

博士論文

BMP signaling regulates retinal cell differentiation  
and morphogenesis in mouse developing retina

(マウス網膜発生後期における BMP シグナルの機能解析)

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## 1. Abstract

**Purpose:** Bone morphogenetic protein (BMP) plays a pivotal role in early retinal development, but its role in the late phase of retinal development has not been known.

**Methods and results:** I found that BMP ligands and BMP receptors (BMPRs) were expressed in the postnatal retina. Furthermore, immunostaining of phosphorylated-Smad 1, 5 and 8 (P-Smad1/5/8) showed that expression was not limited only to the embryonic retina, but P-Smad1/5/8 was expressed also in horizontal and amacrine cells at P3, and in Müller glia and bipolar cells at P10. However, I did not observe the expression in photoreceptors, indicating roles of BMP for cells in the inner nuclear layer. Expression of constitutively active forms of type-I BMPRs (caBMPR-Ia and caBMPR-Ib) during retinal development resulted in increased number of bipolar cells and Müller glia and a decreased number of rod photoreceptors. Dominantly negative forms of type-I BMPRs (dnBMPR-Ia and dnBMPR-Ib) expression in developing retina resulted in decreased number of bipolar cells and Müller glia and increase of rod photoreceptors. I found that basic helix-loop-helix (bHLH) transcription repressor, Hey2 was induced by BMP signaling, and effects of BMP signaling was partly attributed by Hey2. Furthermore, I found that BMP signaling also played a role in the maturation of Müller glia. Application of inhibitor of BMPRs caused inhibition of Müller glial process extension.

**Conclusion:** My results therefore showed that BMP signaling is required for two distinct phases, differentiation of late retinal cell types and maturation of Müller glia.

## 2. List of abbreviations

bHLH : basic helix loop helix

BMP : Bone morphogenetic protein

BMPRs : Bone morphogenetic protein receptors

caBMPRs : Constitutively active forms of BMP receptors

CNS : Central nervous system

dnBMPRs : Dominantly negative forms of BMP receptors

GCL : Ganglion cell layer

GS : Glutamine Synthetase

GS-box : An intracellular glycine/serine-rich domain

Hes : Hairy and enhancer of split

Hey : Hairy/enhancer-of-split related with YRPW motif

Id : Inhibitor of DNA binding

INL : Inner nuclear layer

NBL : Neuroblastic layer

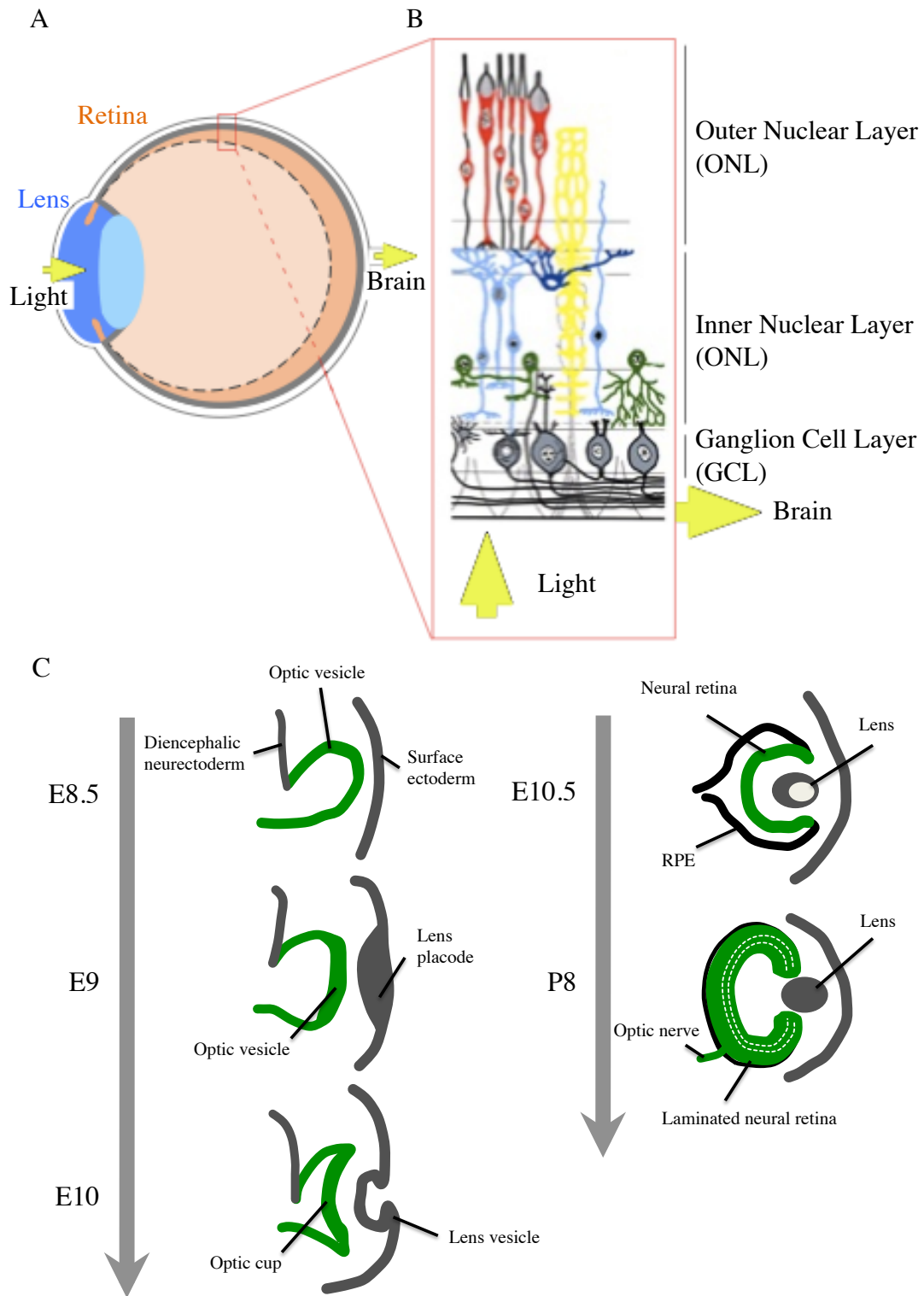
NICD : Notch intracellular domain

ONL : Outer nuclear layer

### 3. Introduction

The vertebrate neural retina is a light sensitive tissue that lines the inner surface of the eye (**Fig.1A**). The neural retina is organized into a laminar structure that is comprised of six types of neurons and glial cells, Müller glia and astrocytes (**Fig.1B**). The neural retina is a part of the central nervous system (CNS) (**Fig.1C**). In the earliest stage of the retinal development, the retina begins to develop as an optic vesicle from the diencephalic neuroectoderm. After the formation of the optic vesicle, it begins to invaginate to form an optic cup. At the same time as the retinal development, the lens placode is formed from the surface ectoderm followed by the formation of a lens vesicle. After that, the lens that is detached from the surface ectoderm and the optic cup finally become the basic structure of the eye. After the formation of the eye structure, retinal progenitor cells initiate to differentiate into various retinal cell types.

In the mice, major retinal cell types are generated from a common population of multi-potent retinal progenitor cells between embryonic day (E) 10 and postnatal day (P) 11 in a conserved temporal order (Marquardt and Gruss, 2002) (**Fig.2**). In the vertebrate retina, ganglion cells, amacrine cells, cone photoreceptors, and horizontal cells differentiate at relatively early stages primarily before birth, while bipolar cells, rod photoreceptors and Müller glia are mainly generated at later stages after birth. As the retinal cell differentiation proceeds, a single layer of the neural retina that is comprised of retinal progenitor cells develops into the laminar structure (**Fig.2**). It has been shown that both the progression of retinal neurogenesis and retinal cell fate specification are controlled by intrinsic cues, such as transcription factors, as well as

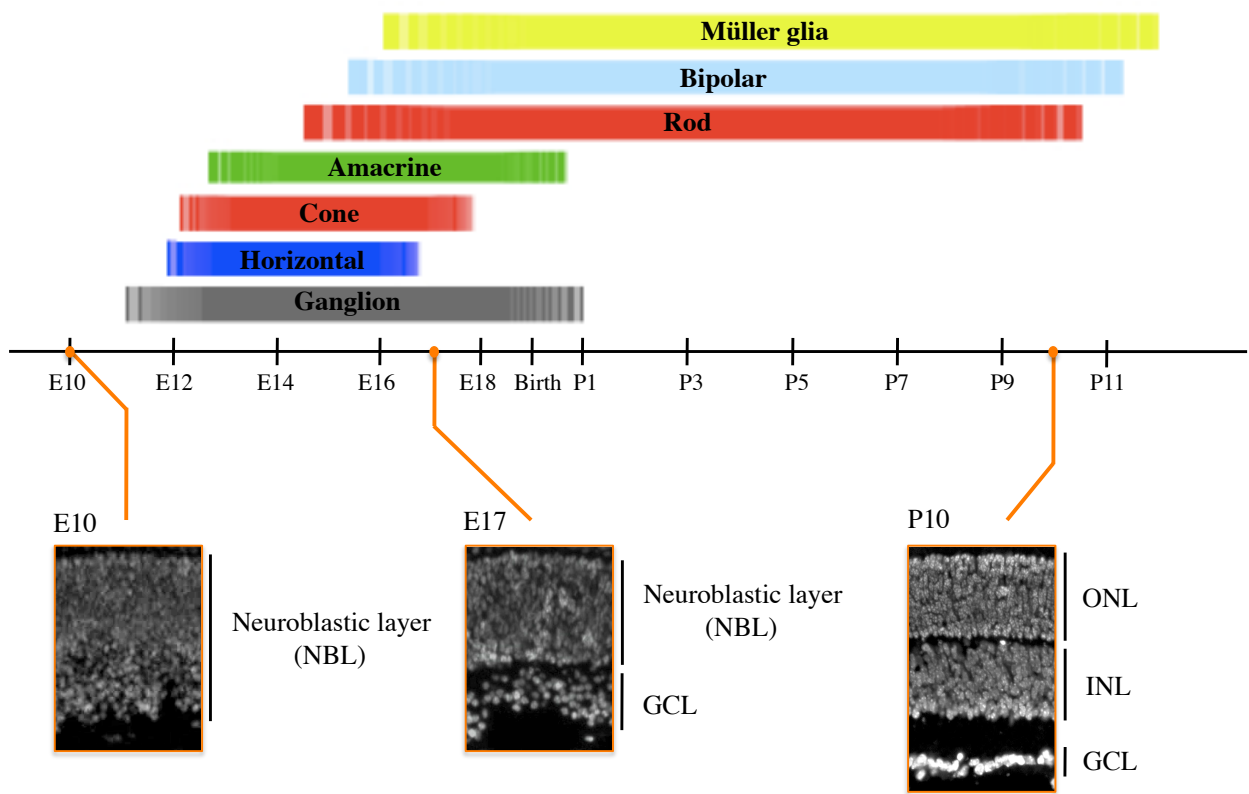


**Fig.1 Schematic diagram of adult mouse eye and mouse eye development**

(A) Adult eye in rodents

(B) Adult retina in rodents

(C) Developmental process of the eye



**Fig.2 Temporal change of retinal progenitor lineage**

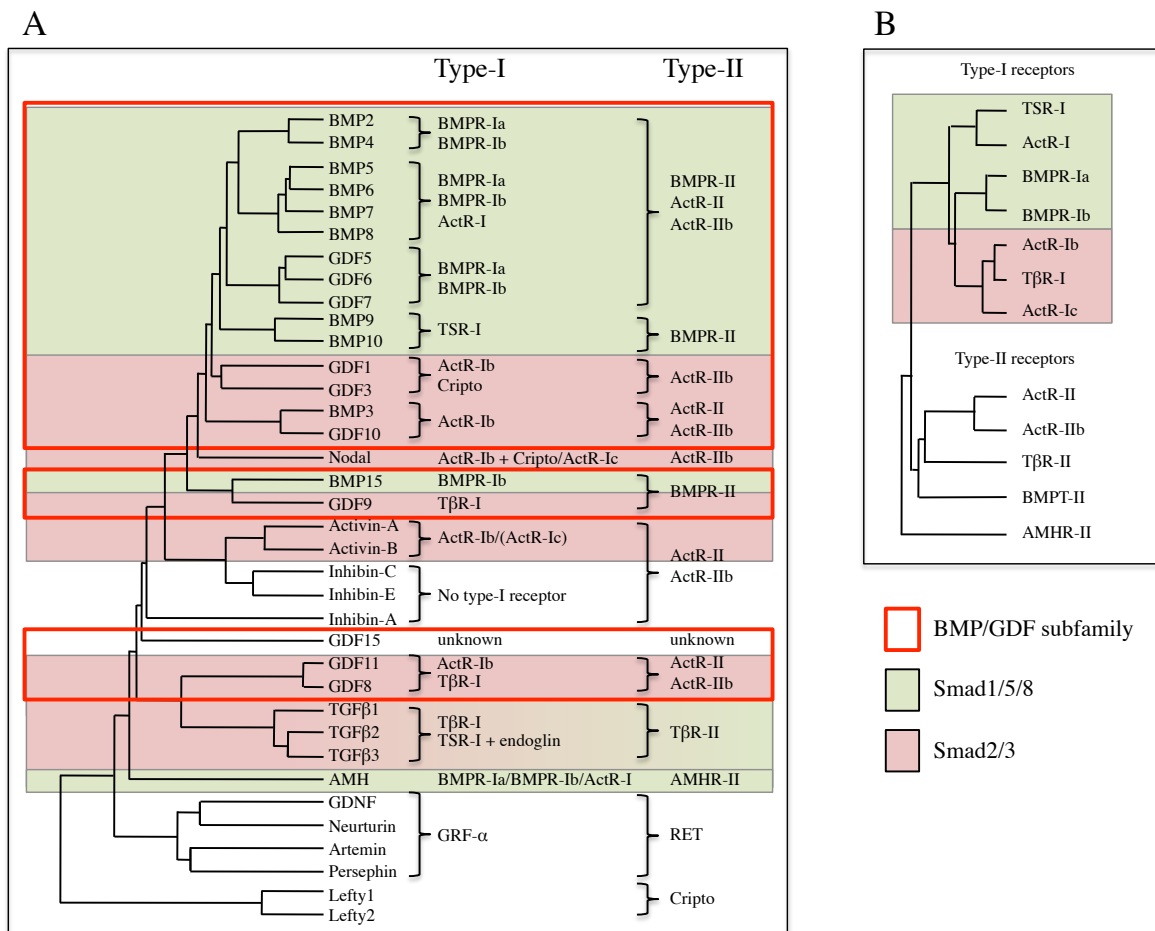
Major retinal cell types are generated from a common population of multipotent retinal progenitor cells between embryonic day (E) 10 and postnatal day (P) 11, in a conserved temporal order. As retinal development proceeds, the neural retina develops into a layered structure consisted of different neuronal types.

by extrinsic signals (Cepko, 1999; Harris, 1997; Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008).

Bone morphogenetic protein (BMP) is a subfamily of the TGF- $\beta$  superfamily that contains more than 20 members (Chen et al., 2004) (**Fig.3A**). BMPs are synthesized as large dimeric proproteins containing an N-terminal signal peptide, a large prodomain and a C-terminal mature part (**Fig.4A**). After the proteolytic processing occurs at the RXXR motif, BMPs finally act as active signaling molecules. These BMPs signal through serine/threonine kinase receptors, composed of type-I and type-II BMP receptors (BMPRs) (**Fig.3B**). BMPRs consist of an N-terminal extracellular ligand binding domain, a single-transmembrane and an intracellular serine/threonine kinase domain (**Fig.4B**). An intracellular glycine/serine-rich domain (GS-box) characteristic for type-I BMPRs (BMPR-I) is essential for kinase and downstream Smad pathway activation. BMP signaling is activated when BMP ligands bind to type-II BMPR (BMPR-II) (**Fig.4C**). After the binding of BMPs to BMPR-II, BMPR-II binds to and phosphorylates GS-box in another type of BMPRs, BMPR-I. Then, the complex of BMPRs phosphorylates Smad1, 5 and 8 (Smad1/5/8). The phosphorylated Smad1/5/8 (P-Smad1/5/8) binds to Smad4 and forms the transcription factor complex. Phosphorylated Smads are translocated into the nucleus where they interact with other transcription factors and regulate the expression of target genes, such as Inhibitor of DNA binding 1 and 3 (Id1 and Id3) (**Fig.4C**).

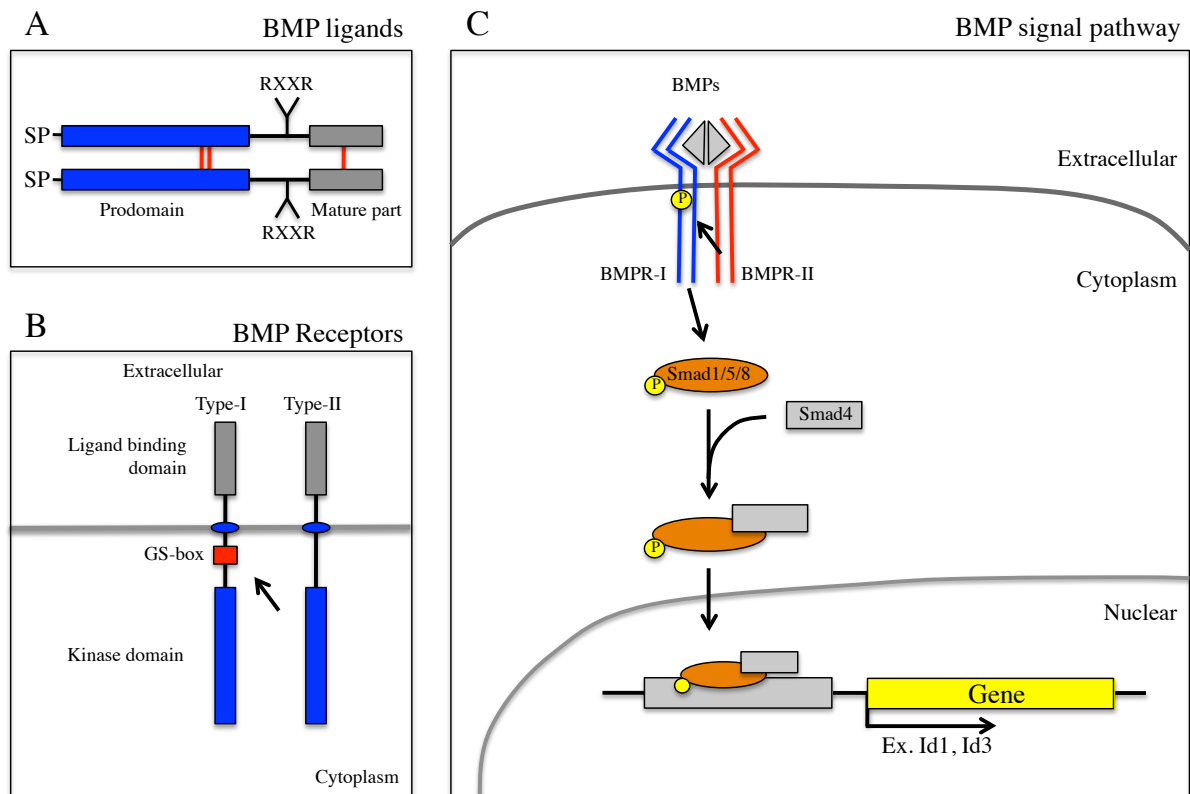
BMP signaling regulates various developmental processes including neural induction, cell proliferation and apoptosis in CNS (Furuta et al., 1997). For example, BMP signaling regulates the initial formation of mouse forebrain (Bachiller et al., 2000), the dorsoventral pattern of spinal cord (Panchision et al., 2001) and the





**Fig.3 Phylogenetic trees of TGF-β superfamily and TGF-β receptors**

(A) Phylogenetic tree of TGF-β ligand superfamily. Ligands circumscribed by red frames belong to BMP/GDF subfamily. (B) Phylogenetic tree of TGF-β receptors. Light-green shed boxes emphasize Smad1/5/8, whereas light-red shed boxes highlight smad2/3 downstream signaling.

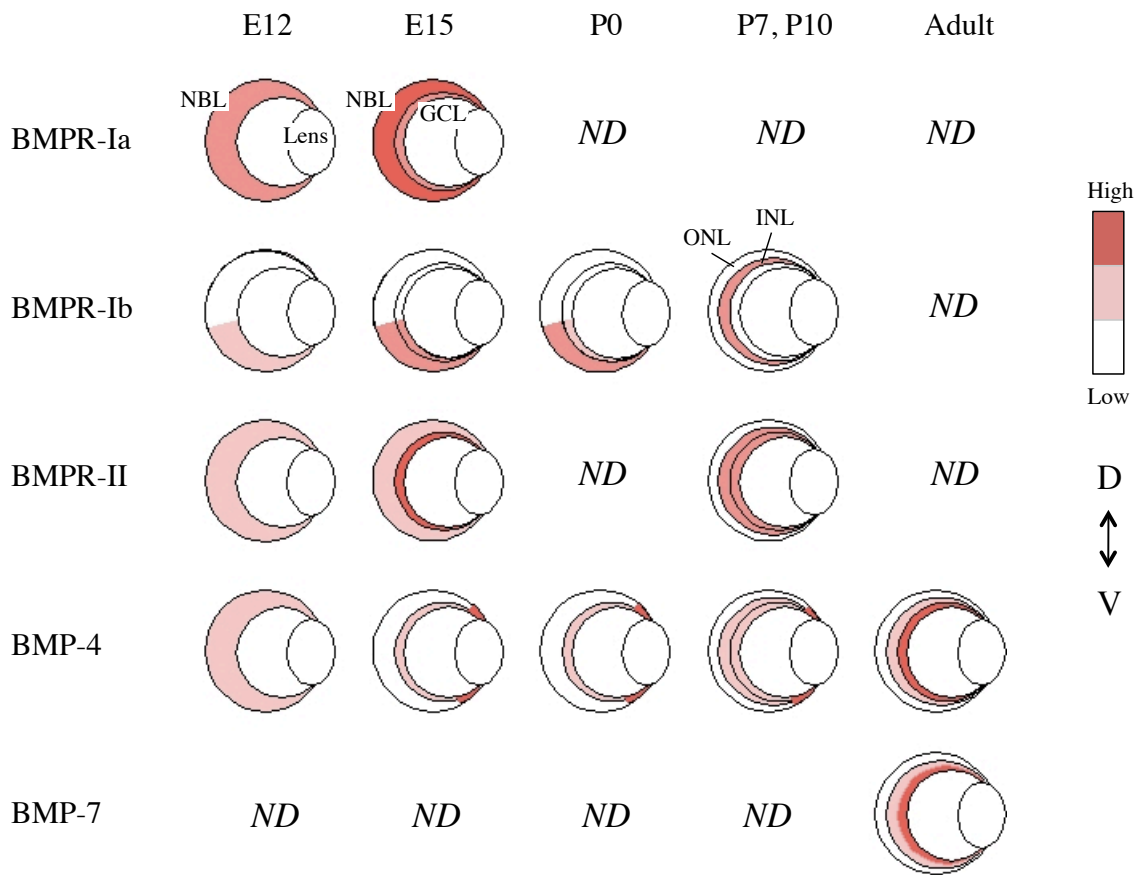


**Fig.4 The architecture of BMP ligands and BMP receptors and the schematic diagram BMP signal pathway**

(A) BMP ligands are synthesized as large dimeric proproteins containing an N-terminal signal peptide, a large prodomain and a C-terminal mature part. (B) BMPRs consist of an N-terminal extracellular ligand binding domain, a single-transmembrane and an intracellular serine/threonine kinase domain. Furthermore, BMPR-I possesses an intracellular glycine/serine-rich domain (GS-box) that is essential for kinase and downstream Smad pathway activation. (C) BMP signaling is conveyed from the cell membrane to the nucleus by the Smad family of proteins. Ligand-binding to BMPR-II promote the complex formation of two types of BMPRs and activation of the receptor complex. The receptor complex phosphorylates the carboxy-terminus of Smad1/5/8. Phosphorylated Smad1/5/8 interact with Smad4, and accumulate in the nucleus, where the Smad complex directly binds defined elements on the DNA and regulates target gene expression together with other factors.

cerebellar granule neuron fate determination (Alder et al., 1999). Because of the above findings, its function in the neural retina that is a part of CNS also attracts great interest. Previous studies showed that BMP-2, BMP-4, BMP-7 and BMPRs including BMPR-Ia, BMPR-Ib and BMPR-II were expressed in mouse retina during embryonic stages (Du et al., 2010; Liu et al., 2003b). The expression pattern of these molecules have been examined with a focus on embryonic stages. Some of these molecules express in a gradient pattern along the dorsoventral axis. BMPR-Ia was expressed at low levels at E9 and the levels increase by E12 (**Fig.5**). At E15, the gene is primarily expressed in NBL and to a lesser degree in GCL. BMPR-Ib, by contrast, has a much more polarized expression pattern at E12. The gene is predominantly expressed in the ventral part of the NBL, rather than in differentiating ganglion cells. In postnatal mice retina, polarity of the expression pattern of BMPR-Ib disappeared by P7. However BMPR-II is barely detectable by E12, and its expression becomes significantly higher after then. BMPR-II is expressed in NBL and GCL at E15 and in postnatal mice retina, the gene is expressed both in the GCL and the middle of the INL. BMP-4 was expressed most highly in the distal part of the retina and developing ciliary body at the embryonic stage. At the same time, there appears to be a low level of expression in the GCL. In addition, BMP-4 is expressed in the ciliary body throughout the first postnatal week. The gene is also expressed in both the GCL and INL at P7.

Because of the above expression pattern of BMPs and BMPRs, the analysis of the function of BMP signaling has been focused on early developmental stages. BMP signaling controls various processes of early retinal development. Because previous studies showed that deletion of Smad4 or BMPR-I in mouse retina caused a loss of



( Cf. Liu. J et al.,2003 and Y. Dua et al.,2010)

**Fig.5 Spatial pattern of BMP ligands and BMPRs during retinal development**

Schematic diagram of spatiotemporal expression of BMPR-Ia, BMPR-Ib, BMPR-II, BMP-4 and BMP-7. ND indicates “no data”.

dorsal retinal character, BMP signaling plays a role in the maintenance of dorsoventral patterning (Belecky-Adams and Adler, 2001; Murali et al., 2005; Murali et al., 2011). In addition, expansion of retinal progenitor cells (Murali et al., 2005) and prevention of apoptosis in vertebrate are also regulated by BMP signaling (Liu et al., 2003b; Murali et al., 2005; Murali et al., 2011). Loss of BMP signaling causes various degrees of microphthalmia. BMP signaling furthermore regulates the differentiation of ganglion cells (Du et al., 2010; Murali et al., 2011) and ganglion cell axon projection through establishment of the dorsoventral expression pattern of related genes in mice retina (Liu et al., 2003b; Murali et al., 2005; Murali et al., 2011). Previous work in my laboratory showed that BMP signaling indirectly controls the differentiation of cone photoreceptors through the regulation of expression level of opsin along dorsoventral axis via regulation of COUP-TF (Sato et al., 2009). As shown above, previous reports focused on BMP function in early stages of development, however, the reports showing BMP function during late stages is few. In addition to these findings, recent reports showed that the activation of BMP signaling could be induced by injury in adult mouse retina (Ueki and Reh, 2013; Ueki and Reh, 2012). These reports revealed that BMP signaling promoted the protection of retinal cells from degeneration.

As shown above, the function of BMP signaling during early developmental stages in mice retina such as morphogenesis and the regulation of differentiation of early retinal cell types has been well studied. Furthermore, its function in adult retina also has been elucidated. Taken together with previous studies, BMP signaling plays a role in a wide range of developmental processes and maintenance of the retina. However, the involvement of BMP signaling in later retinal development, including

retinal cell differentiation and maturation is still unclear. Therefore, I focused on the function of BMP signaling in late phase development in mice retina.

## 4. Material and Methods

### Mouse strain

A mouse carrying a conditional mutant allele for *smad4* (YX. Li et al., 2002) referred to as *Smad4*<sup>fx/fx</sup> mouse was kindly provided by Dr. Y. Furuta (University of Texas). Cre-transgene referred to as *ROSA26-CreER*<sup>T2</sup> was purchased from the Jackson laboratory. *Dkk3-Cre mouse* which was kindly provided by Dr. T. Furukawa (Osaka University) has previously been described (Sato et al., 2007). Tamoxifen induced *Smad4* conditional knock-out (Smad4CKO) mice were obtained by crossing animals carrying *Smad4*<sup>fx/+</sup>; *ROSA26-CreER*<sup>T2</sup> and *Smad4*<sup>fx/fx</sup> genotypes. Smad4CKO mouse whose *Smad4* gene is deleted in retina specifically was obtained by crossing animals carrying *Smad4*<sup>fx/+</sup>; *Dkk3-Cre* and *Smad4*<sup>fx/fx</sup> genotypes.

Adult Institute for Cancer Research (ICR) mice were obtained from Japan SLC Co. The day that the vaginal plug was found was considered as embryonic day 0 (E0), and the day of birth, as postnatal day 0 (P0). All experiments with animals were approved by the Animal Care Committee of the Institute of Medical Science at the University of Tokyo and conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research.

### Plasmids construction

pcDNA3-caBMPR-Ia-HA, pcDNA3-caBMPR-Ib-HA, pcDNA6-dnBMPR-Ia-HA,

pcDNA3-dnBMPR-Ib-HA were kindly provided by Dr. T. Imamura (Tokushima University). The encoding genes, caBMPR-Ia, caBMPR-Ib, dnBMPR-Ia and dnBMPR-Ib were subcloned into pMX-IRES-EGFP. A constitutively active mutant of mouse Notch, NICD (RAMIC) was kindly provided by Dr. T. Honjo (Kato et al., 1997). NICD was also subcloned into pMX-IRES-EGFP.

For the construction of the shRNA expression vector, a target sequence of Hey2 was selected by using siDirect (<http://www.sidirect2.rnai.jp>). The target sequences of Hey2 was: 5'-AAGTCGAAAGACCTAGTTTTATT-3'. Double-stranded DNA for the target sequences was constructed as previously reported using pU6-shRNA expression vector (Yamamichi et al., 2005; Lin et al., 2008)).

For the construction of the luciferase reporter vectors, 5' upstream region of Hey2 was amplified from a genomic DNA of ICR mice by PCR. The resultant fragments were subcloned into the pGL3-Basic (Promega). Subcloned fragments were region from -4054b, -3144b, -1993b, -513b and -276b to first ATG in the exon of Hey2.

### **Electroporation and explant culture**

Retinal explant cultures were prepared as described previously (Tabata et al., 2004). Briefly, retina derived from mice at E17 was placed on a Millicell chamber filter insert (Millipore). Filter was placed into a six-well plate containing 1 ml of explant media and cultured. For in vitro electroporation, dissected retina were transferred to a micro electroporation chamber (Nepagene, model CYU520P5) filled with a DNA solution (1 mg/ml in Hanks' balanced salt solution), and four square



pulses (25 V) of 50 ms duration with 950 ms intervals were applied using an Electroporator CUY21 (Nepa Gene) as described (Matsuda and Cepko, 2004). The electroporated retina were cultured at 34°C on a chamber filter (Millicell).

For BrdU labeling, after 2 days of explant culture of retina, BrdU was mixed into the medium at a final concentration of 1.5 mg/ml at 24 hours prior to fixation. For inhibition of Notch signaling, DAPT was applied into the medium at final concentration of 10µM.

### **Monolayer culture and identification of Müller glia**

Monolayer culture was set up as described previously (Koso et al., 2006). Briefly, explant retina that were cultured for 3 days were treated with 0.25% trypsin and 0.1% DNase (Invitrogen-Gibco) for dissociation into single cells. After the dissociation, the cells were transferred to culture plate coated with poly-l-ornithine (10 µg/ml) and fibronectin (5 µg/ml). The cells were cultured in DMEM/F12 containing N2 supplement (1%), FBS (1%), penicillin, and streptomycin, harvested at 7 or 11 days of culture. For inhibition of BMP signaling, an inhibitor of BMPRs, LDN193189 was applied into the medium at a final concentration of 500nM. To identify Müller glia, the cells were stained with anti-S100β antibody (SIGMA). Both S100β positive- and bipolar shaped cells were judged as Müller glia. Measurement of the process length of Müller glia was performed by using a Zeiss Axio Vision 4.6 microscope.

### **Immunostaining**

The isolated eye and explant retina were fixed with 4% paraformaldehyde (PFA), frozen-sectioned (10 $\mu$ m) after embedment in OCT compound (Miles), and immunostained as described previously (Tabata et al., 2004). Primary antibodies used were as below: monoclonal antibodies against P-Smad1/5/8 (Chemicon International, Temecula, CA, USA); Chx10 (Exalpha Biologicals, Shirley, MA, USA); Islet1 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA); HuC/D (Molecular Probes, Eugene, OR, USA); CalbindinD28K (Sigma, St. Louis, MO, USA); Lim1 (Developmental Studies Hybridoma Bank); Gultamine Synthetase (GS) (Chemicon International, Temecula, CA, USA); S100 $\beta$  (SIGMA); RXR $\gamma$  (ppmx, Tokyo, Japan); PNR (ppmx, Tokyo, Japan); BrdU (Roche Molecular Biochemicals, Penzberg, Germany); Active Caspase-3 (Promega, Madison, WI, USA); polyclonal antibodies against GFP (Clontech, Palo Alto, CA, USA). Nuclei were visualized by staining of DAPI. The first antibodies were visualized by using appropriate Alexa-488 or Alexa-546-conjugated second antibodies (Molecular Probes). Samples were mounted in VectaShield (Vector Laboratories) and analyzed by using a Zeiss Axio Vision 4.6 microscope.

## **RT-PCR**

Total RNA was purified from mouse retina which was cultured for 3 days from E17 using RNeasy Plus Micro (QIAGEN), and cDNA was synthesized using Superscript II (Invitrogen-Gibco). Quantitative RT-PCR (RT-qPCR) was done the SYBR Green-based method using the Roche Light Cycler 1.5 apparatus and

analyzed by the Second Derivative Maximum Method for quantification (Roche Diagnostics). GAPDH, SDHA and  $\beta$ -actin were used as the internal control.

### **Luciferase reporter assay**

HEK293T cells ( $2.5 \times 10^4$  cells /well) were plated in a 24-well culture plate 1 day before transfection with various combination of plasmid DNAs. To assay Hey2 promoter activity, 0.025  $\mu$ g of pGL3-Promoter region of Hey2, 0.0025  $\mu$ g of pRL-Renilla luciferase reporter vector, 0.1125  $\mu$ g of pMX-caBMPR-Ia and 0.1125  $\mu$ g of pMX-caBMPR-Ib were transfected. To assay the crosstalk between BMP signaling and Notch signaling, 0.025  $\mu$ g of pGL3-RBP-J $\kappa$ , 0.0025  $\mu$ g of pRL-Renilla luciferase reporter vector and 0.225  $\mu$ g of some of the plasmids encoding caBMPRs, dnBMPRs, shHey2 and NICD. After 48 hours in culture after transfection, the cells were harvested. Luciferase activity toward a luciferase assay substrate (Promega) was measured with a luminometer (Lumat LB9507, Berthold Technologies). Values were normalized with the renilla signal.

### **Statistical analysis**

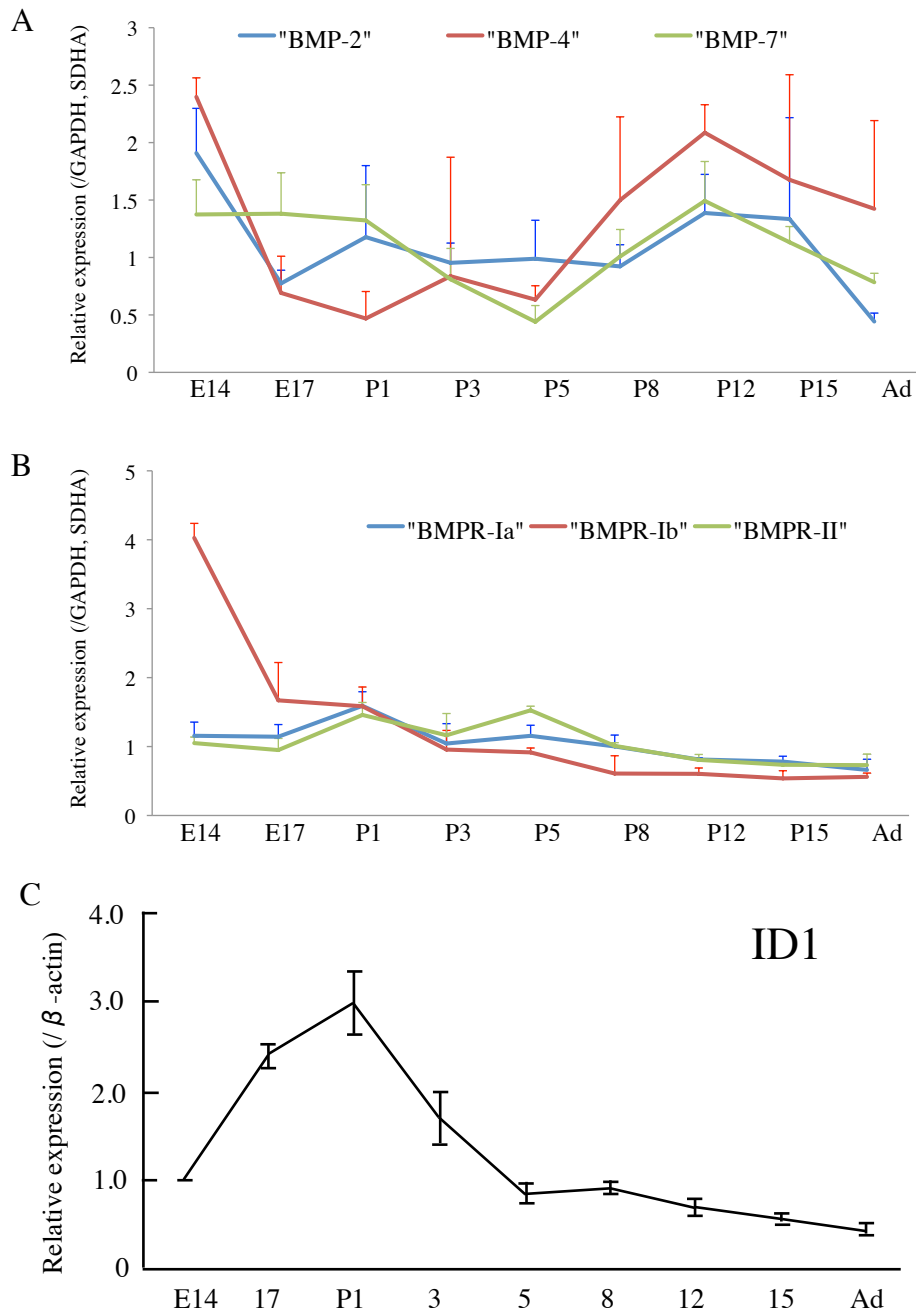
The Mann-Whitney *U* test, a non-parametric test, was used to assess whether the distribution of two samples are the same. Student's t-test was used to test whether means of two normally distributed populations are significantly different or not. Error bars indicate standard error of the mean.

## 5. Results

### *Expression pattern of BMPs and BMPRs in developing retina*

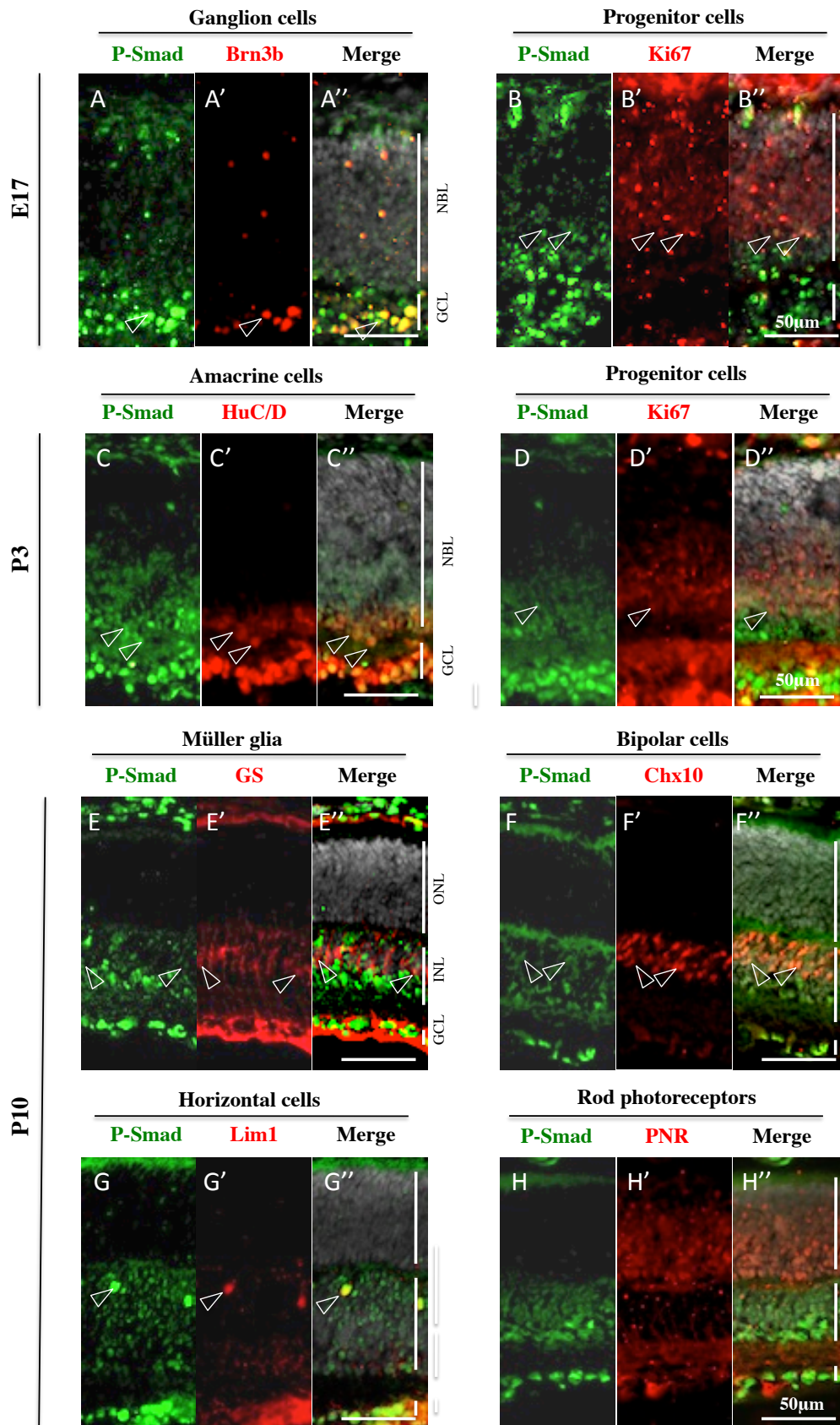
Previous studies reported the expression level and pattern of BMPs and BMPRs in mouse early developing retina (Dewulf et al., 1995; Du et al., 2010; Liu et al., 2003b) (**Fig.5**). In contrast to information in embryonic stages, little has been published about postnatal development. Therefore, I first examined the expression of BMP-2, BMP-4, BMP-7 and BMPRs by RT-qPCR (**Fig.6A, B**). I found the expression of all of these genes from E14 to P15, and in adult. Among them, the expression of BMP-2, BMP-4 and BMP-7 was at a high level at E14, maintained a high level of expression during postnatal stages (**Fig.6A**). However BMPR-Ib was expressed highly at E14, became weak after E17 onwards, and the level was comparable with that of BMPR-Ia and BMPR-II (**Fig.6B**). This result suggested that BMP signaling could function in retinal development during postnatal stages. I then examined whether BMP signaling is really activated or not by examining the expression of a BMP signaling target gene, Id1 by RT-qPCR (**Fig. 6C**). The expression of Id1 was peaked around birth. After birth, the expression level of Id1 decreased gradually and became very low 5 days after birth.

To identify the spatial expression pattern where BMP signaling is activated, I performed co-immunostaining using antibodies anti-P-Smad1/5/8 and cell specific markers. Phosphorylation of Smad1/5/8 can be regarded as an indicator of activity of BMP signaling. At E17, the signal was observed in GCL and the inner side of the NBL (**Fig.7A, B**). P-Smad1/5/8 showed strong expression in Brn3b-positive



**Fig.6 Expression pattern of BMP signaling components and a target gene, Id1 during retinal development**

(A, B) Expression of BMP-2, BMP-4, BMP-7, BMPR-Ia, BMPR-Ib and BMPR-II were examined by quantitative RT-PCR (RT-qPCR) of retina at embryonic day (E) 14, E17, postnatal day (P) 1, P3, P5, P8, P12, P15 and adult. GAPDH and SDHA were used as a control. (C) Expression of Id1 was also examined by RT-qPCR.  $\beta$ -actin was used as a control.



**Fig.7 Identification of retinal cell types where BMP signaling was activated**

(A-H") Co-immunostaining with P-Smad1/5/8 (green) and cell specific markers (red). Frozen sections of mice retina were immunostained at E17, P3 and P10. Brn3b specified ganglion cells (A-A"), Ki67 specified proliferating retinal progenitor cells (B-B", D-D"), HuC/D specified amacrine cells (C-C"), GS specified Müller glia (E-E"), Chx10 specified bipolar cells (F-F"), Lim1 specified horizontal cells (G-G") and PNR specified rod photoreceptors (H-H"). Nucleus was also immunostained by DAPI (gray). Arrow head shows a double-positive cell.

ganglion cells (**Fig.7A-A''**), and weak expression of P-Smad1/5/8 was also detected in Ki67-positive retinal progenitor cells (**Fig.7B-B''**). At P3, signal was also observed in GCL and a subpopulation in NBL (**Fig.7C, D**). The signal was detected in HuC/D-positive amacrine cells and Ki67-positive retinal progenitor cells (**Fig.7C-D''**). At P10, GS-positive Müller glia, Chx10-positive bipolar cells and Lim1-positive horizontal cells were also positive for P-Smad1/5/8 (**Fig.7E-G''**). However, PNR-positive rod photoreceptors did not express P-Smad1/5/8 (**Fig. 7H-H''**).

Taken together, BMP signaling is activated weakly in retinal progenitor cells and strongly in differentiated ganglion, amacrine, horizontal, bipolar cells and Müller glia in developing mouse retina. As shown above, because the expression of P-Smad1/5/8 becomes strong as retinal cell differentiation proceeds, I hypothesized that BMP signaling plays a role in the differentiation of ganglion, amacrine, horizontal, bipolar cells and Müller glia. Therefore, I next focused on the effect of BMP signaling on the differentiation of retinal cells.

#### ***Gain- and loss of function analysis of BMP signaling during retinal development***

To elucidate the function of BMP signaling in retinal cell differentiation during late retinal development, I then performed gain- and loss of function analysis of BMP signaling. Plasmids encoding constitutive active forms of BMPRs (caBMPRs) : both pMX-caBMPR-Ia-IRES-EGFP and pMX-caBMPR-Ib-IRES-EGFP were introduced into isolated retina to activate BMP signaling at E17. Dominantly negative forms of BMPRs (dnBMPRs) : both pMX-dnBMPR-Ia-IRES-EGFP and

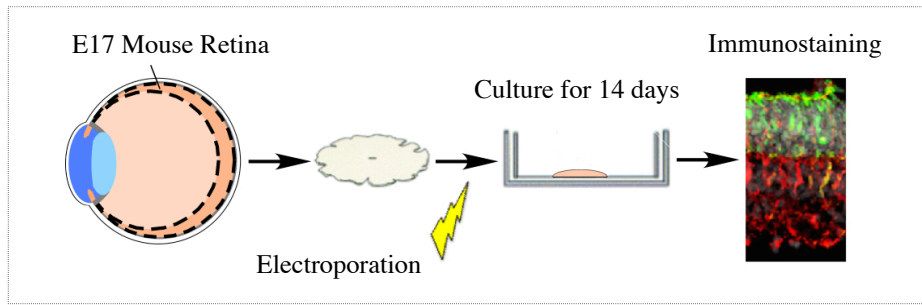


pMX-BMPR-Ib-IRES-EGFP were also introduced into isolated mouse retina to inactivate BMP signaling at E17 by electroporation (**Fig.8A, B**). caBMPRs have a constitutively active mutation in the GS-box and dnBMPRs have a mutation in C-terminal kinase domain (**Fig.8C**). I defined pMX-IRES-EGFP induced retina as a control. The retina was cultured as retinal explants for 14 days. Harvested explant retina was fixed with 4% PFA. After fixation, differentiation was examined by immunostaining using frozen sectioned retina (**Fig.8A**).

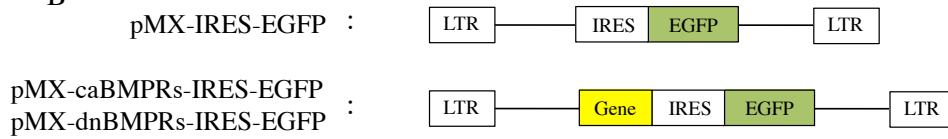
I first confirmed the effect of caBMPRs and dnBMPRs on BMP signaling by immunostaining with P-Smad1/5/8 (**Fig.9**). Introduction of caBMPRs induced the increase of P-Smad1/5/8 signal. On the other hand, introduction of dnBMPRs induced the decrease of the signal.

I next examined sub-retinal localization of EGFP-positive cells. By introduction of plasmids encoding caBMPRs, EGFP-positive cells tended to localize in INL, and a decreased population of EGFP-positive cells in the ONL was observed compared to the control (**Fig.10A, B**). In contrast, by introduction of dnBMPRs, the proportion of cells localized in the INL decreased and those in ONL increased compared to control (**Fig.10A, B**). Furthermore, I examined what types of cells were affected by BMP signaling. To achieve this, co-immunostaining with anti-EGFP antibody and various cell specific markers was performed. In the retina overexpressed with caBMPRs, the proportion of Chx10-positive bipolar cells and GS-positive Müller glia increased and that of PNR-positive rod photoreceptors decreased (**Fig.11A-D**). On the other hand, in retina overexpressed with dnBMPRs, the population of GS-positive Müller glia and Chx10-positive bipolar cells decreased and that of PNR-positive rod photoreceptors increased (**Fig.11A-D**). In contrast to the above result, the proportion

A

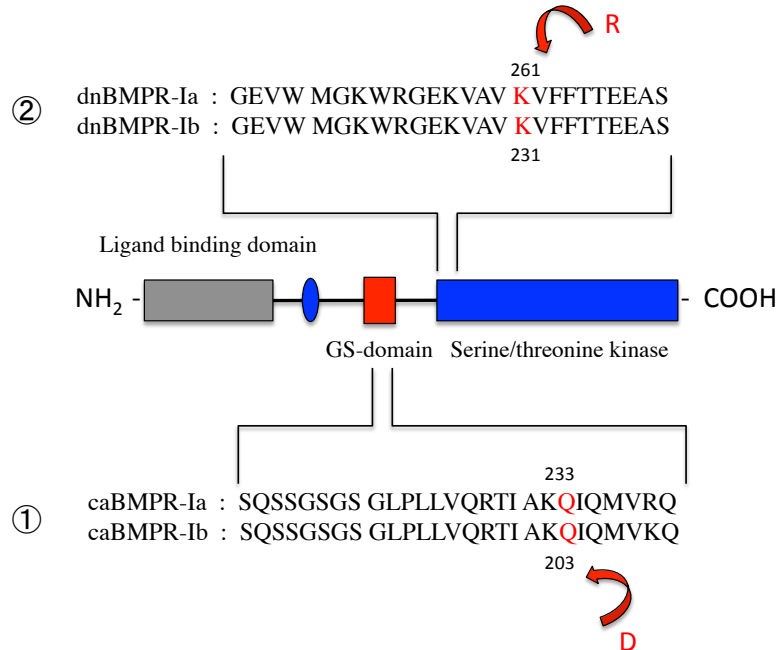


B



- ① Constitutively active BMPR-Ia } **caBMPRs**  
 Constitutively active BMPR-Ib }
- ② Dominant negative BMPR-Ia } **dnBMPRs**  
 Dominant negative BMPR-Ib }

C

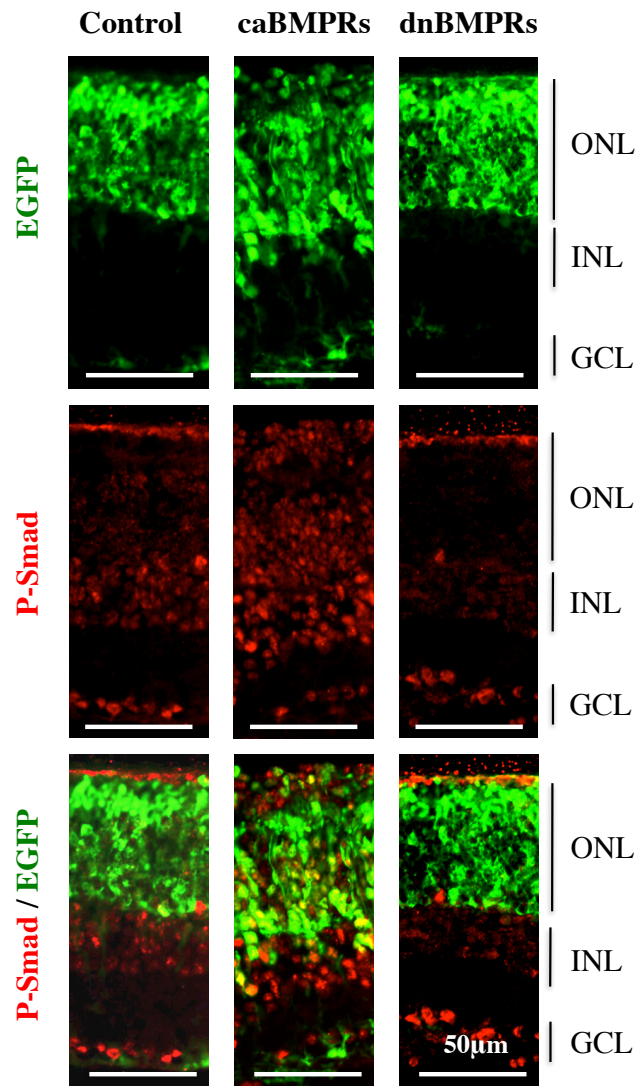


**Fig.8 Schematic diagram of experimental procedure for retinal explant culture and analysis**

(A) Retina was dissected from mouse embryos at E17 and was introduced with the plasmids by electroporation. The explant retina was placed on a

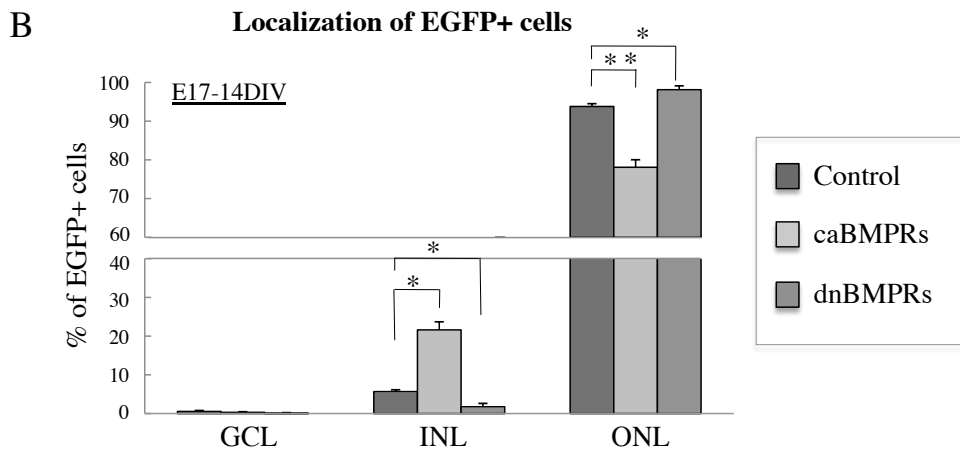
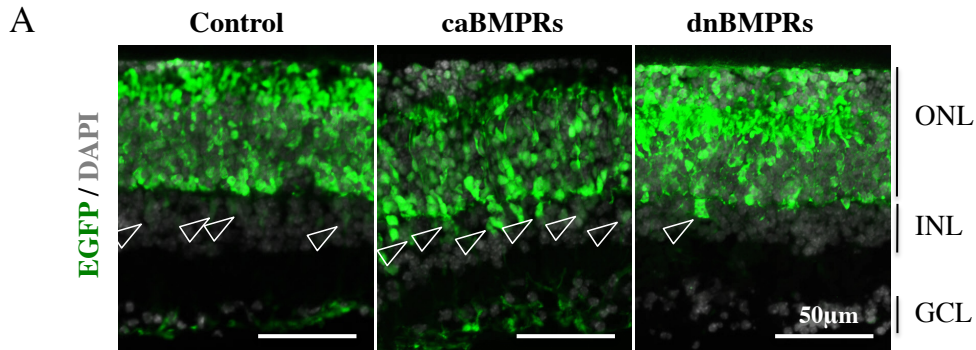
Millicell chamber filter insert and was cultured in a six-well plate for 14 days. After then, explant retina was frozen sectioned and was immunostained. (B) Two types of constitutively active forms of type-I BMPRs (caBMPR-Ia and caBMPR-Ib) or dominantly negative forms of type-I BMPRs (dnBMPR-Ia and dnBMPR-Ib) were encoded in pMX-IRES-EGFP and were expressed under LTR-promoter. (C) caBMPRs have a mutation in GS-box domain and are constitutively active without ligand binding. dnBMPRs have a mutation in kinase domain. This mutation causes dramatic reduction of kinase activity.

E17-14DIV



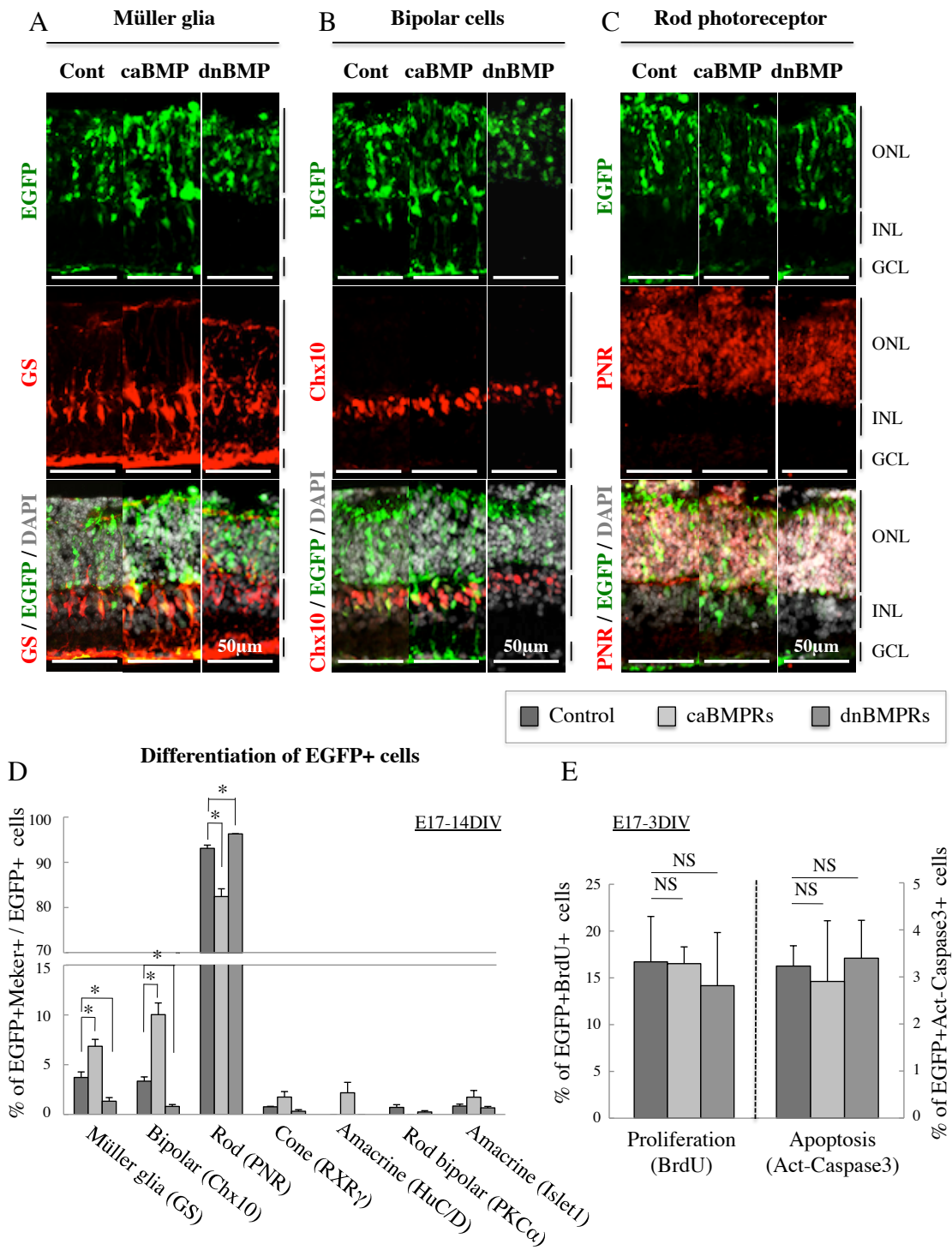
**Fig.9 The effect of caBMPrs and dnBMPrs on the activity of BMP signaling**

Immunostaining with anti-P-Smad1/5/8 antibody was performed in control, caBMPrs and dnBMPrs electroporated retina.



**Fig.10 Layer localization of caBMPRs or dnBMPRs introduced retinal progenitor cells in E17-14DIV retina**

(A) Gene overexpressed cells are labeled by anti-GFP antibody. Arrow head shows a cell existing in INL. (B) Layer localization of EGFP-positive cells was compared among control, caBMPRs and dnBMPRs electroporated retina.



**Fig.11 Differentiation of caBMPs or dnBMPs induced retinal progenitor cells in E17-14DIV retina**

(A-D) Retinal cell differentiation was compared among control, caBMPs and dnBMPs electroporated retina. (E) Retinal cell proliferation and apoptosis were also evaluated by BrdU labeling and Active caspase-3 staining.

of RXR $\gamma$ -positive cone photoreceptors, HuC/D-positive amacrine cells, PKC $\alpha$ -positive rod bipolar cells and Islet1-positive amacrine cells were not changed by the manipulation of BMP signaling (**Fig.11D**). Because the manipulation of BMP signaling affected the proportion of differentiated retinal cell types, I next examined whether this alteration of retinal cell differentiation was attributed to the mitosis or the apoptosis of retinal progenitor cells or not. To examine the effect of BMP signaling on the mitosis and the apoptosis of retinal progenitor cells, I analyzed the mitotic activity and apoptosis by examining BrdU incorporation and expression of Active Caspase-3 (**Fig.11E**). caBMPRs or dnBMPRs were introduced into retina at E17, and the retina was cultured in the presence of BrdU during the last 24 hours, and harvested after 3 days. Proliferation activity and number of apoptosis were comparable among control, caBMPRs and dnBMPRs introduced explant retina (**Fig.11E**).

Taken together, these results suggested that BMP signaling regulates the differentiation of late retinal cell types including bipolar cells, Müller glia and rod photoreceptors without perturbing the proliferation and the apoptosis

### ***Identification of a target gene of BMP signaling***

Previous studies have shown that retinal differentiation is controlled by multiple bHLH genes, which function as intrinsic regulators, and bHLH repressors, such as Hes, Hey and Id genes, which suppress neuronal differentiation (Cepko, 1999; Hatakeyama and Kageyama, 2004). Hairy and enhancer of split (Hes1) and Hairy/enhancer-of-split related with YRPW motif 2 (Hey2) promote generation of

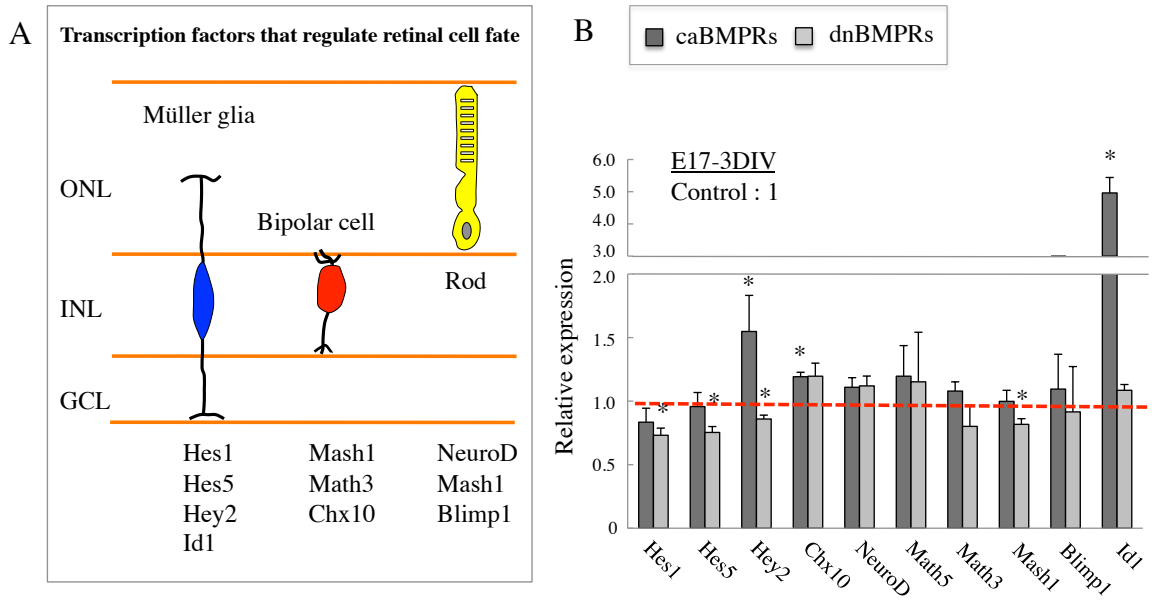
Müller glia in developing mouse retina (Satow et al., 2001a). Id1 also plays a role in differentiation of retinal progenitor cells under BMP signaling (Du et al., 2010). On the other hand, bHLH activators such as Mash1 and Math3 promote neuronal differentiation. Mash1 and Math3 cooperatively regulate neuronal versus glial cell fate determination and regulate the number of bipolar cells and Müller glia in developing mouse retina (Hatakeyama et al., 2001) (**Fig.12A**).

Therefore, I examined the effect of BMP signaling on the expression of various genes involved in the differentiation of late retinal cell types by performing RT-qPCR. caBMPs and dnBMPs were introduced into retina at E17, and cultured for 3 days, and then RNA was purified from the retina. As a result, Hey2 and Id1 expression were strongly upregulated in caBMPs overexpressed retina (**Fig.12B**). The expression of Hey2 was also downregulated in dnBMPs overexpressed retina (**Fig.12B**). But the expression of no other genes was modulated strongly by BMP signaling comparable to Hey2 and Id1.

To examine the expression pattern of Hey2 in developing retina, I performed RT-qPCR at various developmental stages. Consistent with the expression pattern of Id1 that was shown in figure.6C, the expression of Hey2 also showed temporal increase around birth and slight decrease during postnatal stages (**Fig.13A**). In addition to Hey2 expression, the expression of Hes1, which is another bHLH repressor, was also examined. As a result, the expression of Hes1 was not strongly induced around birth compared to Hey2 expression (**Fig.13B**). The expression pattern of Hey2 suggested that Hey2 plays an important role in the differentiation of late retinal cell types that were generated during the postnatal stages.

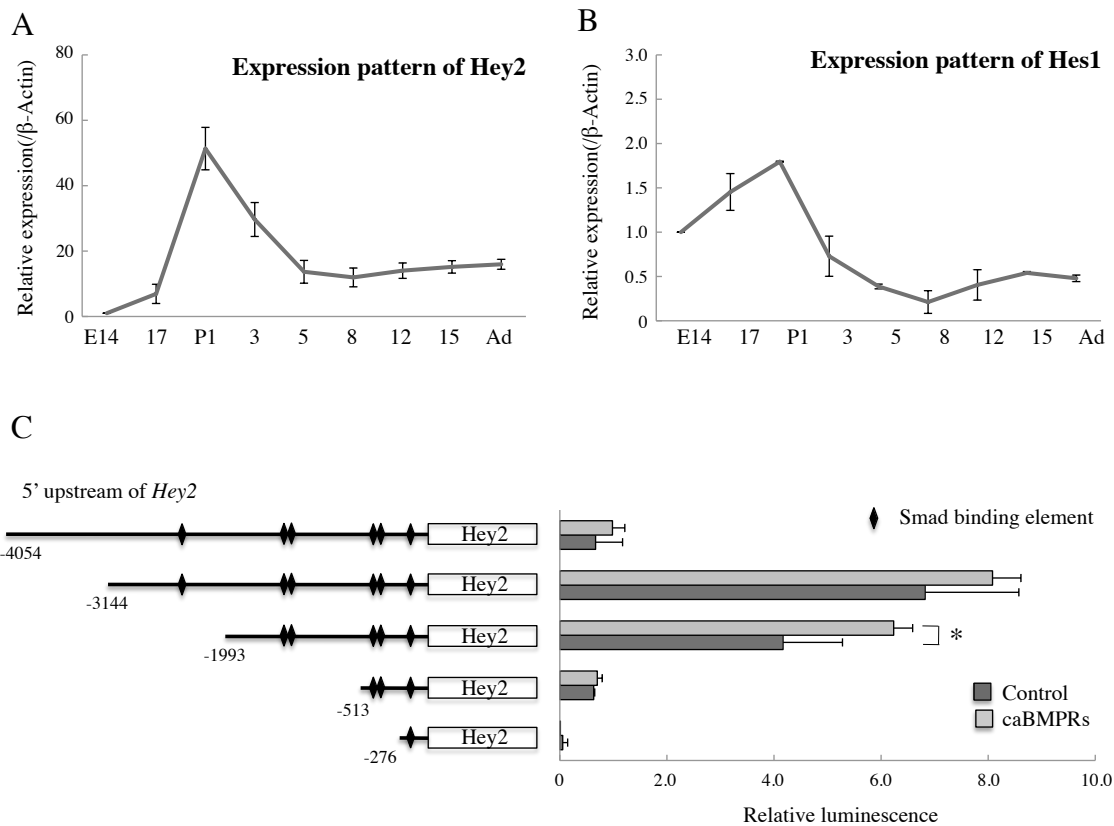
I then tested whether BMP signaling could directly regulate Hey2 promoter





**Fig.12 Variation of expression of various transcriptional factors by the manipulation of BMP signaling**

(A) The relation between the retinal cell differentiation and multiple bHLH transcription factors. (B) The expression levels of bHLH activators and bHLH repressors in caBMPR introduced retina or dnBMPRs introduced retina. The expression levels of genes in control retina were normalized (=1.0).



**Fig.13 The expression pattern of Hey2 and Hes1 and reporter assay of the 5'-upstream region of Hey2**

(A, B) Expression of Hey2 and Hes1 were examined by RT-qPCR of retina at E14, E17, P1, P3, P5, P8, P12, P15 and adult.  $\beta$ -actin was used as a control. (C) Deletion analysis by use of the promoter region of Hey2. Diamond indicates the Smad binding element. The Renilla luciferase gene was used as an internal control.

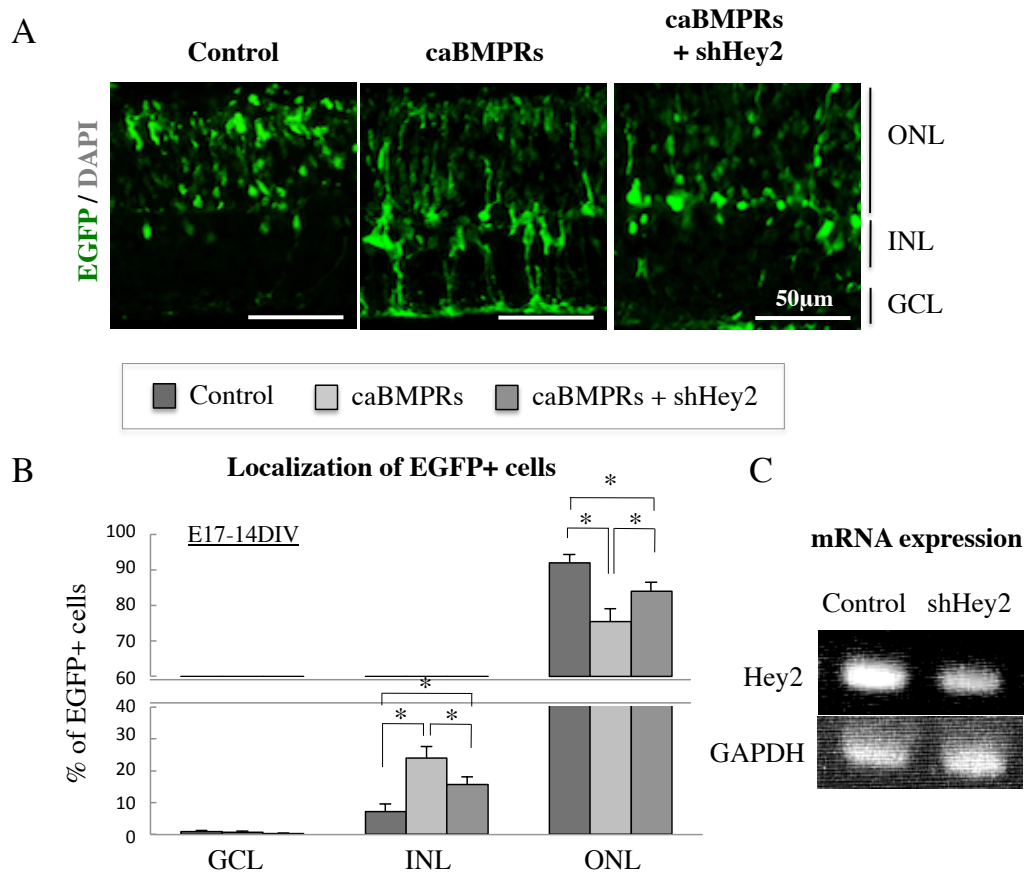
activity or not. Using 5' upstream regions of Hey2 and introducing caBMPRs into HEK293T cells, deletion analysis by use of the promoter region of Hey2 was performed (**Fig.13C**). This experiment revealed that BMP signaling could drive the expression of Hey2 via a region between 0.5kb- and 2.0kb- upstream of Hey2 genes. Furthermore, the region between 3.1kb- and 4.0kb- upstream strongly suppressed the reporter expression.

Taken together, it is suggested that Hey2 is one of target genes of BMP signaling and its expression is partly regulated by BMP signaling via 5'-upstream region of Hey2.

#### ***Rescue of late retinal cell differentiation by suppression of Hey2***

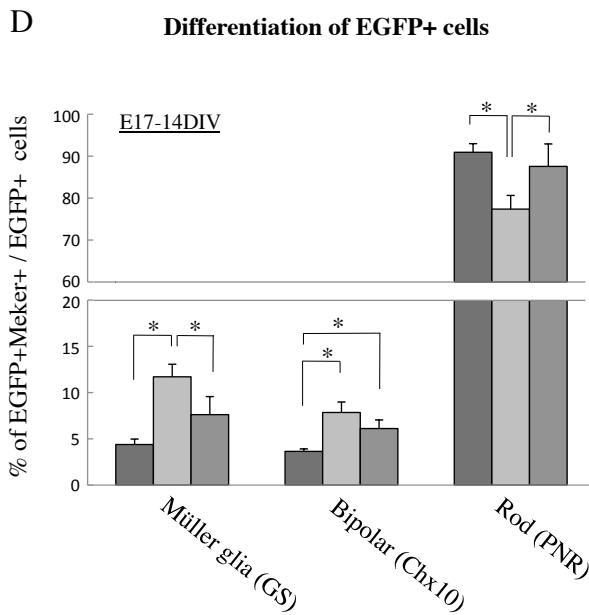
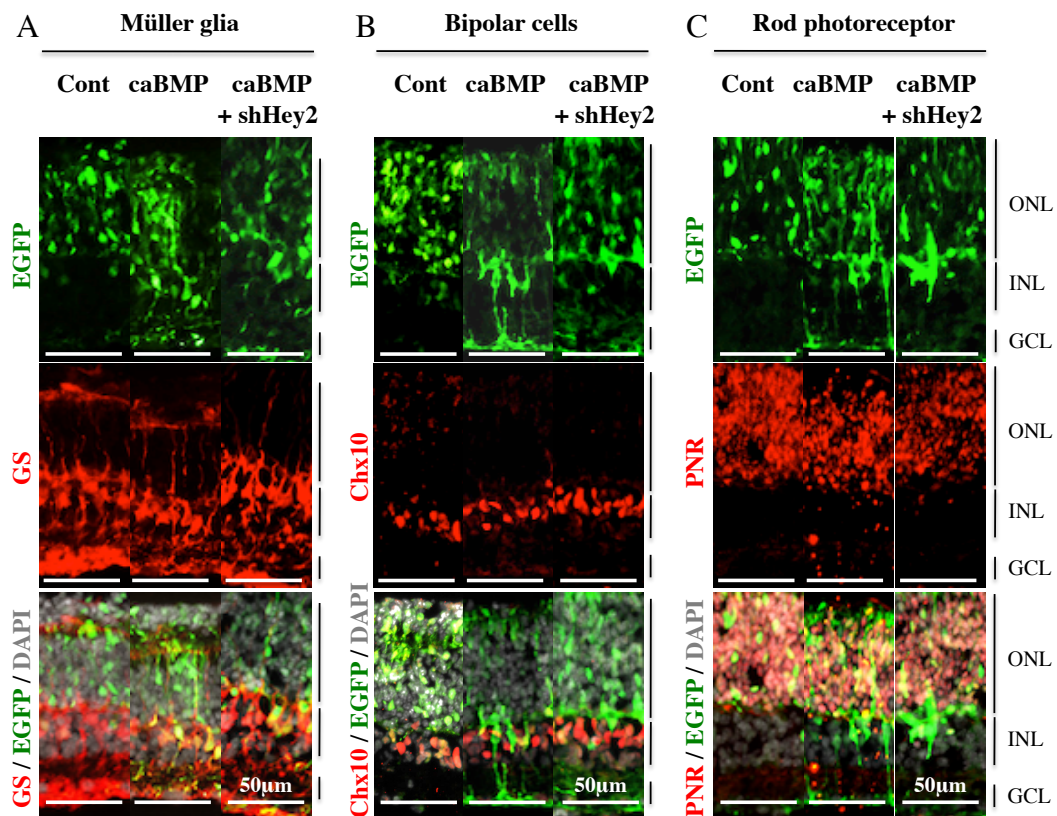
According to above results, I hypothesized that BMP signaling partly regulated the differentiation of late retinal cell types via Hey2. To evaluate this hypothesis, I introduced caBMPRs and pU6-shHey2 which expresses shRNA inducing silencing of Hey2, into explant retina at E17 by electroporation (**Fig.14C**). The explant retina was then cultured for 14 days. Differentiation of retinal cell types was examined by immunostaining using frozen sectioned retina.

I first examined sub-retinal localization of EGFP positive cells, and increased cells in INL and decreased cells in ONL were partially reversed by suppression of Hey2 expression (**Fig.14A, B**). Immunostaining showed that an increased number of GS-positive Müller glia was reversed by introduction of shHey2 (**Fig.15A, D**). Increased number of Chx10-positive bipolar cells was slightly reversed by shHey2 but not statistically significant. Decreased number of PNR was reversed by



**Fig.14 Layer localization of caBMPrs and caBMPrs+shHey2 introduced retinal progenitor cells in E17-14DIV retina**

(A) Gene overexpressed cells are labeled by anti-GFP antibody. (B) Layer localization of the cell was compared among control, caBMPrs and caBMPrs+shHey2 electroporated explant retina. (C) The suppression of Hey2 expression by the introduction of shRNA was examined. To achieve this, shRNA expressing vector was electroporated into the retina at E17. After 3days of culture, RNA was purified from the retina.



**Fig.15 The effect of suppression of Hey2 on the phenotype of caBMPs induced developing retina**

(A-C) Hey2 specific shRNA encoding plasmid was electroporated into mice retina together with caBMPs. The differentiation of Müller glia (A), bipolar cells (B) and rod photoreceptors (C) were examined by immunostaining with cell specific markers. (D) Retinal cell differentiation was examined among control, caBMPs and caBMPs+shHey2 introduced retina.

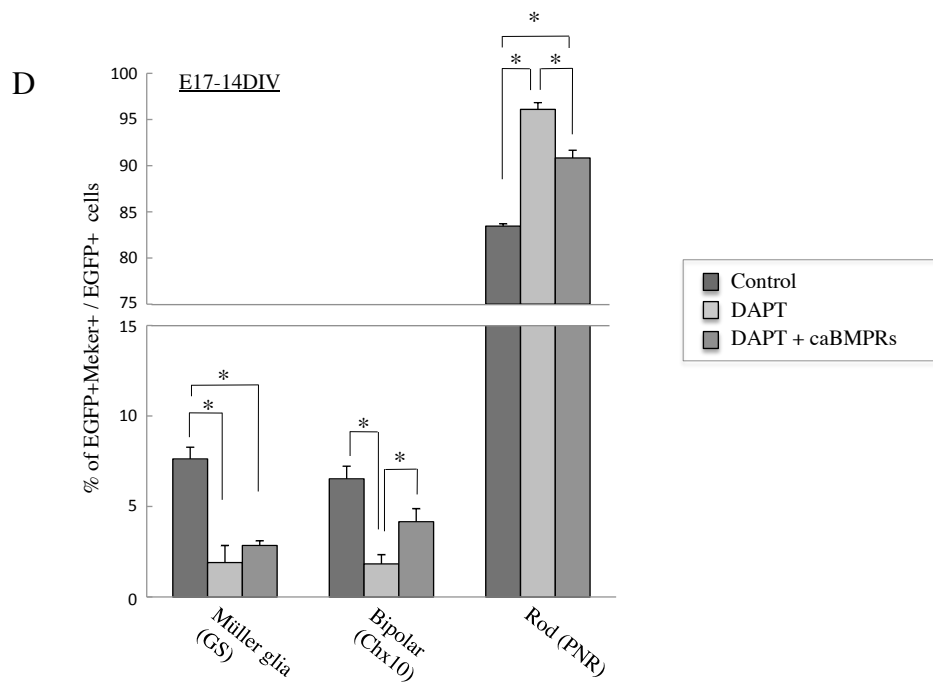
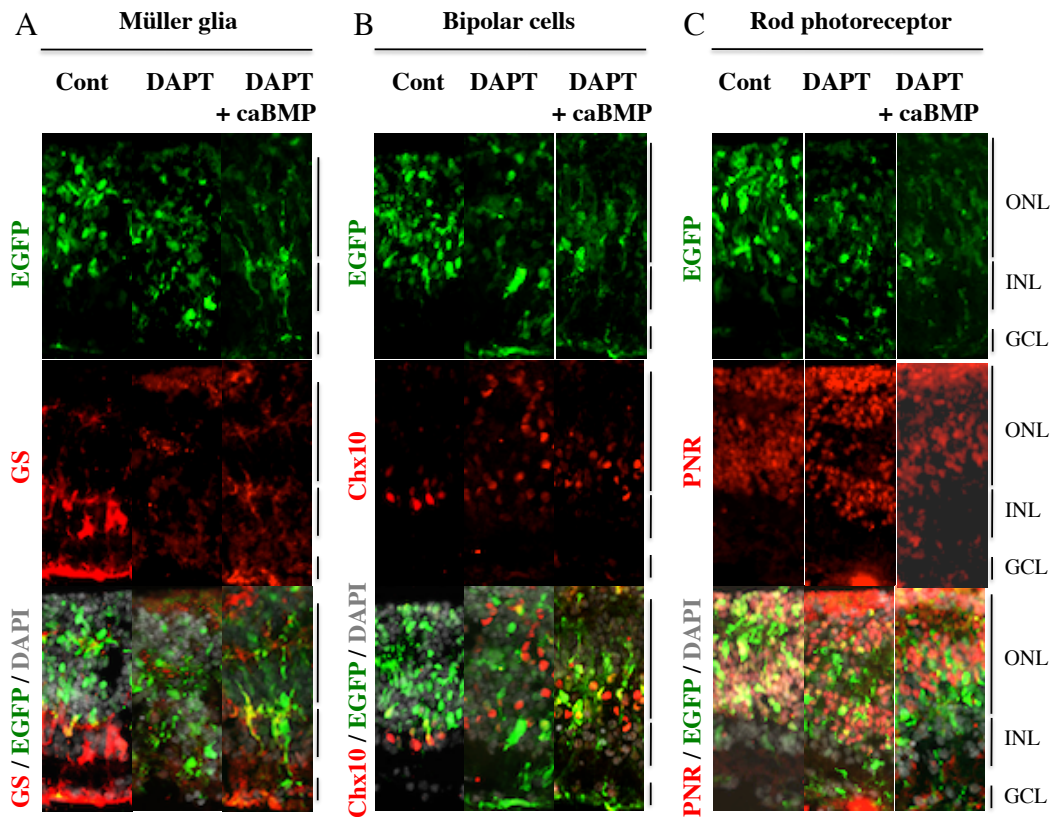
introduction of shHey2 (**Fig. 15B-D**).

This result suggests that Hey2 mediates not all but an important part of BMP signaling to regulate the differentiation of late retinal cell types.

### *Analysis of the crosstalk between BMP signaling and Notch signaling*

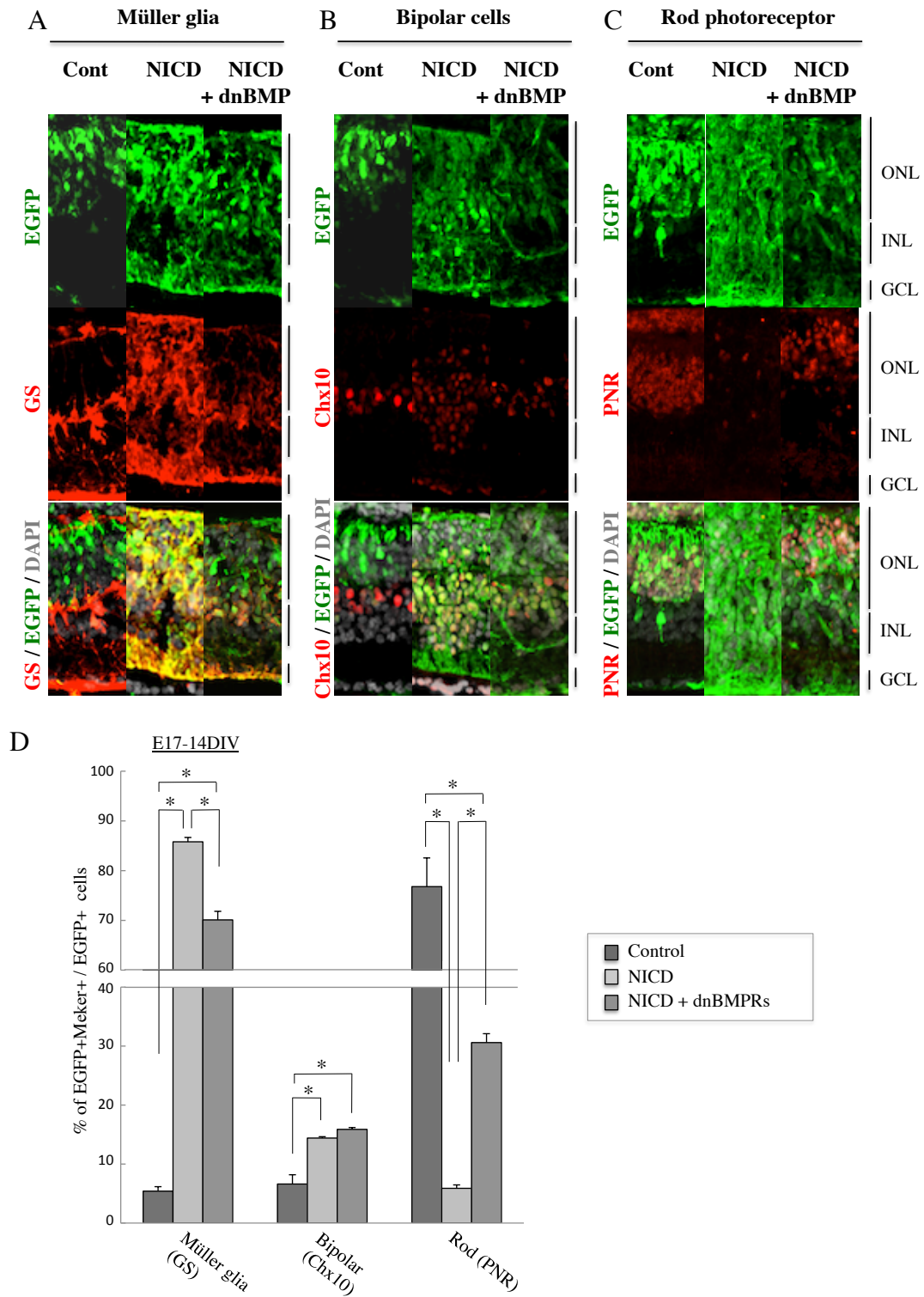
Since Hey2 is well known as a target gene of Notch signaling and is partly involved in the phenotype induced by BMP signaling but not completely, I then explored the possibility of crosstalk between BMP signaling and Notch signaling. I first examined whether active BMP signaling could induce the phenotype shown above in the absence of Notch signaling by treatment of cells with the Notch inhibitor, DAPT. GS-positive Müller glia failed to differentiate, and BMP signaling could not induce Müller glia in the presence of DAPT (**Fig.16A, D**). When I examined Chx10-positive bipolar cells and PNR-positive rod photoreceptors, the differentiation was reversed by BMP signaling but not completely (**Fig.16B-D**). This result suggests that BMP signaling dependently affects to the differentiation of Müller glia and independently affect to the differentiation of bipolar cells and rod photoreceptors from Notch signaling.

To investigate the relation between BMP signaling and Notch signaling more precisely, I next examined whether the suppression of BMP signaling could disturb the phenotype in the activation of Notch signaling by overexpression of Notch intracellular domain, NICD or not. Bipolar cells increased by introduction of pMX-NICD-IRES-EGFP, and suppression of BMP signaling by the introduction of dnBMPRs could not reverse this phenotype (**Fig.17B, D**). When I examined the



**Fig.16 Effect of active BMP signaling on the phenotype of Notch signaling inhibited retina**

(A-C) Effect of treatment of DAPT in combination with caBMPs on mice retinal development was examined after 14 days culture of E17 mice retina. Explant retina was frozen sectioned and immunostained with cell specific markers. (D) Retinal cell differentiation was examined among control, DAPT treated retina and DAPT+caBMPs treated retina.



**Fig.17 Effect of suppression of BMP signaling on the phenotype of Notch signaling activated developing retina**

(A-C) Plasmid encoding NICD was electroporated into mice retina at E17 in combination with plasmids encoding dnBMPs. The retina was cultured for 14 days and was frozen sectioned. Immunostaining was done by use of anti-EGFP antibody and cell specific markers. (D) Differentiation of genes introduced retinal progenitor cells were examined.



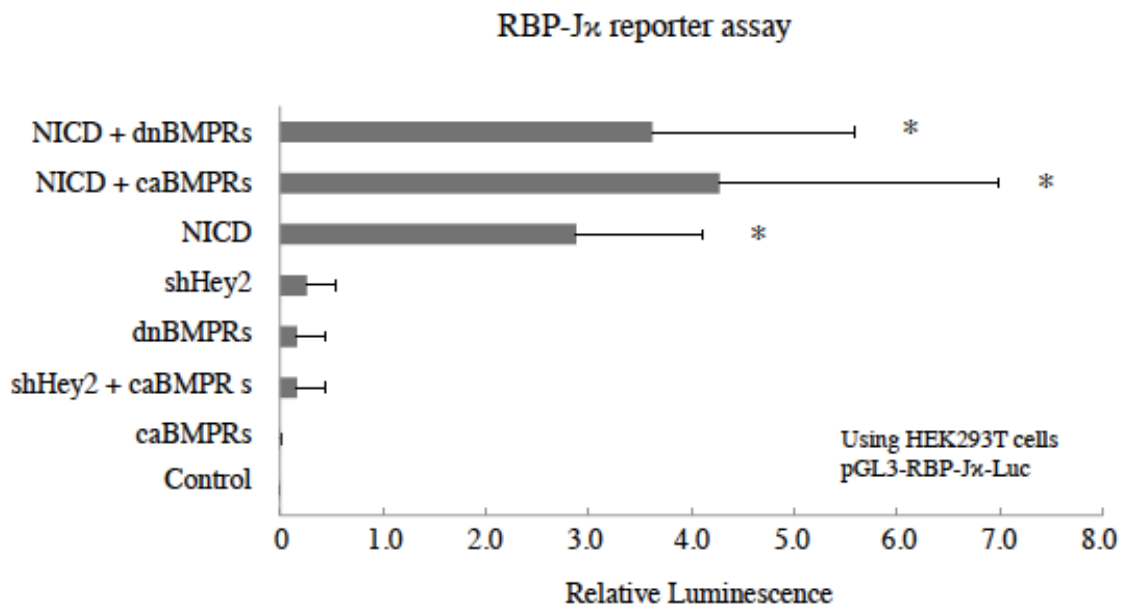
differentiation of Müller glia and rod photoreceptors, the differentiation was partly reversed by suppression of BMP signaling but not completely (**Fig.17A, C, D**).

I then examined the crosstalk between BMP signaling and Notch signaling by using a luciferase reporter plasmid, RBP-J $\kappa$ -luci, which contains the Notch target sequence as an enhancer. RBP-J $\kappa$ -luci was transfected into HEK293T cells together with some of the plasmids encoding caBMPRs, dnBMPRs, shHey2 and NICD. As a result, NICD strongly induced luciferase activity strongly, as expected, and co-transfection of caBMPRs or dnBMPRs did not affect the activity significantly (**Fig.18**). This result suggested that BMP signaling does not directly modulate the Notch signaling pathway.

These results indicate BMP signaling and Notch signaling contribute to the differentiation of late retinal cell types in a redundant manner.

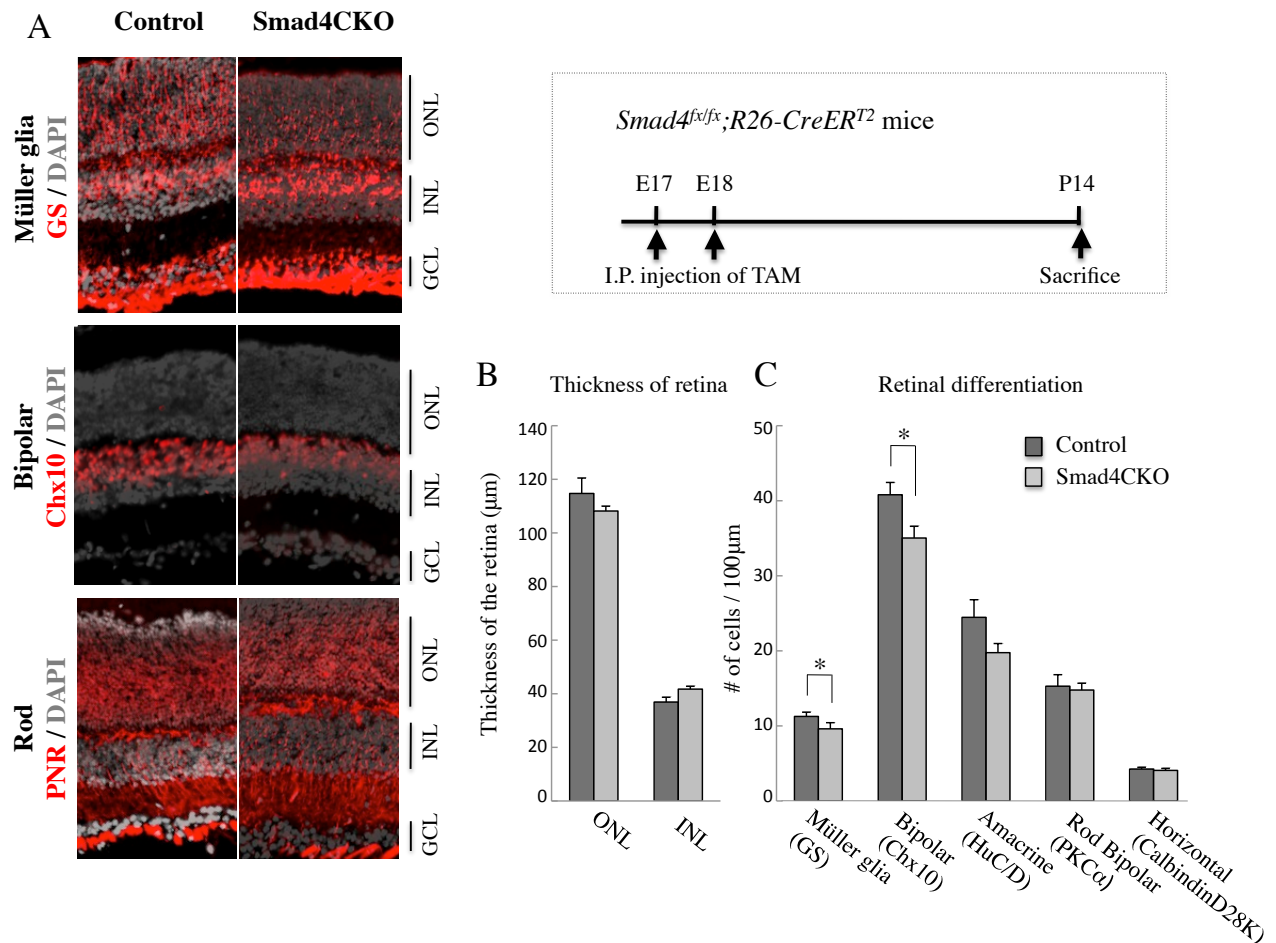
#### ***Analysis of Smad4CKO mouse retina***

To investigate the effect of genetic ablation of BMP signaling on late retinal development, I then generated *Smad4<sup>flx/flx</sup>;ROSA26-CreER<sup>T2</sup>* (Smad4CKO) mice by intercrossing *Smad4<sup>flx/flx</sup>* and *Smad4<sup>flx/+</sup>;ROSA26-CreER<sup>T2</sup>* mice. To suppress activity of BMP signaling at E17 similar to the explant experiment, tamoxifen was injected into pregnant mice both at day E17 and E18 of embryogenesis. Smad4CKO mice were sacrificed at P14. After that I compared retinal development of Smad4CKO mice with that of control mice by immunostaining. In Smad4CKO mice, the thickness of both the ONL and INL in the retina were measured. As a result, both of were comparable between control mice and Smad4CKO mice (**Fig.19A, B**). Because the



**Fig.18 The crosstalk between BMP signaling and Notch signaling using a Notch target sequence**

The crosstalk between BMP signaling and Notch signaling was examined by using a luciferase reporter plasmid, RBP-J $\kappa$ -luciferase. RBP-J $\kappa$ -luciferase was transfected into HEK293T cells together with some of the plasmids encoding caBMPRs, dnBMPRs, shHey2 and NICD. The Renilla luciferase gene was used as an internal control.



**Fig.19 Immunostaining with various cell specific markers in tamoxifen injected *Smad4<sup>fx/fx</sup>;ROSA26-CreER<sup>T2</sup> mice retina***

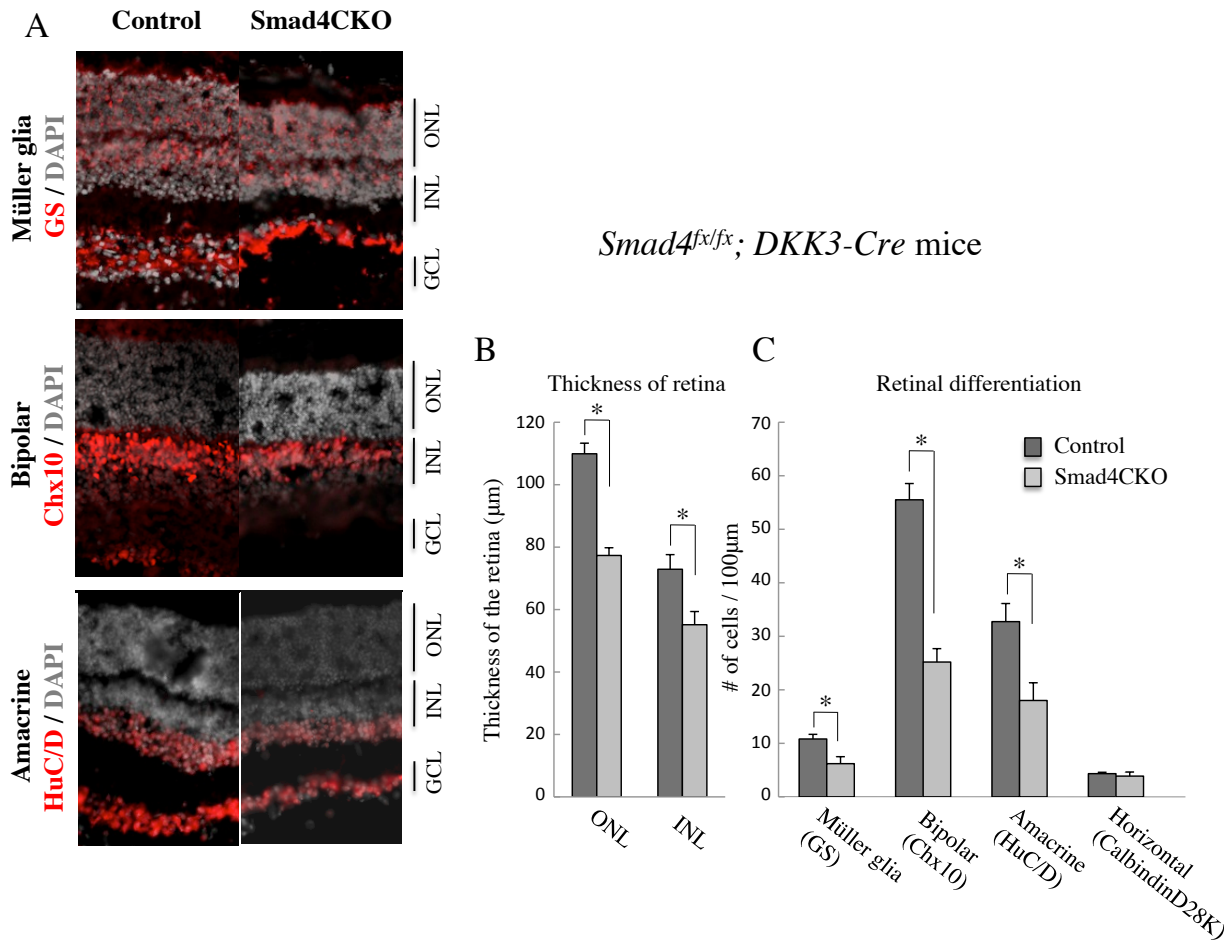
(A, C) Retinal cell differentiation of *Smad4<sup>fx/fx</sup>;ROSA26-CreER<sup>T2</sup> mice retina* was analyzed by immunostaining at P14. Immunostaining with GS, Chx10, HuC/D, PKCα and CalbindinD28K was performed. The number of marker-positive retinal cells was counted (C). (B) ONL and INL thickness were compared between control and Smad4CKO mice.

ONL is mainly comprised of rod photoreceptors, my result suggests that the number of rod photoreceptors is not increased but maintained in *Smad4*CKO mice. I furthermore counted the number of retinal cell types in INL. Chx10-positive bipolar cells and GS-positive Müller glia in *Smad4*CKO mice were decreased compared to those in control mice (**Fig.19A, C**). Other cell types such as amacrine cells, rod-bipolar cells and horizontal cells were not changed.

I then generated *Smad4<sup>flx/flx</sup>;Dkk3-Cre* mice. Previous work showed that the activity of Cre-recombinase was detectable from E10 and genetically restricted to the retina during embryogenesis in *Dkk3-Cre* mice (Sato et al., 2007). Because the ablation of *Smad4* starts at early stage, more severe abnormality was observed in the mice compared to *Smad4<sup>flx/flx</sup>;ROSA26-CreER<sup>T2</sup>* mice. As a result, except for CalbindinD28K-positive horizontal cells, the number of Chx10-positive bipolar, HuC/D-positive amacrine cells and GS-positive Müller glia were reduced compared to control retina (**Fig.20A, C**). Consistent with this result, the thickness of both INL and ONL in the retina was also reduced in *Smad4<sup>flx/flx</sup>;Dkk3-Cre* mice (**Fig.20A, B**).

### ***Analysis of the function of BMP signaling in the Müller glial process extension***

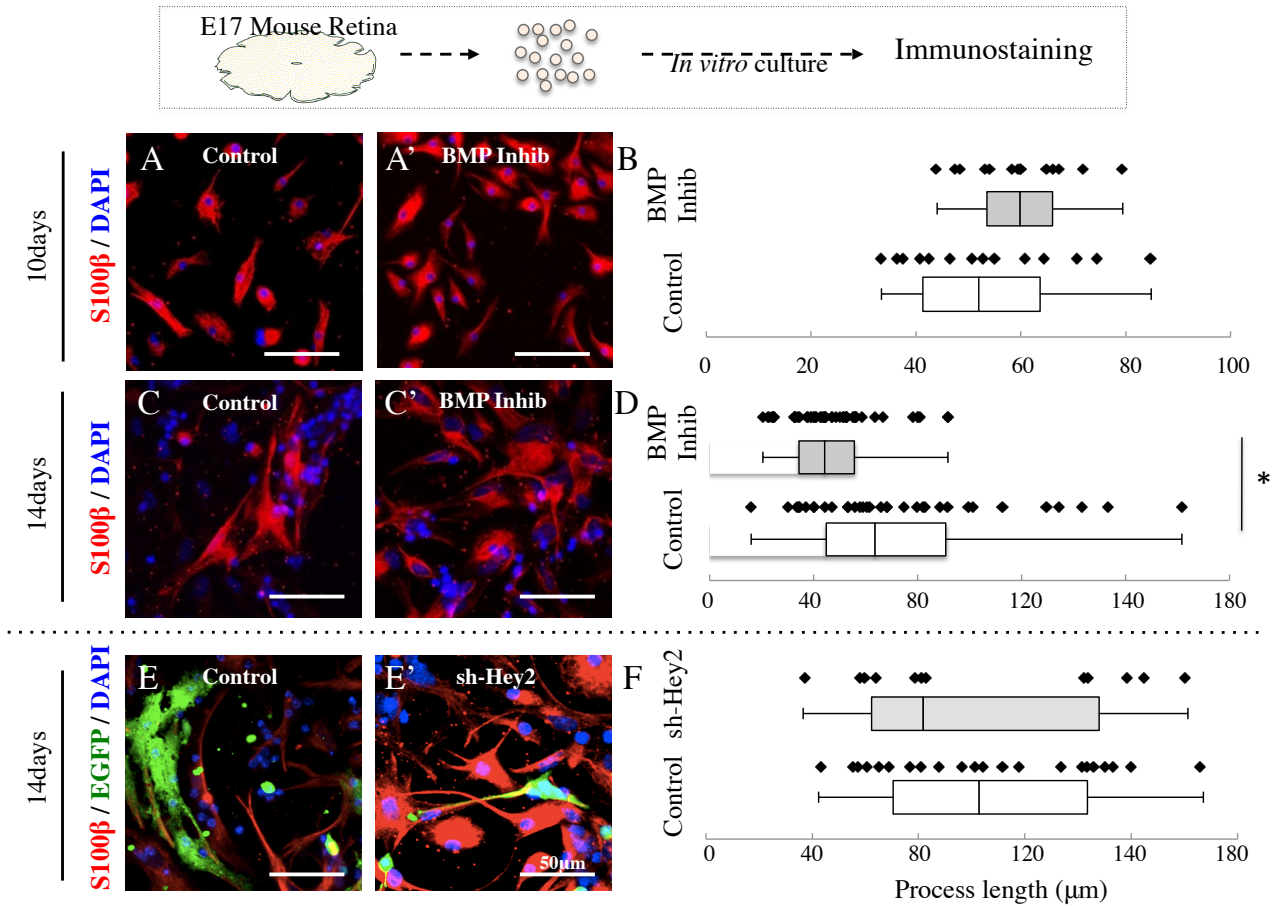
Because caBMPRs introduced Müller glia have long straight processes and dnBMPRs introduced Müller glia have disordered processes as shown in figure.11A, I finally examined whether BMP signaling plays a role in maturation of Müller glia. To achieve this issue, I dissected retina from E17 mouse eye and cultured for 3 days. The retina was then dissociated into single cells, and retinal cells were cultured for 7days or 11 days in the absence of BMP signaling by treatment of cells by an



**Fig.20 Immunostaining with various cell specific markers in *Smad4<sup>flx/flx</sup>;Dkk3-Cre* mice retina**

(A, C) Retinal cell differentiation of *Smad4<sup>flx/flx</sup>;Dkk3-Cre* mice retina was analyzed by immunostaining at P14. Immunostaining with various cell specific markers including GS, Chx10, HuC/D and CalbindinD28K was performed. The number of marker-positive retinal cells was counted (C). (B) ONL and INL thickness were compared between control and Smad4CKO mice.

inhibitor of BMPRs, LDN193189. I then defined retinal cells that are S100 $\beta$ -positive and bipolar in shape as Müller glia and then I measured the process length of the cells. When I compared the process length of Müller glia in control and LDN193189, there was not a significant difference at 10 days after the dissection (**Fig.21A-B**). However, there was a significant difference at 14 days after the dissection (**Fig.21C-D**). From this result, my study suggested that BMP signaling controls the process length of Müller glia. In addition, I examined whether this process extension was regulated via Hey2 or not, by measuring the processes of Müller glia in which shHey2 was introduced. To mark the cells in which the plasmid was successfully introduced, I co-introduced pMX-IRES-EGFP together with the pU6-shHey2 expression vector. As a result, suppression of Hey2 did not affect Müller glial process extension (**Fig.21E-F**). Because of this result, process extension of Müller glia is regulated by BMP signaling, but is independent of Hey2.



**Fig.21 Effect of inhibition of BMPRs on Müller glial process extension**

(A-D) Retina taken from E17 mice were dissociated and cultured in the presence or absence of BMPRs inhibitor, LDN193189. Müller glia was identified by S100 $\beta$  (red) and bipolar shape, their morphology was observed by immunostaining after 10 days (A, A') and 14 days (C, C') of culture. And then, the processes of Müller glia were measured (B, D). (E-F) The effect of shHey2 on Müller glial process extension was also examined. EGFP (green) and S100 $\beta$  (red) -positive bipolar shape cells were defined as Müller glia in which the plasmid introduced (E, E'). Processes of Müller glia were measured (F).

## 6. Discussion

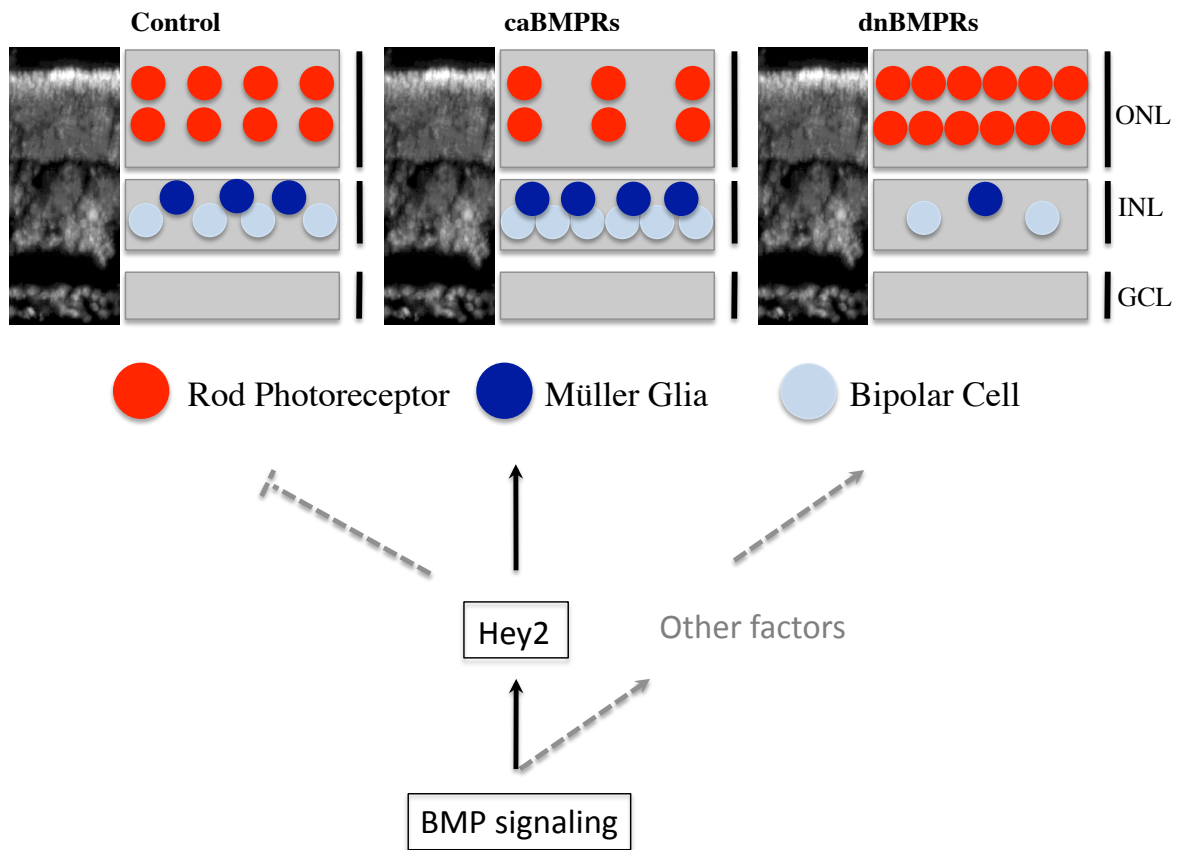
The present study showed that BMP signaling regulates the differentiation of late retinal cell types, such as bipolar cells, Müller glia and rod photoreceptors, and this regulation was partly controlled via bHLH repressor, Hey2 (**Fig.22**). In addition, BMP signaling contributed to Müller glial process extension. Taken together, I unveiled that BMP signaling plays important roles not only in early developing retina but also in late retinal development; the differentiation and the maturation of late retinal cell types.

### *Mechanisms of BMP signaling action in late retinal development*

As shown in figure.7, immunohistochemistry with anti-P-Smad1/5/8 antibody and various cell specific markers revealed that BMP signaling was activated weakly in retinal progenitor cells and strongly in ganglion cells, amacrine cells, horizontal cells, bipolar cells and Müller glia, but not detected in ONL that was comprised of rod and cone photoreceptors. Previous reports have shown the expression of BMP-4 is predominantly expressed in GCL. On the other hand, BMPR-Ia, BMPR-Ib and BMPR-II are predominantly expressed in NBL, GCL and INL (Dewulf et al., 1995; Du et al., 2010; Liu et al., 2003b), suggesting that BMP-4 that is secreted from GCL activating BMP signaling to regulate the differentiation of retinal cells in GCL and INL in an autocrine and/or paracrine manner.

Previous research showed that sonic hedgehog is secreted by differentiated ganglion cells and plays a role to negatively regulate ganglion cell genesis (Zhang et





**Fig.22 Schematic diagram showing the function of BMP signaling in late retinal cell differentiation.**

BMP signaling regulates the differentiation of Müller glia, bipolar cells and rod photoreceptors. The regulation is partly dependent on bHLH transcriptional repressor, Hey2.

al., 2001). This molecular mechanism is important to support the well-ordered retinal cell differentiation. I suggest that BMP signaling also play a role to put the retinal development in order as well as sonic hedgehog signaling.

### *Action mechanisms of BMP signaling for late retinal differentiation*

I found that BMP signaling modulates the fate determination of retinal progenitor cells, especially Müller glial differentiation and maturation. Overexpression of *Drm/Gremlin*, BMP antagonist, in Chick retina does not affect differentiation of neuronal cells but rather alters the laminar organization of the retina in chick (Huillard et al., 2005). These alterations correlate with the absence of differentiating Müller glia. This report suggested that the inhibition of BMP signaling suppressed differentiation and/or maturation of Müller glia during retinal development.

I found that promotion of Müller glial differentiation and suppression of rod photoreceptors differentiation that are induced by active BMP signaling are partly depend on *Hey2*. A previous study showed that overexpression of *Hey2* promoted the generation of Müller glia and suppressed the generation of rod photoreceptors (Satow et al., 2001b). At the same time, the results also suggested that the differentiation of bipolar cells does not depend on *Hey2*, and other mechanisms under BMP should be considered to explain the phenomenon. A recent study revealed that *Id1* and *Id3*, which are known as primal target genes of BMP signaling, also induced the Müller glia and bipolar cell fate (Mizeracka et a., 2013). Because I showed that the induction of the expression of *Id1* occurred in caBMPRs electroporated retina, it is possible that *Id1* also contributes to the differentiation of

Müller glia, bipolar cells and rod photoreceptors.

### ***Direct regulation of enhancer region of Hey2 by BMP signaling***

A luciferase reporter assay using a series of deletion constructs of the Hey2 promoter revealed that BMP signaling induced the high activity of the Hey2 promoter via the 2.0kb- upstream region of Hey2 transcription initiation site. On the other hand, 0.5kb- and 0.27kb upstream genomic region of Hey2 did not induce luciferase activity. This suggested that the 5'-upstream region between 0.5kb and 2.0kb is important for Hey2 expression mediated by BMP signaling. I found a potential Smad4 binding element in Hey2 promoter region by use of TRANSFAC (<http://www.gene-regulation.com/pub/databases.html>). Because Smad4 binding elements exist between 0.5kb and 2.0kb upstream region of Hey2, it is possible that BMP signaling regulates the expression of Hey2 via the binding of Smad complex to these elements. Furthermore, the 4.0kb upstream region of Hey2 showed weak expression of Hey2 compared to 2.0kb- and 3.1kb upstream regions. This result suggested that a suppressor binds to the region from 3.1kb to 4.0kb and suppressed the expression of Hey2.

Hey2 is also a primal target gene of Notch signaling (Iso et al., 2001). A recent study showed that Hey1 and Hey2 were regulated by not only Notch signaling but also BMP signaling (Korchynskiy et al., 2003; Itoh et al., 2004; Morikawa et al., 2011). However induction of Hey1 by BMP signaling is weak, BMP signaling promotes expression of Hey1 synergistically under active Notch signaling. This synergistic regulation depends on the interaction of Smads1/5/8 and RBP-J $\kappa$ /CBF-1

(Itoh et al., 2004). Therefore these results suggested that BMP signaling and Notch signaling share their target genes and regulate the expression in a coordinated manner. I revealed that Id1 was strongly expressed just before birth and during the first postnatal week in retina. In addition, Notch signaling is activated at the same stages in retina. Taken together, I hypothesize that Hey2 regulates the differentiation of late retinal cell types, and the expression of Hey2 in retina at late developmental stage was achieved by both BMP signaling and Notch signaling.

### ***The interaction between BMP signaling and Notch signaling***

By gain- and loss-of-function analyses of BMP and Notch signaling, I found that active BMP signaling could not reverse the differentiation of GS-positive Müller glia in the absence of Notch signaling, but inactivation of BMP signaling could reverse the differentiation in NICD overexpressed retina. The results suggest that Notch signaling is critical for Müller glial differentiation. Chx10-positive bipolar cells showed the recovery of differentiation by the activation of BMP signaling in DAPT treated retina, and no recovery of differentiation by the inactivation of BMP signaling in NICD overexpressed retina. Active BMP signaling can partly induce the differentiation of Chx10-positive bipolar cells instead of Notch signaling. At the same time, Notch signaling induces the differentiation of bipolar cells. In contrast to above two cell types, the differentiation of PNR-positive rod photoreceptors reversed by active BMP signaling in the absence of Notch signaling. In addition, inactivation of BMP signaling also reverses the phenotype of rod photoreceptors in NICD overexpressed retina. Therefore, the differentiation of rod cells is highly susceptible

to BMP signaling compared to Müller glia and bipolar cells.

### ***Smad4 CKO mouse model confirmed roles of BMP in late retinal development***

The retinal development in *BMPR-Ia;BMPR-Ib* double mutant mice and *Smad4<sup>fx/fx</sup>;Six3-Cre* mice were analyzed previously (Liu et al., 2003b; Murali et al., 2011). Mouse embryos that lack the function of both BMPR-Ia and BMPR-Ib in the embryonic retina, exhibit elevated retinal cell death, followed by deficits in cell proliferation. In addition, expression of the proneural gene Math5 is not detected in the *BMPR-Ia;BMPR-Ib* double mutant retina. In *Smad4<sup>fx/fx</sup>;Six3-Cre* mice retina, elevated cell death was observed predominantly in the ventral part. However the expression of Math5 was maintained in the mice. Furthermore, defects in the targeting of RGC axons and the ablation of retinal spatial patterning were detected in both *BMPR-Ia;BMPR-Ib* double mutant mice and *Smad4<sup>fx/fx</sup>;Six3-Cre* mice (Murali et al., 2011; Murali et al., 2005). I first focused on late stage retinal development and revealed that Smad4 played a role in late phase of retinal development by the analysis of *Smad4<sup>fx/fx</sup>;ROSA26-CreER<sup>T2</sup>* mice. In addition, ablation of Smad4 at E17 reduced the number of Müller glia and bipolar cells. On the other hand, the thickness of the ONL was comparable between control mice and Smad4CKO mice. Because the ONL is comprised of a comparatively small number of cone photoreceptors and a large number of rod photoreceptors which comprise about 90% of ONL, results suggests that the genetic deletion of Smad4 at E17 does not effect the number of rod photoreceptors. However the effect of Smad4 deletion on the proliferation and apoptosis is still unclear, these results are similar to the results of the retinal explant

experiment.

Analysis of *Smad4<sup>flx/flx</sup>;Dkk3-Cre* mice revealed that the thickness of both INL and ONL were reduced in *Smad4<sup>flx/flx</sup>;Dkk3-Cre* mice compared to control mice. Furthermore, not calbindinD28K-positive horizontal cells but Chx10-positive bipolar, HuC/D-positive amacrine cells and GS-positive Müller glia were reduced in *Smad4<sup>flx/flx</sup>;Dkk3-Cre* mice. Because severe reduction of retinal cell number was observed in the mice, it is suggested that the ablation of Smad4 from early stage induces defect of the proliferation and/or the apoptosis of retinal progenitor cells.

### ***Regulation of Müller glial process extension by BMP signaling***

Previous studies have shown that polymerization of cytoskeleton such as actin and microtubules played an important role in morphogenesis. It was shown that microtubules were especially important for morphogenesis of radial glia (Li et al., 2003). Radial glia lost their bipolar process and become polygonal shapes by the addition of microtubules inhibitor, NCD or taxol. Furthermore, another study showed that BMP signaling regulated the neurite extension accompanying microtubule stabilization (Podkowa et al., 2010). From these reports, I hypothesized morphology of Müller glia was also regulated by BMP signaling. According to the previous study, BMP-7 induced protrusion formation in N1E115 cells, which are mouse neuroblastoma cells (Podkowa et al., 2010). Furthermore, this regulation is dependent on the non-canonical BMP signaling pathway, which shows that BMP-7 activates c-Jun N-terminal kinases (JNKs) in the tips of dendrites, that BMP signaling regulates microtubule stabilization, and that the binding of JNK to

BMPR-II carboxy-terminal tail is required for BMP-induced microtubule stabilization and dendritogenesis in cortical neurons. In addition to non-canonical BMP signaling that is via JNKs, BMP signaling is comprised of some other Smad-independent pathways, including p38/MAPK and ERK. I revealed that the process extension of Müller glia was regulated by BMP signaling, but was independent of Hey2. This result supports the idea that the process extension of Müller glia is regulated by non-canonical BMP signaling or by other target genes that are related to the formation or stabilization of the cytoskeleton.

## **8. Acknowledgement**

I am most grateful to Dr. Sumiko Watanabe, Dr. Shinya Satoh and Dr. Hideto Koso for supervising my PhD studies. I thank Dr. Yukihiro Baba, Dr. Toshiro Iwagawa and Dr. Rika Saito for helpful discussions and suggestions. I would like to thank all the people in S.W.'s lab for helping my experiments. Finally, I thank my parents for their support throughout my life.



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