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FUNCTION OF BONE MORPHOGENETIC PROTEIN SIGNALING DURING MOUSE DEVELOPMENT

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1. ABSTRACT

Bone morphogenetic proteins (BMPs) play pleiotropic roles during development and after birth in many different organisms. BMPs are members of TGF-beta superfamily. There are more than 20 members with three type II receptors and three type I receptors. Genetic approaches using the mouse as a model system revealed many of the functions of BMPs. Particularly, results obtained through loss-of-function analyses of BMP ligands and their receptors are reviewed in this article.

2. INTRODUCTION

Bone morphogenetic proteins (BMPs) were originally identified by their ability to induce ectopic bone formion. Recent findings reveal that BMPs have many functions during embryogenesis and after birth. In this article, I will discuss the function of BMP signaling during early mouse development. BMPs are processed and secreted growth factor molecules. Their receptors play important roles. I will mention the structure of BMP receptors, and summarize attempts done in recent years to uncover the function of BMP receptors during mouse embryogenesis. I will not discuss germ cell formation, gonadogenesis, lung development, and some other organogenesis processes where BMPs also play critical roles due to space limits. Please see excellent review articles for these issues (1-3, 163).

3. BMP AND THEIR RECEPTORS

3.1. BMPs are the members of TGF-beta superfamily

BMPs were originally identified by their ability to induce ectopic bone formation when implanted into muscle (4). Bone formation occurs through a series of endochondral events initiated by chemotaxis of mesenchymal stem cells into the muscle implantation site (5-6). These cells proliferate and differentiate into chondrocytes, whose matrix is calcified and subsequently replaced by the deposition of bone. Molecular cloning has revealed that BMPs are members of the TGF-beta superfamily (7). Currently, over twenty members of BMPs are identified from different species to form the largest subfamily in the TGF-beta superfamily (1, 3). This subfamily also includes differentiation factors (GDFs), *nodal* and *lefty* in vertebrates, *decapentaplegic*, 60A, and *screw* in *Drosophila*, and *Daf-7* in *Caenorhabditis elegans*.

Recent studies of several organisms suggest that BMPs have other roles during embryogenesis, notably in dorsoventral and/or anterior-posterior axis formation. In *Drosophila melanogaster*, mutations in *decapentaplegic* (*dpp*), which is believed to be a homologue of *Bmp2* and *Bmp4*, cause dorsoventral patterning abnormalities at the blastoderm stage (8). The finding that human BMP4 can rescue the embryonic lethality of *dpp*-null mutants (9) suggests that BMPs may have a comparable role in vertebrate pattern formation. Additionally in *Xenopus laevis*, BMP4 can act as a posterior-ventralizing factor in animal cap explant and blastocoele implant assays (10-11). As discussed below, results from loss-of-function experiments in mouse revealed that members of the BMP subfamily play critical roles during body pattern formation and organogenesis (12-30).

3. 2. Receptors for BMPs

As BMPs are secreted proteins, characterization of their receptors and signal transduction pathways is an

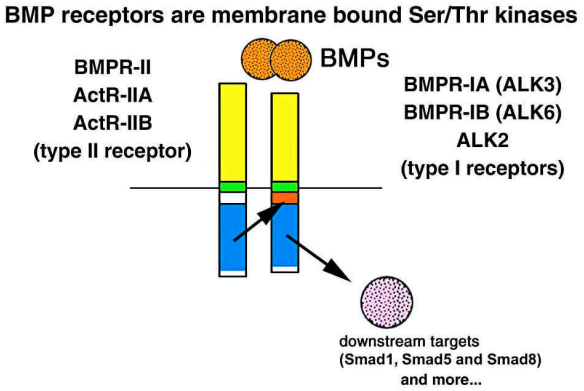

Figure 1. BMP receptors are membrane bound Ser/Thr kinases

important step in understanding the role of these proteins during development. Recently, type I and type II receptors for TGF-beta superfamily ligands have been cloned and shown to have conserved Ser/Thr kinase domains (31-40) (Figure 1). In *C. elegans*, *Daf-4* encodes a type II receptor that can bind both human BMP2 and BMP4 (41). Mutations in *Daf-4* inhibit dauer larva formation (42). In *Drosophila*, *saxophone* (*sax*), *thick veins* (*tkv*) and *punt* were cloned and shown to encode type I (*sax* and *tkv*) or type II (*punt*) TGF-beta superfamily receptors. It is believed that these proteins are functional Dpp receptors because their mutant phenotypes are similar to those of *dpp* mutants (43). Moreover, in mammalian cells, Tkv binds *Dpp* and *Punt* binds BMP2 in the presence of either Tkv or Sax (44-49).

Upon ligand binding, both type I and type II receptors form hetero-multimers, most likely a tetramer consisting of two pairs of type I and type II receptors (50). Type II receptors phosphorylate a short stretch of amino acids called a GS box in type I receptors to activate their kinase activity. Then type I receptors phosphorylate downstream targets such as Smads (2, 51). In vertebrates, seven type I receptors and five type II receptors have been found so far. Type II receptors are named by their ligands, i.e. ActR-IIA (activin type II A receptor), BMPR-II (BMP type II receptor), whereas type I receptors are frequently referred as ALKs, i.e. ALK1, ALK2 (36). ALK stands for Activin receptor-Like Kinase, named after the first cloned receptor for TGF-beta superfamily, that is Activin type II receptor (31, 37). Among ALKs, ALK2 was initially believed to be a type I receptor for TGF-beta (35), but biochemical results suggested that it was a type I receptor for activin (34). However, based on recent careful characterization, ALK2 is now believed to be a type I receptor for BMPs (52-53). Including ALK2, there are now three type I receptors (BMP type IA receptor, BMPR-IA, or ALK3, BMP type IB receptor, BMPR-IB, or ALK6, and ALK2), and three type II receptors (BMPR-II, ActR-IIA and ActR-IIB) for BMPs. The specificity of signaling is primarily determined by type I receptors (54). Interestingly, however, the specificity of ligand binding is altered by the combination of type I and type II receptors (55) (Figure 2).

Besides type I receptors for BMPs, ALK1 and ALK5 are type I receptors for TGF-beta, and ALK4 and ALK7 are the type I receptors for Nodal (55). ALK4 also act as a type I receptor for activin (alternatively known as Activin type IB receptor or ActR-IB).

Downstream targets of the receptor complexes have been extensively studied. A group of genes called Smads are major downstream targets of type I receptors (51, 55-56). Biochemical studies revealed that type I receptors for BMPs phosphorylate Smad1/5/8, whereas type I receptors for activin/TGF-beta phosphorylate Smad2/3 (51, 55-56). These phospholylated Smads form heterooligomers with Smad4 to translocate to the nucleus and alter the gene expression (51, 55).

BMP signaling is also regulated "before receptor." Cystein-rich extracellular proteins such as noggin and chordin bind BMP ligands to prevent their interaction to the receptors (57, 58). Together with the various combinations of receptors, regulation of BMP signaling is very complex. Therefore, a genetic approach, in which the functions of various components of the BMP pathways are altered during development, will be needed to understand the function of these genes in each tissue studied.

3.3. Structure and strategy

Targeted gene disruption of the BMP receptors is one approach to understand their function. Structural features of the BMP receptors also allow us to have two additional approaches. One is to overexpress truncated receptor for dominant negative action and the other is to introduce point mutation in the amino acid of the GS box, thus creating a constitutively active receptor that signals without ligand. Receptors form multimers through Cys residues in their extracellular region. A mutant receptor that has a truncation at the juxtamembrane domain will be able to interact with ligands and other receptors, but cannot transduce signals. Therefore, overexpression of truncated forms of receptors could block specific signals from corresponding wild type receptors (Figure 3). This approach is easier and less time consuming than targeted mutation via embryonic stem cells, but interpretation of the results is more difficult because the dominant negative receptor may block more than one of the BMP signaling pathways and are thus less specific in their affects.

Type I receptor kinases are activated when Ser or Thr residues in their GS box are phosphorylated. However, an amino acid change in the GS box (i.e. Thr to Asp) makes the kinase activity constitutive. These forms of type I receptors can send signals without ligands or type II receptors (59) (Figure 3). Therefore, if these mutant forms of receptors can be expressed in a tissue-specific manner in transgenic mice, it is possible to acquire different types of information regarding *in vivo* function of the signaling in addition to the knockout mice.

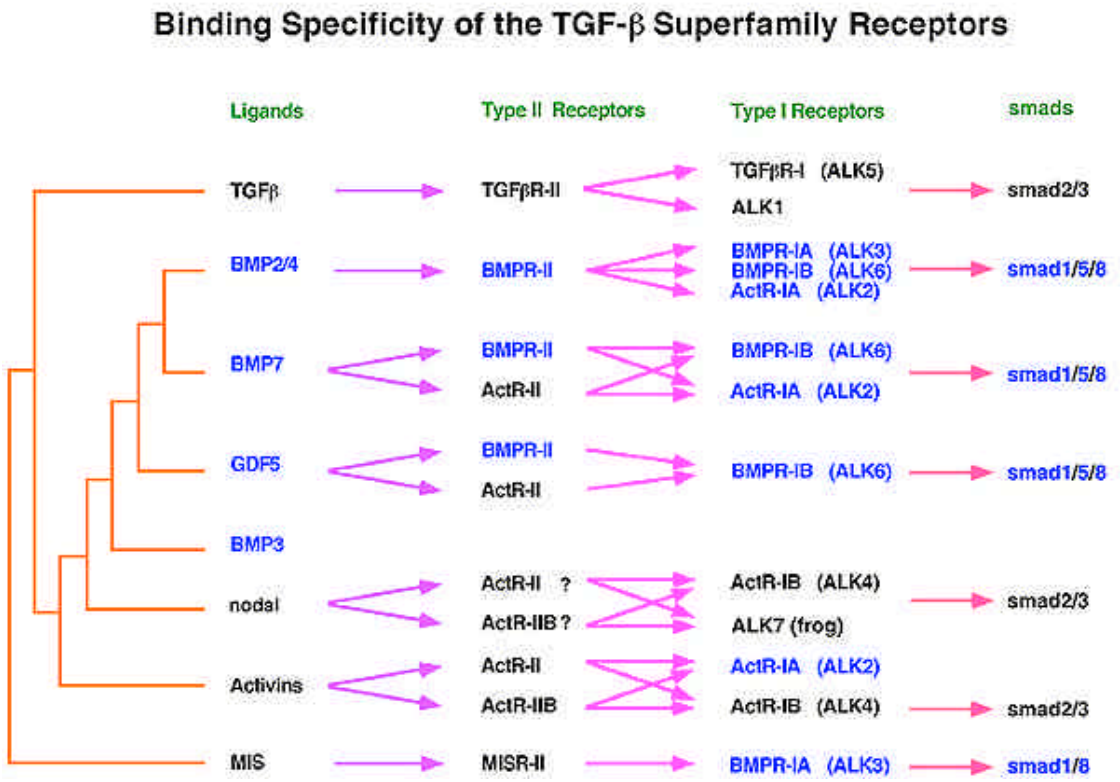

Figure 2. Binding Specifity of the TGF-beta superfamily receptors.

4. FUNCTION OF BMP DURING EARLY EMBRYOGENESIS

4.1. Gastrulation defects and extraembryonic tissues

Mouse embryos have symmetrical structure when they start to develop. They establish the first axis of polarity, proximal-distal polarity, prior to implantation. At the blastocyst stage (E 3.5), the inner cell mass (ICM) is formed on one side of round embryos. The side opposite of the ICM is the side of the mouse embryo that implants into the uterine wall. The proximal side of the mouse embryo, the side of the mouse embryo where ICM locates, will be the future dorsal side whereas the opposite side, called the distal side, will be the future ventral side. Soon after implantation, cells in the embryo proliferate and form a three-dimensional body plan, consisting of an anterior-posterior polarity and left-right polarity, in addition to the dorsal-ventral polarity (60). Once polarities are determined, each part of the embryo differentiates into various tissues and organs to establish the identities for the different parts of the body. This process is called establishment of body plan. The molecular mechanisms involved in these processes are largely unknown. Several BMP ligands, receptors, and signaling molecules are expressed during early stage of embryonic development. Because of this, many of the null mutant mice of these genes generated via conventional gene targeting methods results in early embryonic lethality (see below).

Bmpr-1a (alternatively known as *Alk3* or *Bmpr*) is expressed ubiquitously in epiblast and the extraembryonic region at gastrulation (61). Disruption of *Bmpr-1a* causes early embryonic lethality without mesoderm formation (61). Since homozygous ES cells cannot be established from blastocysts of heterozygous intercrosses (Mishina, unpublished observation), BMP signaling through BMPR-IA may be essential for the growth of ES cells. This is consistent with the fact that *Bmpr-1a* mutant embryos show growth defects prior to gastrulation (61).

Bmp4 ligand starts to be expressed as early as E3.5 in ICM (62). At the stage of gastrulation, *Bmp4* is highly expressed in the primitive streak and in the extraembryonic tissues (17, 63-65). Homozygous mutant embryos for *Bmp4* show various phenotypes most likely due to variation in the genetic background, but none of them can survive beyond E9.5 (17, 63, 66). Most severe ones showed gastrulation defect similar with *Bmpr-1a* mutant embryos (17). Chimeric analysis using tetraploid embryos revealed that *Bmp4* expression in the extraembryonic region (trophoblast and primitive endoderm) can largely rescue the gastrulation defects shown in *Bmp4* null embryos, but *Bmp4* expression in the extraembryonic mesoderm is essential for the formation of primordial germ cells and development of allantois (67).

Bmp2 starts to be expressed at E6.0 in the extraembryonic region (65). Later BMP2 is expressed in the extraembryonic mesodermal cells lining the chorion and amnion after gastrulation (18). Unlike *Bmpr-1a* mutant embryos, *Bmp2* mutant embryos can gastrulate, and they

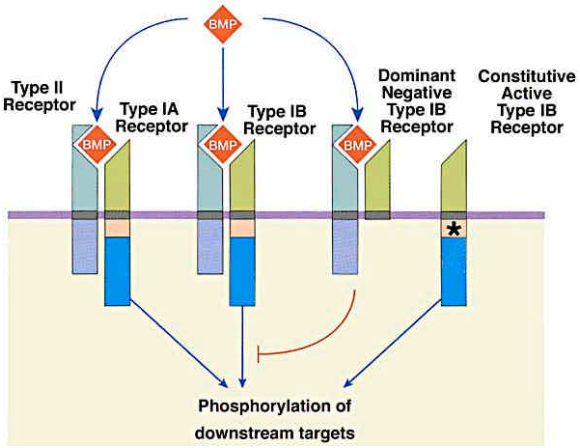

Figure 3. Constitutece Active Type I receptors send signals without ligands or type II receptors. Dominant Negative Type I receptors block BMP signaling.

show abnormal development in the extraembryonic region such as failure of the fusion of preammniotic canal and die by E8.5 (18). Even though BMP2 and BMP4 share more than 90% identity in their amino acid sequence, the phenotype of *Bmp2* homozygous mutant embryos are different from that of *Bmp4* mutant embryos, most likely due to the difference of their regulation and tissue specific expression patterns.

BMPR-IA binds BMP4 and BMP2, but as described above, phenotypes of *Bmp2* and *Bmp4* mutant embryos are similar to, but less severe than that of *Bmpr-1a* mutant embryos. One possible explanation for these differences is that in the absence of one ligand the other ligand can provide compensatory functions. For example the *Bmp5* and *Bmp7* double homozygous mutant embryos die around E10.5 due to the failure of chorioallantoic fusion, whereas individual null mutants do not show overt phenotype in mesoderm formation (12, 15-16, 68). Generation of *Bmp2* and *Bmp4* double homozygous mutants could provide some answers. However, compound heterozygous mutants of *Bmp2* and *Bmp4* show reduced fertility (65, Mishina, unpublished observation). This makes generation of double homozygous mutant embryos difficult.

Recently, a conditional allele of *Bmpr-1a* was generated (69-70). Exon 2 of *Bmpr-1a* was floxed and deletion of this exon is enough to disrupt gene function. The Mox2-Cre (MORE) mouse line is a Cre transgenic line that expresses Cre recombinase in an epiblast-specific manner (71). Breeding of the floxed allele of *Bmpr-1a* with MORE should generate mosaic embryos of wild type extraembryonic region and mutant embryonic region of *Bmpr-1a*. Interestingly, the mutant embryos do not show overt abnormality up to E7.5 and gastrulate normally (72). This suggests that BMP signaling through BMPR-IA plays a critical role in the extraembryonic region before (and during) gastrulation.

Alk2 is expressed only in visceral endoderm at the stage of gastrulation (73-74). Then *Alk2* is expressed in embryonic portions of the head mesenchyme and heart primodia (74). Targeted disruption of *Alk2* causes embryonic lethality at the stage of gastrulation, but its phenotype is less severe than that of *Bmpr-1a* (74-75). Gastrulation is initiated and mesodermal wings are formed, but their development is arrested at mid/late streak stage (74-75). Interestingly, even the mutant *Alk2* embryos develop thickened primitive streak. Expression of Brachyury, widely used as a molecular marker of mesoderm induction, is very low compare to wild type embryos of the same age (75). Unlike *Bmpr-1a*, null ES cells for *Alk2* can be established from homozygous mutant embryos and chimeric studies were done. Gastrulation phenotype is rescued by wild type extraembryonic tissues, indicating that BMP signaling through ALK2 plays critical roles in the extraembryonic region prior to, and during gastrulation as BMPR-IA does (74-75). Recently, a conditional allele of *Alk2* in which exon 7 was floxed has been established (76). Tissue-specific disruption of *Alk2* should reveal various functions of BMP signaling through the ALK2 receptor at later stage of development.

Bmpr-2 encodes the type II BMP receptor that forms heteromers with BMPR-IA, BMPR-IB, or ALK2 (55). Disruption of *Bmpr-2* causes severe embryonic lethality that mimics the abnormalities of *Bmpr-1a* mutant embryos (77). Mutant *Bmpr-2* embryos fail to gastrulate and no signs of mesoderm formation are observed (77). Interestingly, the mutant embryos still express visceral endoderm markers such as *HesX1*, but no spatial expression pattern was examined (77). These observations suggest that BMPR-II and BMR-IA form heteromultimers to transduce BMP signaling at the stage of gastrulation, presumably in the extraembryonic region.

The above hypothesis is well supported by the phenotype of the mutant embryos of *Smad1*, one of the major downstream targets of BMP receptor complexes. *Smad1* is first highly expressed in the visceral endoderm at E6.5. Then, *Smad1* is expressed in both embryonic and extraembryonic tissues after gastrulation (78). Mutant embryos for *Smad1* show ruffles in the visceral endoderm adjacent to the primitive streak (78-79). Embryos die by E10.5 due to the failure of chorioallantoic fusion and these abnormalities cannot be rescued by wild type epiblast (78-79).

Nodal is a distant member of the BMP subfamily and utilizes different signaling pathways, which consists of *Alk*4 or *Alk*7 in combination with ActRIIA or ActRIIB, and downstream targets, Smad2 and Smad3 (55, 80). Nodal was originally disrupted by retroviral insertion in ES cells, and the disrupted gene was then identified as a member of TGF-beta superfamily (13). Mutant embryos die without overt gastrulation. However unlike Bmpr-1a mutant embryos, the embryos form randomly positioned patches that express Brachyury (81). Recently, it was shown that the amount of nodal expression plays a critical role during pattern formation. Mutant embryos that still express limited amount of nodal can undergo gastrulation, but show anterior-posterior patterning defects (82). Moderate amount of nodal leads to defects in left-right asymmetry (82).

Several lines of evidences suggest that ALK4 is a type I receptor for nodal during mouse development (80, 83-84). Indeed, phenotypes of *Alk4* mutant embryos resemble that of nodal null embryos (85). ActR-IIA and ActR-IIB are believed to be type II receptors for Nodal because double homozygous mutant embryos for both type II receptors die during gastrulation without mesoderm formation (86-87).

Smad2 is believed to be a major downstream target of ALK4 signaling (51, 55-56 88). Mutant embryos for *Smad2* cause patterning abnormalities during gastrulation, but unlikely *Bmpr-1a* or *Bmpr-2* mutants, *Smad2* null embryos can form extraembryonic mesoderm (88-89).

4.1.1. Cavitation soon after implantation

Soon after implantation (E5.0), the ICM starts to form a cavity inside to establish a cup shape structure. This process is known as cavitation and the resulted cavity is called as the proamniotic cavity. BMP signaling is believed to play a role during cavitation as a death signal (62). Both embryonic carcinoma (EC) cells and embryonic stem (ES) cells form embryoid bodies when their differentiation is promoted using suspension culture (90). Embryoid bodies are valid models to study early morphological events of mouse development, because they spontaneously differentiate in culture to mimic some of the aspects of early embryogenesis, i.e. differentiation of primitive endoderm (visceral and parietal endoderm) and cavitation.

Interestingly, expression of Bmp4 in embryoid bodies coincides with differentiation of visceral endoderm and subsequent cavitation. However, a mutant EC cell line that develops only parietal endoderm and fails to cavitate does not express Bmp4 (62). Addition of BMP2, BMP4, or BMP7 in culture media induces the cavitation of the mutant EC cells and restores the expression of marker genes for visceral endoderm (62). Moreover, overexpression of a dominant negative form of BMPR-IB prevents cavitation of a wild type EC cell line (62). Taken together with *in vivo* expression pattern of BMPs, these cavitation experiments with wild type and mutant EC cells suggest that BMP4 produced in the embryonic ectoderm stimulates the differentiation of visceral endoderm. In combination with BMP2 produced by the visceral endoderm and other unknown visceral endoderm-derived factors, BMP4 causes apoptotic cell death to form the proamniotic cavity (62).

The above hypothesis is consistent with the observation that both Bmpr-1a and Alk2 homozygous mutant embryos show defects in visceral endoderm (61, 74-75). However, none of the mutant embryos for Bmp2, Bmp4, Bmpr-1a, Bmpr-1b, Alk2, or Bmpr-2 show overt abnormalities in the process of cavitation (17, 18, 61, 74-75, 77, 91-92). One of the possibilities to explain this inconsistency is that BMP ligands expressed in maternal tissues compensate for the deficiency of ligand production in the visceral endoderm. Another possibility, though not mutually exclusive, is that receptors for BMPs are expressed in very low levels at the stage of cavitation and disruption of one receptor can be rescued by others. Generation of double (or triple) homozygous mutant embryos for BMP receptors should reveal this possibility.

4.1.2. Left-right asymmetry

Establishment of the asymmetry of the left-right (L-R) axis is important for normal vertebrate body plan, especially for the asymmetric positioning and morphogenesis of internal organs (93-94). Evidence is accumulating that BMP signaling is a " right determinant" in the chick and *Xenopus* systems (93). In *Xenopus*, ALK2 is shown to be the receptor responsible for transducing the BMP signal for determining right-hand identity (95). However, in mammals little is known about BMP function in the development of L-R asymmetry, particularly the identity of the receptor(s) that are involved in L-R asymmetry (96).

Nodal is the first member among TGF-beta superfamily shown to play a critical role in establishment of L-R asymmetry. Nodal is expressed in both sides of the node at early somite stages. The expression domain for nodal in the left side is larger than that in right side. Later nodal is expressed only in left lateral plate mesoderm (13, 81, 97-98). Compound mutants with nodal receptors and its downstream target, Smad2, also show right isomerism, thus demonstrating that Nodal is a left determinant (88). Homozygous mutant embryos for ActR-IIB show heterotaxia (99), and compound mutants of *Actr-2a* and *Actr-2b* show various degrees of L-R defects (86-87). These combined results suggest that Nodal binds to ActR-IIA or ActR-IIB, and ALK4 to signal to Smad2 during establishment of L-R asymmetry.

Distal members of the BMP subfamily, Lefty1 and Lefty2, are also right determinant, presumably interacting with nodal signaling (see below). *lefty1* is expressed in the notochord and presumptive floor plate (midline) and believed to form a "midline barrier" to prevent overflow of left-side signals to the right side (26). The midline barrier is still conceptual, but it may explain the phenotype of *lefty1* mutant embryos well, since lefty1 null embryos show bilateral expression of *nodal*, that is on both sides of lateral plate mesoderm (26). Like *nodal*, but unlike *lefty1*, *lefty2* is expressed only in the left lateral plate mesoderm with slightly larger expression domain than *nodal* (26). Biochemical and genetic studies suggest that Lefty proteins compete with the same receptor complex as nodal. Lefty1 and lefty2 inhibit nodal signaling (100). Indeed, wider expression of *nodal* is observed in the *lefty2* mutant embryos (28, 162). Both Lefty1 and Lefty2 are required for fine-tuning of Nodal activity to establish proper L-R asymmetry.

Gdf1, another distal member of BMP subfamily, is expressed in the epiblast, node and lateral plate mesoderm in a symmetrical manner (29). Homozygous mutant embryos for *Gdf1* show random situs and right isomerism of the lung due to the absence of expression of *nodal*, *lefty1* and *lefty2* (29). These results suggest that GDF1 is an upstream factor of Nodal in L-R asymmetry at the LPM. Nodal expression in the node remains normal in the *Gdf1* mutant embryos (29). Receptors and signaling molecules for Gdf1 are unknown.

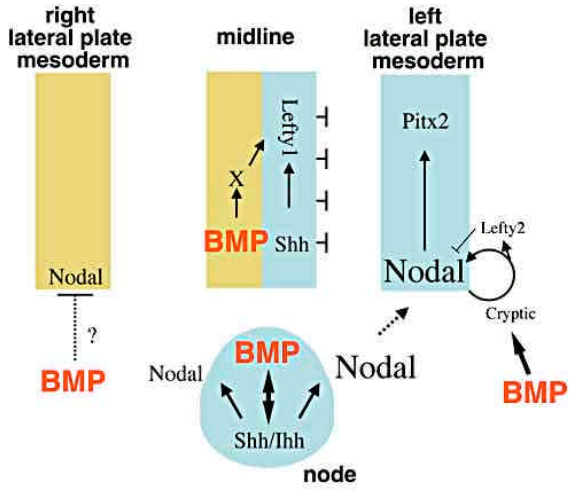

Figure 4. Function of BMP signaling in L-R asymmetry.

In early stage chick embryos, BMP treatment suppresses the expression of nodal (101-102). Based on these findings, BMPs are believed to act as right-hand determinants in L-R asymmetry. As mentioned above, expression of *caAlk2* in frog embryos leads to left isomerism and support this idea (95). Recently, a loss of function study for *Smad5* showed that Smad5 is essential for the establishment of the right-hand identity (103). The *Smad5* mutant embryos show bilateral expression of *nodal* and *lefty2* in the lateral palate mesoderm, but no expression of *lefty1* is observed in the midline (103). BMP(s)/ALK2/Smad(s) pathway are most likely component in the establishment and/or the maintenance of the L-R asymmetry in mammals as well.

Function of BMP signaling in L-R asymmetry may be more complex than expected (Figure 4). It is known that the administration of BMPs suppresses nodal expression and leads to left isomerism. But recent careful experiments have demonstrated that administration of BMPs in lateral plate mesoderm of chick embryos at slightly later stage induces nodal expression (104-105). BMPs induce cripto that is a cofactor for nodal signaling. Cripto also enhance a positive feedback loop of nodal expression (104-105). These controversial results can be explained as follows. Recently, it is reported that the interaction between BMP and sonic hedgehog (SHH) in Hensen's node plays a key role in L-R patterning in the chick (106). Overexpression of BMP signaling in Hensen's node may lead to suppression of SHH signaling. Decreases SHH signaling at this stage leads to down regulation of nodal. Whereas overexpression of BMP in the lateral plate mesoderm leads to direct up-regulation of nodal. In the mouse, hedgehog signaling (both SHH and Indian hedgehog) has been shown to act as a left determinant in the node, although HH signaling itself seems to be symmetric (107). Therefore, BMP and HH signaling most likely interact with each other to establish the L-R asymmetry in the mouse node. Since ALK2 is expressed in midline, but not in the lateral plate mesoderm (108), ALK2 may be a BMP receptor at the node and help establish the L-R asymmetry. Instead, BMPR-IA may be a BMP receptor at the lateral plate mesoderm and functions in maintaining L-R asymmetry.

4.2. BMP function during organogenesis

4.2.1. Heart formation

During mouse development, the heart is the first organ to develop. At the presomite stage, left and right mesodermal layers expand and meet at the anterior part of the embryos (109). At the 1-2 somite stage, endocaridal elements form a pair of tubes, and then, the bilateral heart tubes fuse at the midline after 4 somite stage. Subsequently, extensive cardiac morphogenesis, including heart looping and septa formation, is carried out. BMPs have been shown to play important roles in cardiac development in *Drosophila*, fish, frog and chick (109). Particularly, chick explant studies demonstrate that BMPs are essential signals from myocardium to endocardial cushion formation that eventually gives rise to the atrio-ventricular (AV) valves (110).

In *Bmp2* mutant embryos, heart development is delayed and also occurs in presumptive exocoelomic cavity (18). These results indicate that BMP2 is important for proper cell movement of cardiac precursors.

An epiblast-specific mutant for *Bmpr-1a* was generated using Mox2-Cre (MORE) transgenic mice. These epiblast specific *Bmpr-1a* mutant embryos gastrulate almost normally, but no signs of heart development were found in histological sections (72). Early heart mesoderm markers, like NKX2.5, GATA4 or dHAND, are expressed at E7.5 in presumptive heart region. However, at E8.5, those markers are no longer expressed due to failure to form the heart. These results suggest that BMP signaling is essential when heart mesoderm differentiates to form heart tissue.

During AV valve formation from endocardial cushion, it is known that TGF-beta2 and TGF-beta3 play important roles (111-113). To understand the involvement of BMPs in this process, myocyte-specific disruption of *Bmpr-1a* was carried out using a MHC-Cre mouse (114-115). Mutant embryos die by E15.5 showing defects in the interventricular septum, trabeculae, and AV cushion (115). Increased level of TUNEL positive cells suggests that BMP-IA signaling is essential for survival of cardiac myocytes (115). Interestingly, expression of TGF-beta2 is severely reduced in the mutant heart (115). These results suggest that BMP signaling in cardiac myocytes is essential for their survival and expression of TGF-beta2. TGF-beta2 acts as a paracrine factor from caridac myocytes to endocaridum for development of AV cushion. ALK2 also plays roles in AV cushion development in chick (116). BMP signaling through ALK2 cannot overcome the disruption of BMPR-IA signaling in this process.

4.2.2. Neural induction and differentiation of neural tissues

In vertebrate embryos, neuroectoderm differentiates from ectoderm soon after gastrulation. This process is called neural induction. The neural ectoderm undergoes rapid proliferation and forms a neural tube.

During mid-gestation, the neural tube establishes dorsal-ventral and anterior-posterior polarities. These polarities depend on, at least in part, signals from adjacent tissues. The central nervous system (CNS) arises from this development (117). Neural crest cells that are precursors of the peripheral nervous system (PNS) start to differentiate from the neuroepithelium and overlaying ectoderm around E8. The neural crest cells first emerge around the dorsal region of the neural tube, then migrate into various parts of the body to form different types of tissues including the PNS, melanocytes, and odontoblasts, and much of the cranial facial bones and cartilage.

Some of the BMP ligands are highly expressed in the ectoderm, and several studies suggest that BMPs play important roles in neural induction and neural differentiation. In *Xenopus*, blockage of BMP signaling by overexpression of dominant-negative forms of BMP receptors or signaling molecules leads to fate changing from neuroectoderm to surface ectoderm (118-120). Overexpression of extracellular proteins that restrict BMP signaling also show similar results (121-124). In mouse, overexpression studies using dominant-negative forms of receptors have not been reported partly due to the technical difficulties. Mutant mice for some of the extracellular binding molecules do not show overt abnormalities during neural induction (125-128). Conditional alleles of *Bmpr-1a*, *Alk2*, and *Bmp4* with combination of proper Cre transgenic mice should address this issue (70, 76, 129).

BMPs are also believed to play critical roles during neural differentiation. For instance, *in vitro* experiments demonstrated that neural crest cell differentiation into sympathetic neurons is depended on BMPs (130). However, the effects of BMPs reported so far are very diverse and sometimes controversial. One of the ways to determine how BMPs are effecting neural development is to focus on the signaling components of the BMP pathway and alter their activities *in vivo*. Mouse embryos that carry a constitutive active form of *Bmpr-1a* (*caBmpr-1a*) driven by nestin promoter show over proliferation of the neural tube and dorsalization (132). Overexpression of *caBmpr-1b* with the nestin promoter promotes premature differentiation of neural tissues (132). These results suggest that BMP signaling through different type I receptors maintains some of their specificity.

Availability of conditional alleles for BMP receptors allows us to analyze neural tissue specific-disruption of BMP signaling. As an example, telencepharon-specific disruption (dorsal region) of *Bmpr-1a* has recently been carried out. (133). Mutant embryos lack choroid plexus, the most dorsal structure of the neural tube (133). Molecular analysis revealed that primodia of the choroid plexus are formed, but fail to differentiate and remain in a proliferative state (133). This is consistent to the results obtained from *caBmpr-1a* overexpressing mice, that BMP signaling through the BMP receptor 1a plays a critical role in the growth and formation of dorsal structure of the neural tube.

4.2.3. Skeletalgenesis and limb patterning

Since the first report that BMPs can induce ectopic bone formation (4), many researchers have investigated how BMPs and their signaling molecules play roles in bone formation. The spontaneous mutations *short ear* and *brachypodism* that show abnormal bone shapes are assigned to *Bmp5* and *Gdf5*, respectively (12, 14), providing the first genetic evidences that BMPs are involving in skeletalgenesis. Recently, *Gdf5* is identified as the responsible gene for two human hereditary diseases, acromesomelic chondrodysplasia, Hunter-Thompson type, and chondrodysplasia, Grebe type (134-135). *Bmp7* homozygous mutants die soon after birth due to the kidney failure, but these animals also show skeletal defects (15-16). The majority of the heterozygous mutants for *Bmp4* do not show an overt phenotype, but some of them show extra digits and shortening of skull (136). Interestingly, inactivation of *Bmp3* leads to higher bone density (30) solving the puzzle why recombinant BMP3, unlike other BMP members, does not have the ability to induce ectopic bone formation.

The roles of each BMP receptor in the signaling processes were first studied in chick with overexpression of mutant forms of receptors. Both *Bmpr-1a* and *1b* are expressed in chick limb bud with different pattern. Overexpression of a dominant negative form of *Bmpr-1a* in chick limb bud suppresses apoptosis of the interdigit mesenchymal cells (137). In contrast, overexpression of a dominant negative form of *Bmpr-1b* does not lead overt abnormalities, but co-overexpression of dominant forms of *Bmpr-1b* and *Bmpr-2* leads shortening of digits (138). Overexpression of constitutive active forms of BMP receptors in chick cause more dramatic effect than that of dominant negative forms. Overexpression of *caBmpr-1b* leads ectopic chondrogenesis and overgrowth of chondrocytes, whereas, overexpression of *caBmpr-1a* increases growth and delays differentiation of chondrocytes (139). These results suggest that IB signaling regulates condensation of mesenchyme and IA signaling regulates growth and differentiation of chondrocytes to hypertrophic chondrocytes, at least, in chick. The stage at which the chick retrovirus is applied can also have a profound affect on the outcome in these limb growth and differentiation assays.

Recently, two mutant mouse lines for *Bmpr-1b* were generated through different approaches to show abnormalities in the bones in digits (91-92). The proximal and middle phalanges are fused and reduced in *Bmpr-1b* mutant mice (91-92). This phenotype resembles to that in *Gdf5* mutant mice, *brachypodism*. In *Bmpr-1b* mutant mice, mesenchymal condensation for chondrogenesis is not affected, but differentiation and proliferation of chondrocytes are largely reduced specifically in the phalangeal region by E13.5 (91-92). Double homozygous mutant mice of *Bmpr-1b* and *Gdf5* closely resemble phenotype of *brachypodism* suggesting that GDF5 is one of the major ligands for BMPR-IB *in vivo* (91-92), which is consistent with biochemical results (140). In contrast, double homozygous mutant mice of *Bmpr-1b* and *Bmp7* show more severe phenotype suggesting that BMP7 can interact with other type I receptors during skeletalgenesis in limb (91).

A transgenic mouse line that express a dominant-negative form of *Bmpr-1b* in osteoblast-specific manner

shows similar, but more severe abnormalities in skeletal system than *Bmpr-1b* deficient mice (141). In these mice, bone mineral density, bone volume, and bone formation rate are severely reduced, and responses to BMP2 of primary osteoblasts isolated from calvaria of the transgenic mice were significantly inhibited (141). These results suggest important functions of BMPs in bone remodeling after birth. Having a more severe phenotype in this transgenic mouse than *Bmpr-1b* deficient mouse implies that a dominant negative form of BMPR-IB may alter several of the BMP pathways in addition to that from BMPR-IB. Interestingly, a transgenic mouse line that express a dominant-negative form of *Bmpr-1a* in osteoblast-specific manner shows similar, but a different phenotype suggesting that different forms of truncated receptor can block BMP signals with different specificity (Mishina and Nakashima, unpublished observation).

Since homozygous mutations of the most potent BMP ligands and their receptors cause early embryonic lethality, little is known of the function of BMPs during skeletalgenesis and bone remodeling. Conditional alleles for these genes should be valuable tools to uncover the function of BMPs during skeletalgenesis. As a prelude to this long waited story, the apical ectodermal ridge (AER)-specific disruption of *Bmpr-1a* in developing limb bud reveals that BMP signaling is required for proper dorsoventral pattern formation in limb bud (142). The limb bud is an excellent system to study the 3-dimensional morphogenetic events since its structure has anterior-posterior, dorso-ventoral, and proximal-distal polarities. In the AER-specific mutant embryos of *Bmpr-1a*, expression of AER marker genes such as Fgf8 is reduced (142). Strikingly, expression of *engrailed-1* that is critical to establish the ventral identity of limb is completely missing, and the ventral side is dorsalized in the mutant limb (142). This is the first example that BMP signaling through BMPR-IA plays essential roles during early patterning events in limb development. Similar phenomena are found in chick limb bud, suggesting that the function of BMP in specification of ventral identity in limb bud is conserved in different species (143).

4.2.4. Reproductive tract

In mammals, both XX and XY individuals develop one pair of undifferentiated gonads and two pairs of genital duct systems, the Müllerian ducts and the Wolffian ducts. In XY individuals, the Müllerian ducts are positively regressed during embryogenesis and male-specific reproductive tissues such as vas deference and epididymis develop from the Wolffian ducts. A member of TGF-beta superfamily, Müllerian inhibiting substance (MIS, alternatively known as Anti-Müllerian hormone, AMH), and that has moderate homology to the BMPs, plays essential roles in regression of the Müllerian ducts (144). During embryogenesis, MIS expression is detected only in Sertoli cells in male and the highest levels of MIS are detected during the period of Müllerian duct regression (145). Transgenic female mice that expressed human MIS show regression of the Müllerian ducts, and homozygous mutant males for MIS retain the tissues derived from the Müllerian ducts, such as the uterus, and develop into male pseudohermaphrodites (146-147). The type II receptor for MIS (MISR-II) is expressed surrounding the mesenchyme of the Müllerian ducts (38-39). Homozygous mutant mice for MISR-II are a phenocopy of the MIS ligand mutant mice (148). The MISR-II is the only receptor for MIS even in the presence of pharmacologic levels of MIS (149). This is the only case where receptor-deficient mice are a phenocopy of the ligand deficient mice among TGF-beta superfamily members that have been studied.

Alk2 is one of the candidates for the type I receptor for MIS because Alk2 is expressed in the mesenchyme surrounding the Müllerian ducts (152) and its constitutive active form can repress the aromatase promoter activity in HEK-293 (human embryonic kidney) cells (151). This indicates that ca-Alk2 can send a signal similar to the MIS signal because MIS is known to repress aromatase biosynthesis (152-153). This idea was confirmed using either anti-sense oligomers for Alk2 or a dominant negative form of Alk2 in embryonic carcinoma-derived P19 cells (154-155). Moreover, antisense oligomers for *Alk2* can block MIS dependent regression of the duct in an *in vitro* culture of rat urogenital ridges (155).

Interestingly, a genetic approach successfully demonstrated that BMPR-IA is a type I receptor essential for MIS signaling (156). *Bmpr-1a* expression is specifically disrupted in the surrounding mesenchyme of the Müllerian duct using a Cre knock-in mouse to the MIS type II receptor locus (156). Resulted mutant male mice show persistence of the Müllerian duct that is a phenocopy of MIS ligand or MIS receptor mutant males (147-148, 156). These results clearly show that BMPR-IA is involving in MIS signaling pathway conceivably forming a complex with MISR-II. Even though ALK2 cannot compensate for the lack of BMPR-IA in the mesenchyme for MIS-induced duct regression, it does not necessary mean that ALK2 is not involving in the MIS signaling. Direct genetic evidence using conditional *Alk2* allele will answer this question.

5. PERSPECTIVE

In this article, I summarized recent achievement in mouse genetics to uncover the function of BMPs, particularly during pattern formation and organogenesis. Intensive studies in human genetics have also revealed that mutations in BMP ligands, receptors, and signaling molecules are involved in the pathogenesis of chondrodysplasia, hereditary haemorrhagic telangiectasia, primary pulmonary hypertension, and tumorigenesis (Juvenile Polyposis) (133-134, 157-161). The above-cited studies suggest that the BMPs play an important role in human development. Functional studies utilizing mutant mice, including conditional gene knockout (69) should draw our attention to the mouse as one of the best animal models for human diseases.

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