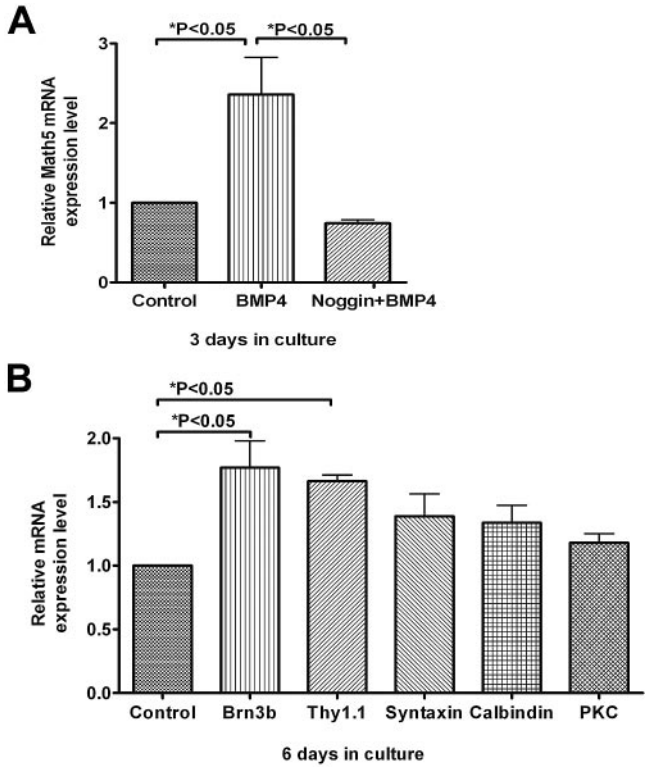

FIGURE 5. (**A**) Real-time PCR results showed that BMP4 significantly increased Math5 mRNA expression in RPCs after 3 days in culture, whereas there was no upregulation of Math5 mRNA expression when the RPCs were pretreated with Noggin. (**B**) After 6 days in culture, the mRNA expression levels of the RGC markers Brn3b and Thy1.1 were significantly increased, whereas the mRNA expression levels of inner nuclear cell markers such as syntaxin, calbindin, and PKC showed no significant change ($^*P < 0.05$).

Smad1/5/8-Induced BMP4 Upregulation of Id1–3 Expression

The luciferase assay result revealed that Id1 promoter activity was significantly increased after treatment with BMP4 at 20 ng/mL (Fig. 9A). BMP4 treatment significantly increased Id1–3 mRNA expression (Fig. 9B). BMP4 treatment also increased Id1–3 protein expression after 30 minutes, reaching a peak at 6 hours (Fig. 9C). The increase of Id1–3 protein expression correlated with the corresponding increase of pSmad1/5/8 (Fig. 8A).

Noggin Blockade of the BMP/Smad/Id Signaling Pathway and Its Effects on Retinal Cell Differentiation

The effect of BMP4 on the upregulation of Id1 promoter activity was blocked after preincubation with the BMP antagonist Noggin (Fig. 9A), and there was no upregulation of Id1 mRNA (Fig. 10A) or Id1–3 proteins (Fig. 10B) in the Noggin pretreatment group. Noggin also blocked the effect of BMP4 on the phosphorylation of Smad1/5/8 proteins (Fig. 10B). Further,

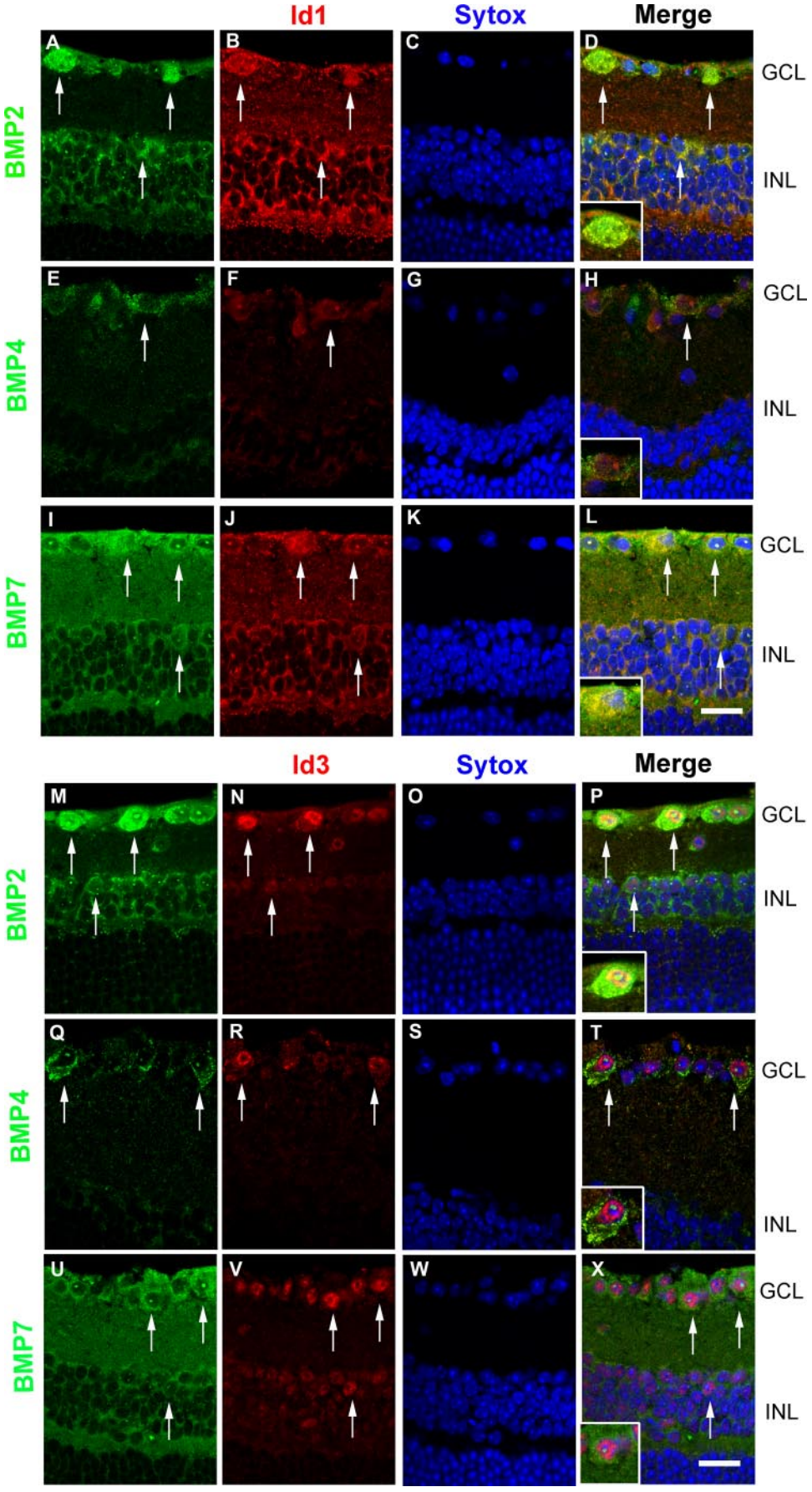

Figure 6. Confocal micrographs of co-localization of BMP2, -4, and -7 with Id1 and -3 proteins in the adult mouse retina. BMP2, -4, and -7 (**A**, **E**, **I**, *arrows*) were co-localized with Id1 (**B**, **F**, **J**, *arrows*), mainly in the GCL and some in the INL (**D**, **H**, **L**, *arrows*). BMP2, -4, and -7 (**M**, **Q**, **U**, *arrows*) were co-localized with Id3 (**N**, **R**, **V**, *arrows*), mainly in the GCL and some in the INL (**P**, **T**, **X**, *arrows*). Scale bar, 20 μm.

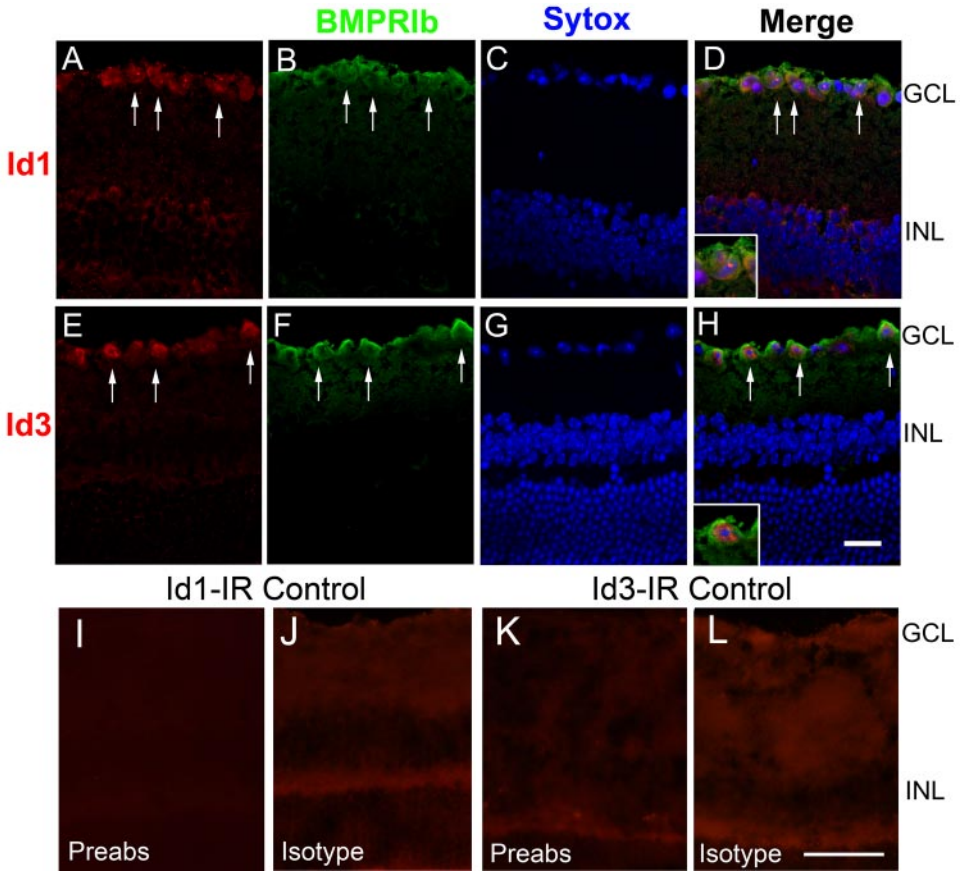

FIGURE 7. Confocal micrographs of adult mouse retina showing co-localization of BMPRIb proteins with Id1 and Id3 proteins. BMPRIb proteins (**B**, **F**, *arrows*) were co-localized with Id1 and Id3 proteins (**A**, **E**, *arrows*) in the GCL (**D**, **H**, *arrows*) and Id3 antibody immunoreactivity (IR) control in adult mouse retina. Preabsorption controls with the antigen and the same antibody resulted in the complete loss of the Id1- and -3-immunoreactivity (**I**, **K**), and incubation with concentration-matched IgG isotype control revealed no specific Id1 and -3 staining (**J**, **L**). Scale bar: (**A**–**H**) 20 μm; (**I**–**L**) 50 μm.

Noggin pretreatment blocked the effects of BMP4 on the upregulation of Math5 expression (Fig. 5A).

DISCUSSION

Previous work has shown that BMPs and BMP receptor mRNA had spatially restricted expression patterns, with the receptors found primarily in the ventral retina and ligands and binding proteins localized to the other regions of the retina in the early chick eye.[21] It is also documented that BMPRIb and -I were distributed in the mouse and chick retina, mainly in the GCL and the optic nerve fiber layer.[22] In this study, we examined the expression patterns and expression levels of BMP and BMPR mRNAs and proteins and the pSmad1/5/8 proteins in mouse retina from the embryonic to the adult stage.

The expression pattern of BMPs and BMPRs in the developing mouse retina was consistent with possible roles of BMPs in regulating retinal cell proliferation and differentiation. The high expression level of BMP signaling molecules during the embryonic stage suggested that it may play essential roles during early retinal development. For example, it was demonstrated that BMP4, which is expressed strongly in the optic vesicle during the early embryonic stage, has crucial roles in the eye formation[23,24]; and BMP7-knockout mice have severe eye defects.[25] BMPs and BMPRs can still be detected in the adult stage albeit at a low level, suggesting that they may still function in the adult retina. Another study has revealed that adult BMP4 heterozygotes have defects in a variety of ocular phenotypes and show many different eye abnormalities.[26] In our study, we also found that exogenous BMP4 could promote RPC differentiation into a neuronal lineage. Previous work has shown that neurogenesis was promoted by BMPs.[27–29] Furthermore, our results demonstrated that BMP4 can promote RPC differentiation mainly into RGCs. This observation was consistent with our in vivo results, which showed that BMP4 was primarily expressed in the RGCs in the GCL.

How BMP activities within their microenvironment influence the outcomes of RPC differentiation remains unknown. It has been reported that Id proteins are important targets of BMP signaling and that they are responsible for performing the biological activities of BMPs through Smad proteins. Our previous work showed that Id1, -2, -3, and 4 were all expressed in the developing and adult mouse retina. The embryos of Id1 and -3 double-knockout mice showed smaller retinal sizes compared with wild-type animals, demonstrating that Id proteins are involved in the regulation of retinal development (Du Y, Yip HK, unpublished results, 2009). In this study, the results demonstrated, for the first time to our knowledge, that BMP proteins and BMPRIb proteins were co-localized with Id1 and -3 proteins, mainly in the RGCs in GCL of the adult mouse retina, suggesting that Id proteins are regulated by BMP/Smad signaling in the mouse retina.

Using RPC culture, we showed that RPCs endogenously expressed BMP/Smad/Id signaling pathway–related proteins, BMPs, BMPRs, and Id proteins. Treatment of RPCs with BMP4 upregulated expression of Id1, -2, and -3, with concomitant phosphorylation of Smad proteins. The BMP antagonist Noggin blocked the effect of BMPs on the phosphorylation of Smad proteins and the upregulation of Id proteins, which further blocked BMP4-mediated induction of the differentiation of RPCs into RGCs. These results suggest that the BMP/Smad/Id signaling pathway exists in RPCs and may influence retinal cell proliferation and differentiation during normal retinal development.

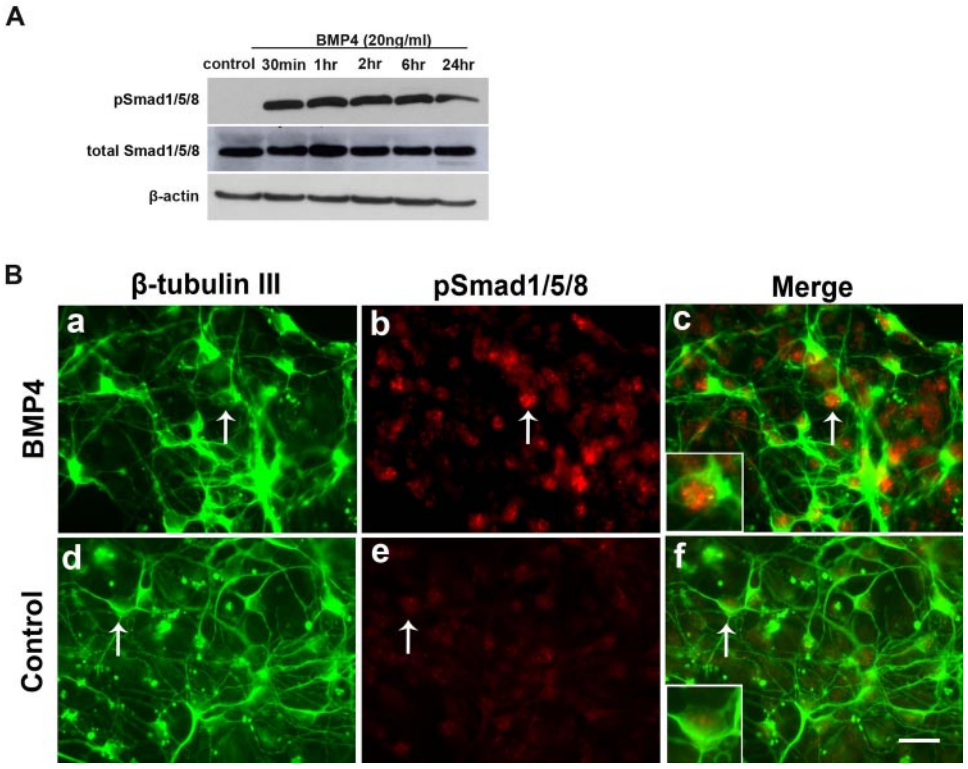

FIGURE 8. Id expression by BMP4 was regulated through Smad1/5/8 proteins. (**A**) pSmad1/5/8 proteins were detected 30 minutes after BMP4 treatment, whereas total Smad1/5/8 protein levels remained unchanged. (**B**) Immunocytochemistry showed that pSmad1/5/8 proteins were detected in the β-tubulin III$^+$ RPCs (*arrows*, **Ba**, **Bd**) in the BMP4-treated group (*arrows*, **Bb**, **Bc**), whereas there were no pSmad1/5/8 proteins in the control group (*arrows*, **Be**, **Bf**). Scale bar, 20 μm.

BMPs have been demonstrated to exert various effects on eye development through activation of Smad proteins,[30,31] but the downstream target molecules are still unknown. For the first time, our study demonstrated that BMP4 can promote RPC differentiation into the neuronal lineage in vitro. We propose that Id proteins are potential targets responsible for BMP signaling in regulating retinal cell development, according to the evidence that both BMPs and Id proteins play important roles in retinogenesis, BMPs and Id proteins are coexpressed in the mouse retina in vivo, and BMP4 may regulate the expression of Id proteins through pSmad1/5/8 proteins. Whether Id proteins are indeed responsible for the effects of BMPs on inducing RPC differentiation into neuronal cells needs further study. Our study proposes a potential signaling pathway (BMP/Smad/Id) in regulating retinal cell differentiation and cell phenotypic determination. Understanding the mechanisms and the gene regulation in retinal cell specification helps us to understand retinal develop-

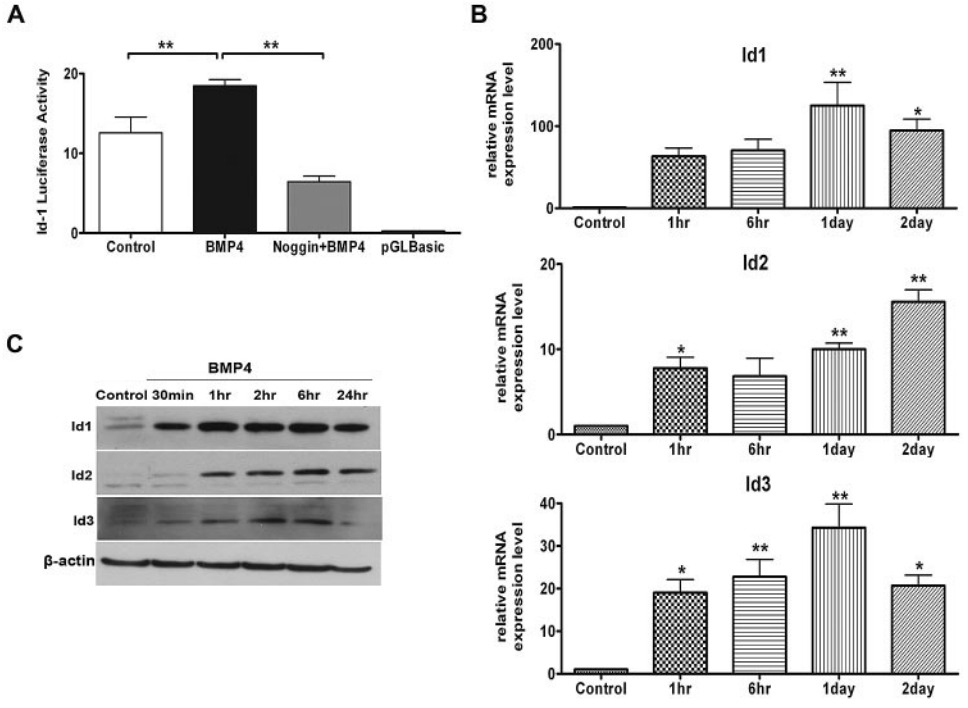

FIGURE 9. BMP4 regulated Id1–3 expression. (**A**) Luciferase assay results showed that Id1-promoter activity increased significantly after treatment with BMP4 at a dose of 20 ng/mL in RPCs, whereas pretreatment with Noggin, a BMP antagonist, blocked the BMP4-mediated upregulation of Id1 promoter activity. (**B**, **C**) Id1–3 mRNA and protein expression levels were significantly upregulated after treatment with BMP4 in cultured RPCs ($^*P < 0.05$; $^{**}P < 0.01$).

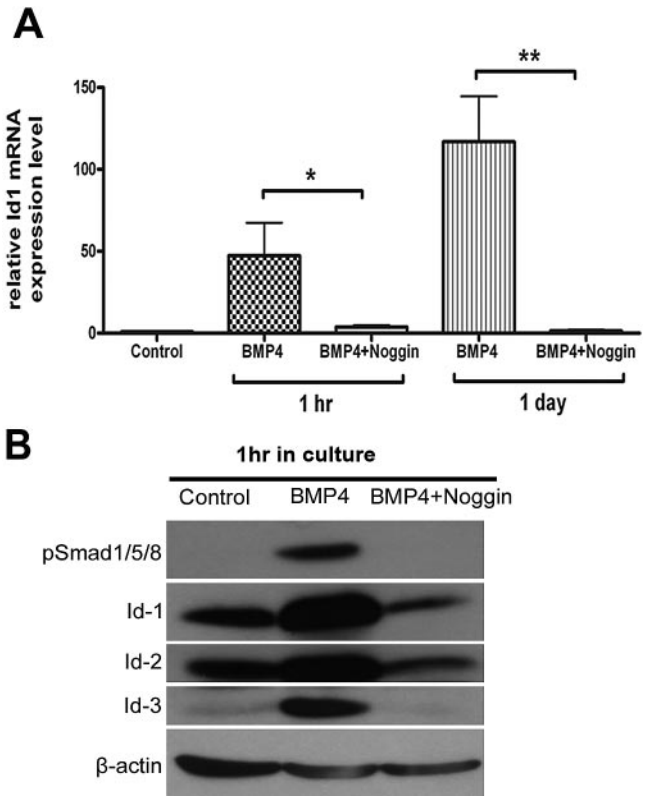

FIGURE 10. Noggin blocked the effects of BMP4 on Id regulation. (**A**) The effect of upregulation of Id1 mRNA via BMP4 was blocked by pretreatment with Noggin. (**B**) There was no phosphorylation of Smad1/5/8 proteins and no increase in expression of Id1, -2, and -3 protein in the Noggin pretreatment group ($^{*}P < 0.05$; $^{**}P < 0.01$).

ment and to design future therapy to treat clinical retinal degenerative diseases.

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