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BRAIN RESEARCH

Short Communication

BMP4 expression in the developing rat retina

Yuka Maruyama[a,b], Sumiko Mikawa[b], Yoshihiro Hotta[a], Kohji Sato[b,*]

[a]Department of Ophthalmology, Hamamatsu University School of Medicine, Hamamatsu, Japan
[b]Department of Anatomy and Neuroscience, Hamamatsu University School of Medicine, Hamamatsu, Japan

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BMP, bone morphogenetic protein
TGFβ, transforming growth factor β
BMPR, bone morphogenetic protein receptor
IR, immunoreactivity
GFAP, glial fibrillary acidic protein

ABSTRACT

We investigated the expression of bone morphogenetic protein-4 (BMP4) in the developing retina. At E19, we found very intense BMP4 immunoreactivity (IR) in the nerve fiber layer. At P1, the inner plexiform layer exhibited very strong BMP4-IR. Thereafter, abundant BMP4 expression was kept to the adult period. These results suggest that BMP4 plays pivotal roles in the retina not only in the early embryonic period but also in the late embryonic and postnatal periods, and even in the adult.

Bone morphogenetic protein-4 (BMP4) is a member of the transforming growth factor β (TGFβ) superfamily (Hogan, 1996). BMP4 signaling has been shown to play an important role in multiple biological events, including neural induction (Harland, 2000), tissue patterning (McMahon et al., 1998), epithelial–mesenchymal interactions underlying organogenesis (Kulessa et al., 2000), lineage selection (Mabie et al., 1999), and in the creation of stem cell "niches" in developing and adult organs (Lim et al., 2000; Watt and Hogan, 2000). BMP4 is synthesized as a large precursor and subsequently cleaved to yield a carboxy-terminal mature protein. BMP4 exerts its biological functions by interacting with membrane bound receptors belonging to the serine/threonine kinase family including bone morphogenetic protein receptor I (BMPRIA, BMPRIB) and type II (BMPRII) (Sasai and De Robertis, 1997). These receptors form heteromeric complexes of type I and type II receptors in which type II is the ligand binding subunit which phosphorylates type I resulting in intracellular cascade events. The functions of BMP4 are also regulated in the extracellular space by secreted antagonistic regulators such as noggin, chordin, follistatin, neurogenesin-1, which are thought to bind BMP4 and prevent their interaction with their receptors (Cho and Blitz, 1998; Ueki et al., 2003).

Also in the early stages of the developing eye, BMP4 has been reported to play pivotal roles, including lens formation (Furuta and Hogan, 1998), topographic retinotectal projection

* *Corresponding author*. Fax: +81 53 4352288.
E-mail address: ksato@hama-med.ac.jp (K. Sato).

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(Sakuta et al., 2001) and apoptotic cell death (Trousse et al., 2001). Although BMP4 expression has been well described in the early stages of the developing eye (Dudley and Robertson, 1997; Furuta and Hogan, 1998), little information is available for BMP4 expression in the late embryonic period and postnatal period. On the other hand, some reports have reported that in postnatal periods BMP4 is involved in the survival of inner retinal neurons and proliferation of Müller glia (Fischer et al., 2004; Liu et al., 2003), suggesting the possibility that BMP4 has additional roles in the late period and postnatal period of the developing retina. In the present study, we thus investigated the expression of BMP4 in the developing retina.

Since the amino acid sequences of BMP2 and BMP4 are very similar, we first investigated whether anti-BMP4 antibody (NCL-BMP4) can discriminate between them using ELISA method. As shown in Figs. 1A and B, the antibody could specifically recognize only BMP4 protein in a dose-dependent manner. In addition, western blotting with the antibody exhibited a single band of about 19 kDa, the size consistent with a previous report (Wozney, 1989, Fig. 1C). Furthermore, pre-absorption of the antiserum with BMP4 completely abolished the immunostaining (Figs. 1D and E). These data indicate that the antibody specifically recognizes BMP4 protein.

At E15, the retina is composed of the inner neuroblastic layer and outer neuroblastic layer (Fig. 2A). Honeycomb-like BMP4 immunoreactivity (BMP4-IR) was observed in both layers (Fig. 2A). In addition, the surface of the inner neuroblastic layer, presumably the prospective nerve fiber layer, showed relatively intenser staining than the other parts (arrowheads in Fig. 2B).

At E19, we could observe three new layers originated from the inner neuroblastic layer, i.e. the nerve fiber layer, ganglion cell layer and inner plexiform layer (Fig. 2C). Interestingly, we found very intense BMP4-IR in the nerve fiber layer (Figs. 2C and D), suggesting that axons of ganglion cells express abundant BMP4 protein. In addition, the inner plexiform layer exhibited strong BMP4-IR, where ganglion neurons, amacrine cells and bipolar cells make synapses at that time. In the ganglion cell layer, inner and outer neuroblastic cell layers, weak honeycomb-like BMP4-IR continued to be observed (Fig. 2C). Nuclear staining showed that BMP4-IR was detected also in the extracellular space in the inner neuroblastic layer (Fig. 4E).

At P1, very intense BMP4-IR was observed in the inner plexiform layer (Figs. 2E and F). In addition, the ganglion cell layer also exhibited strong BMP4-IR (Figs. 2E and F). Interestingly, although cell bodies of ganglion cells did not show detectable amounts of BMP4-IR until E19, we could detect strong BMP4-IR in ganglion cell bodies at P1 (arrowheads in Fig. 2F). The nerve fiber layer continued to exhibit strong BMP4-IR. The inner neuroblastic layer and outer neuroblastic layer showed weak BMP4-IR (Fig. 2E).

At P7, the intensity of BMP4-IR in ganglion cell bodies further increased (Fig. 3A), in addition, the nerve fiber layer also continuously showed strong BMP4-IR (Fig. 3B). In contrast, the intensity of BMP4-IR in the inner plexiform layer drastically decreased (Fig. 3B). The inner nuclear layer exhibited

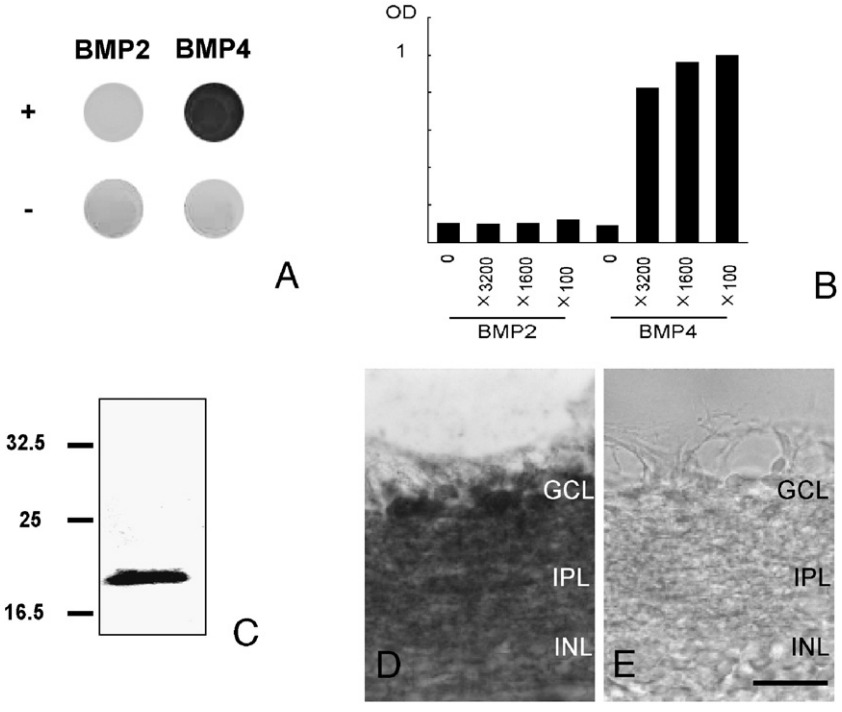

Fig. 1 – Specificity of anti-BMP4 antibody. (A) Wells added with NCL-BMP4 antibody (+) show intense ELISA reaction only when coated by BMP4 protein, but not BMP2 protein. Wells without the first antibody (–) show no ELISA reaction against both proteins. (B) NCL-BMP4 specifically recognizes only BMP4 protein in a dose-dependent manner. (C) Western blot analysis using NCL-BMP4 shows a single band of about 19 kDa. Photomicrographs of pre-absorption tests; control (D), pre-absorbed (E). Note that immunoreactivities in the ganglion cell layer, inner plexiform layer and inner nuclear layer are completely abolished by pre-absorption test. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar=20 μm (D and E).

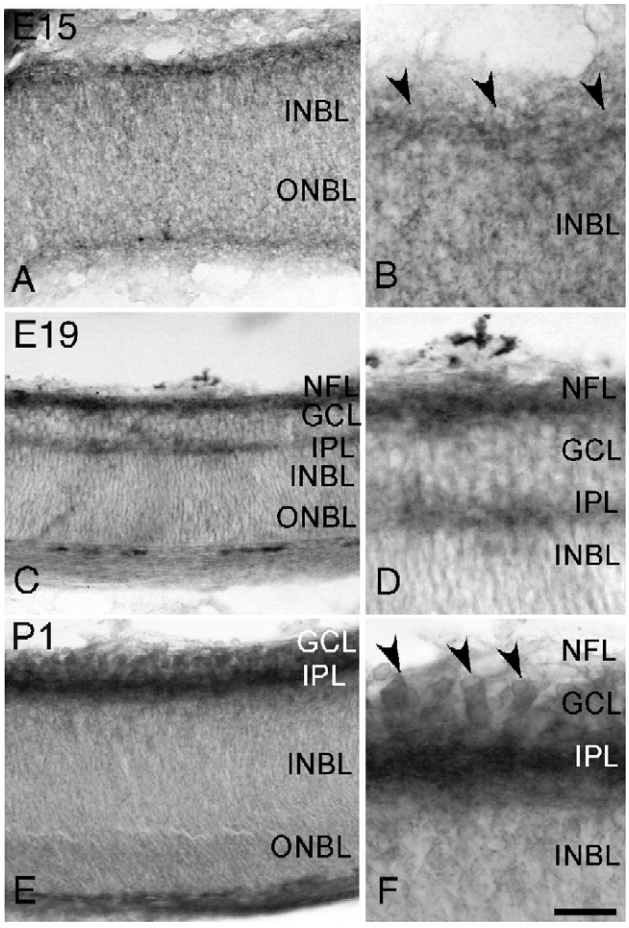

Fig. 2 – BMP4 expression in the developing rat retina (E15–P1). (A, B) At E15, the surface of the inner neuroblastic layer showed abundant BMP4 expression (arrowheads in panel B). (C, D) At E19, intense BMP4 expression was observed in the nerve fiber layer and inner plexiform layer. (E, F) At P1, intense immunoreactivity was observed in the inner plexiform layer. In addition, strong BMP4-IR was also detected in ganglion cell bodies (arrowheads in panel F). INBL, inner neuroblastic layer; ONBL, outer neuroblastic layer; NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer. Scale bar = 50 μm (A, C and E), 20 μm (B, D and F).

moderate BMP4-IR (Fig. 3B). At this time point, the outer neuroblastic layer has already been separated into the outer plexiform layer and outer nuclear layer. Both layers showed weak BMP4-IR (Fig. 3A).

At P14, the ganglion cell layer and nerve fiber layer still kept high levels of BMP4-IR (Figs. 3C and D). The inner plexiform layer also continued to express abundant BMP4 protein. The inner nuclear layer and outer plexiform layer showed moderate BMP4-IR. Weak BMP4-IR was also seen in the outer nuclear layer and photoreceptor layer (Fig. 3C).

At P21, the ganglion cell layer still kept high levels of BMP4-IR (Figs. 3E and F). In contrast, the expression level in the nerve fiber layer decreased to a moderate level (Fig. 3F). The inner plexiform layer, inner nuclear layer, outer plexiform layer and outer nuclear layer showed moderate BMP4-IR. The level of BMP4 expression in the photoreceptor layer was continuously weak (Fig. 3E). After P21, substantial changes were not observed for BMP4-IR (data not shown). The relative intensity of BMP4-IR in each layer of the developing rat retina is summarized in Table 1.

To investigate the possibility that astrocytes in the nerve fiber layer also express BMP4-IR, we performed double fluorescence immunohistochemistry using anti-GFAP (glial fibrillary acidic protein) antibody. We found that GFAP positive cells were simultaneously BMP4-IR positive (Figs. 4A–C), indicating that astrocytes express BMP4 protein. Additional nuclear staining clearly showed that BMP4-IR was observed in the cell bodies and processes of astrocytes (arrowheads in Fig. 4D). Quantitative analysis showed that almost all GFAP-IR positive cells simultaneously expressed BMP4-IR.

This is the first report providing detailed BMP4 expression patterns in the developing retina. We show here that BMP4 is

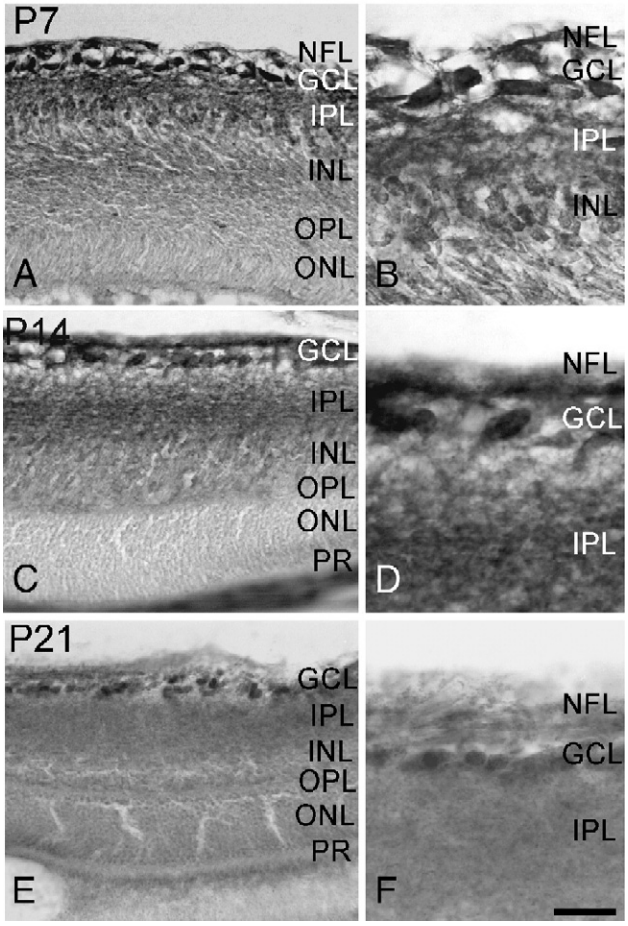

Fig. 3 – BMP4 expression in the developing rat retina (P7–P21). (A, B) At P7, the intensity of BMP4-IR in ganglion cell bodies further increased. (C, D) At P14, the ganglion cell layer and nerve fiber layer still kept high levels of BMP4 expression. (E, F) At P21, the ganglion cell layer still kept high levels of BMP4 expression, while the expression level in the nerve fiber layer decreased to a moderate level. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PR, photoreceptor layer. Scale bar = 50 μm (A, C and E), 20 μm (B, D and F).

Table 1 – Distribution and intensity of BMP4 in the developing rat retina

Time	Area						
	INBL					ONBL	
E15			+			+	
	NFL	GCL	IPL	INBL			
E19	++++	+	+++	+		+	
P1	+++	+++	++++	+		+	
				INL	OPL	ONL	
P7	+++	++++	+++	++	+	+	
						ONL	PR
P14	+++	++++	+++	++	++	+	+
P21	++	++++	++	++	++	++	+
Adult	++	++++	++	++	++	++	+

Relative intensities were estimated by visual comparison of immunostained slides: +, low; ++, moderate; +++, strong; ++++, very strong. INBL, inner neuroblastic layer; ONBL, outer neuroblastic layer; NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PR, photoreceptor layer.

abundantly and differentially expressed throughout the development of the retina. These results suggest that BMP4 plays pivotal roles not only in the early embryonic period but also in the late embryonic and postnatal period, and even in the adult retina.

BMP4 expression in the late embryonic period and postnatal period has been partially investigated using in situ hybridization method. Liu et al. have reported in the mouse retina that BMP4 mRNA was detected in the ganglion cell layer at E15 and that BMP4 mRNA was expressed in the ganglion cell layer and inner nuclear layer at P7 (Liu et al., 2003). These data are consistent with our present results.

Rapaport et al. have reported that in the rat retina 50% of total ganglion cells have been generated at E13, amacrine

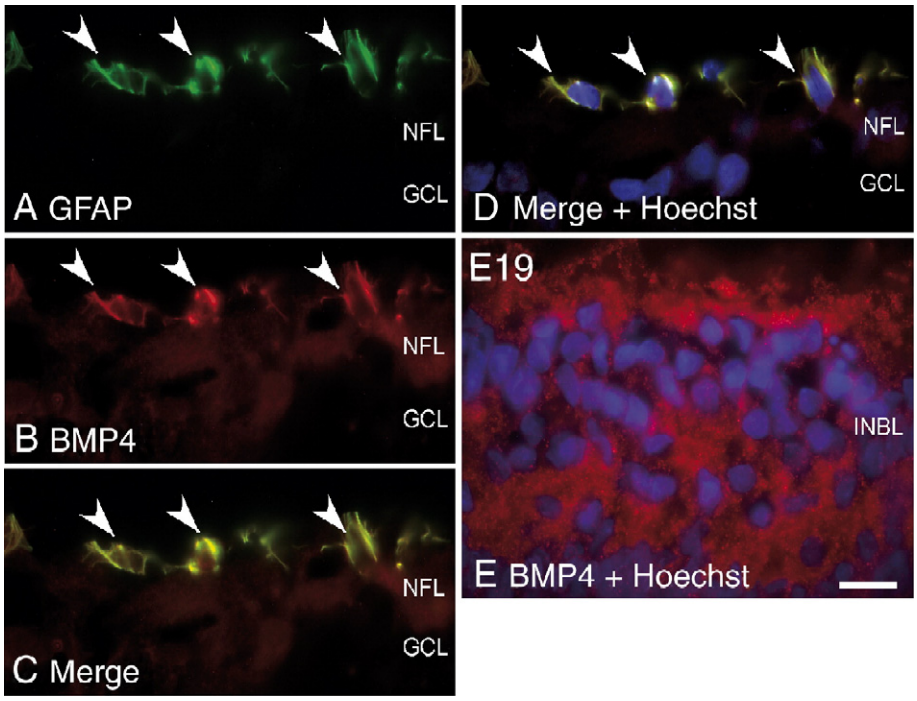

Fig. 4 – Double-staining study showing that GFAP positive cells in the nerve fiber layer (A) are also positive for BMP4-IR (B) in a merged photomicrograph (C) at P49. (D) Additional nuclear staining clearly shows that BMP4-IR is observed in the cell bodies and processes of astrocytes (arrowheads in panel D). (E) Nuclear staining shows that BMP4-IR is detected also in the extracellular space in the E19 inner neuroblastic layer. NFL, nerve fiber layer; GCL, ganglion cell layer; INBL, inner neuroblastic layer. Scale bar=20 μm.

cells at E18 and bipolar cells at P3 (Rapaport et al., 2004). In addition, Dhingra et al. have reported that, in the inner plexiform layer, syntaxin and synapsin I, marker proteins of synapses appeared at P1 and P2, respectively (Dhingra et al., 1997). These data indicate that ganglion neurons, amacrine cells and bipolar cells elongate their dendrites and make synapses each other around P1. On the other hand, in the central nervous system, the involvement of BMPs in dendrogenesis and synaptogenesis has been reported. Addition of BMP7 to cultured hippocampal neurons results in a rapid and profound acceleration of dendritic growth and enhances synaptogenesis (Withers et al., 2000). In addition, enhancement of dendritic growth due to BMPs has been demonstrated for sympathetic (Lein et al., 1995) and cerebral cortical neurons (Le Roux et al., 1999). Interestingly, in the present study, we observed very intense BMP4-IR in the inner plexiform layer at P1. Taken together, like other regions, BMP4 expressed in the inner plexiform layer around P1 might also play a pivotal role in regulating dendritic branching and synapse formation.

In the present study, we also found abundant BMP4 expression in ganglion cells and their axons throughout the retinal development. Interestingly, a BMP4 antagonist, ventroptin, and BMP receptor 1b have been reported to be involved in the mechanisms of retinotectal projection (Sakuta et al., 2001; Liu et al., 2003), suggesting that BMP4 abundantly expressed in ganglion cells and their axons might be involved in the pathway finding mechanisms of the axons of ganglion cells.

Fischer et al. have reported that in the adult chick intraocular injections of BMP4 on the days immediately before a neurotoxic insult greatly reduced the number of Müller glia that reentered the cell cycle and that BMP4 reduced the amount of cell death induced by NMDA (Fischer et al., 2004). Furthermore, in the rodent retina, the absence of BMP receptor 1b results in elevated apoptotic cells in the amacrine cell layer, suggesting that BMP-mediated signaling is normally required to promote the survival of inner retinal neurons (Liu et al., 2003). In the present study, we observed abundant BMP4 expression in the internal nuclear layer, raising the possibility that BMP4 may act as a survival factor there. Interestingly, Close et al. have reported that another ligand of TGFβ superfamily, TGFβ2, is also highly expressed by inner retinal neurons at P4 and regulates proliferation of postnatal progenitors and Müller glia (Close et al., 2005), suggesting that both BMP signaling and TGFβ singling play pivotal roles in the postnatal retina.

We also found that retinal astrocytes express BMP4 protein. In the central nervous system, Peretto et al. have reported BMP4 expression in astrocytes in the olfactory bulb (Peretto et al., 2002), supporting our finding. What is the role of BMP4 secreted from astrocytes? During the development, many ganglion cells extend their axons in the nerve fiber layer, and most astrocytes are situated in this layer. Thus, one possibility is that BMP4 secreted from astrocytes might control axonal pathfinding. Another possibility is that BMP4 secreted from astrocytes affects astrocytes themselves. For example, in the central nervous system, BMP4 promotes astrogliogenesis and inhibits oligodendrocyte preprogenitors or precursors from becoming immature oligodendrocytes (Gross et al., 1996; Mabie et al., 1997). In addition, Gomes et al. have reported that transgenic overexpression of BMP4 increases astroglial and decreases oligodendroglial lineage commitment (Gomes et al., 2003). These data raise the possibility that BMP4 signaling somehow controls the functions of astrocytes also in the retina.

Male Wistar rats at various ages (at embryonic days 15 and 19 and postnatal days 1, 7, 14, 21 and 49; $n=3$ at each time point) were used. For immunochemistry, the embryos were immersed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4 °C overnight. The postnatal rats were perfused transcardially under deep diethylether anesthesia with saline followed by 0.1 M PB containing 4% paraformaldehyde. The eyes of the postnatal rats were removed rapidly, postfixed in the same fixative for 2 h at 4 °C. All samples were immersed in 30% buffered sucrose at 4 °C overnight. Frozen sections (12 μm in thickness) were cut on a cryostat. All experiments conformed to the Guidelines for Animal Experimentation at Hamamatsu University School of Medicine on the ethical use of animals.

For immunoperoxidase staining, the sections were treated with 10% normal goat serum, 2% bovine serum albumin (BSA) and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature and incubated further in monoclonal mouse anti-BMP4 (1:100 dilution; NCL-BMP4, Novocastra, Newcastle, UK) overnight at 4 °C. The antigen used to produce this antibody is the recombinant mouse bone morphogenetic protein-4. After washing 0.1 M PB, sections were incubated in amino acid-polymerized goat anti-mouse IgG with peroxidase complex (Histofine Simple Stain Rat MAX-PO; Nichirei, Tokyo, Japan) for 2 h at room temperature. After washing 0.1 M PB, immunoreaction was visualized with 3,3′-diaminobenzidine (Wako, Osaka, Japan). For double immunofluorescence, sections were treated with 10% normal goat serum, 2% BSA and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature and incubated further in mouse anti-BMP4 antibody (1:20; Novocastra) and goat anti-GFAP antibody (1:50; Santa Cruz Biotechnology) at 4 °C overnight. After washing with 0.1 M PB, sections were incubated in Alexa Fluor 594 chicken anti-mouse IgG (1:500; Molecular probes, Eugene USA) and Alexa Fluor 488 chicken anti-goat IgG (1:500; Molecular probes) for 1.5 h at room temperature. After immunofluorescence staining, sections were incubated with a nuclear dye Hoechst 33258 (0.1 μg/ml in PBS; Sigma, St. Louis, USA) for 5 min at room temperature, washed with PBS. The sections were dried in dark and coverslipped with Permafluor Mountant Medium (Thermo Shandon, Pittsburgh, PA, USA). For pre-absorption controls, 1 nmol BMP4 protein (R&D System, Minneapolis, USA) was added to the diluted primary antiserum (1: 100) at 4 °C overnight.

Rat eyes were homogenized in a 50 mM Tris–HCl (pH 7.4) buffer containing protease inhibitor cocktail (Nakarai Tesque, Kyoto, Japan) to avoid degradation of proteins and solubilized by adding 4× SDS-PAGE sample buffer containing 4% 2-mercaptoethanol. After gentle sonication, SDS-PAGE (12.5% acrylamide) was performed, and proteins were transferred to PDVF membrane (Immobilon-P;Millipore, Billereca, USA) by electroblotting (40 V 1 h at 4 °C) using a transfer buffer (10 mM MOPS, 4 mM sodium acetate [pH 7.5, 20% ethanol, 0.1% SDS]). Blots were blocked with 5% BSA in 50 mM Tris-buffered saline

containing 0.05% Tween-20 (TTBS) for 2 h at room temperature and then incubated with mouse monoclonal anti-BMP4 antibody (1:200; Novocastra) in TTBS containing 1% BSA. After washing with TTBS, blots were incubated with horseradish peroxidase-linked sheep anti-mouse Ig (1:1500; Amersham Bioscience, Piscataway, USA) at 4 °C overnight. Following several washes with TTBS, signals were detected with an ECL western blotting detection kit (Amersham Bioscience).

Solutions of 4 μg/ml of the antigen (BMP4; R&D Systems, Inc., Minneapolis, USA and BMP2; PeproTech EC, London, UK) in 0.1 M carbonate buffer (pH 9.6) were prepared, 50 μl of the antigen solutions was added to each well (96-well EIA/RIA Plate; Corning Incorporated, Corning, USA), and then was incubated overnight at 4 °C. After washing the plate with 0.01 M PBS, the wells were filled with 3% BSA in 0.1 M carbonate buffer and then were incubated overnight at room temperature. After washing the plate three times with 0.01 M PBS, 50 μl of anti-BMP4 antibody (1:100–1:3200) was added to each well and then was incubated for 2 h at room temperature. After washing the plate three times with PBS, 50 μl of horseradish peroxidase conjugated sheep anti-mouse Ig (1:4000; Amersham Bioscience Corp., Piscataway, USA) was added to each well, and then was incubated for 2 h at room temperature. After washing the plate three times with 0.01 M PBS, 100 μl of ABTS substrate solution (Roche Applied Science, Basel, Switzerland) was added to each well and then was incubated for 30 min at room temperature. To quantitate the binding, the results were read at 405 nm using the microplate reader.

Acknowledgments

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