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BONE MORPHOGENETIC PROTEIN-3 FAMILY MEMBERS AND THEIR BIOLOGICAL FUNCTIONS

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1. ABSTRACT

Bone morphogenetic protein-3 and 3b (BMP-3 and BMP-3b) together represent a unique subgroup of the BMP family. BMP-3b shares 82% amino acid identity with BMP-3 in the mature region (ligand domain), but only 37% in the pro-region (pro-domain). In osteoblasts, BMP-3 and 3b have similar antagonistic activity against BMP-2, but they are differentially regulated. In developing embryos, BMP-3 and 3b have different dorsalizing activities. BMP-3b triggers secondary head formation in an autonomous manner, whereas BMP-3 induces aberrant tail formation. Loss-of-function analysis demonstrates that coordinated activity of xBMP-3b and cerberus, a head inducer, are required for head formation in *Xenopus* embryos. At the molecular level, BMP-3b antagonizes both nodal-like proteins (Xnr1 and derrière) and ventralizing BMPs (BMP-2 and ADMP), whereas BMP-3 only antagonizes ventralizing BMPs. Moreover, BMP-3b, but not BMP-3, associates with the monomeric form of Xnr1, a nodal-like protein. These molecular features of BMP-3 and 3b are due to their distinct pro-regions. These findings suggest that the processing of precursor regions and assembly of BMP-3 and 3b are important in various developmental processes and organogenesis.

2. INTRODUCTION

Bone morphogenetic proteins (BMPs) constitute a subgroup of proteins which belong to the transforming growth factor-ß (TGF-ß) superfamily. BMPs were originally identified as proteins that induce endochondral bone formation in adult mammals (1). Today, over 15 members of the BMP family have been isolated (2). They are expressed in a variety of tissues and have diverse effects on different cell types during embryogenesis and in adult life (3-5). BMPs are synthesized as large precursor proteins that are cleaved at a characteristic R-X-X-R consensus site to release mature domains containing a characteristic motif of seven conserved cysteine residues (4). The biologically active proteins are homo- or heterodimers of the mature domains (3-5). BMPs bind BMP receptors (types I and II) and signal through Smad proteins (6).

Several antagonists of the BMP family, discovered in *Xenopus* embryos, act as dorsalizing factors and neural inducers (7). One such antagonist, cerberus, is secreted from the organizer to induce head formation (8). This effect is due to its specific inhibition to BMP-4, nodal and Wnt family proteins (9). BMPs and their antagonists regulate the axial patterning of vertebrate embryos (10).

BMP-3 (also called osteogenin) and 3b (also called GDF-10) are structurally different members of the BMP family (1, 11-13). Studies of BMP-3 and 3b have frequently been contradictory and their precise biological activity remains obscure. BMP-3b was originally isolated from the rat femur, and BMP-3 was co-purified with BMP-2, a molecule that induces bone formation in adult animals (1, 11). Neither recombinant BMP-3 nor BMP-3b, has osteogenic activity, although native BMP-3 (osteogenin) purified from bovine bone has osteogenic activity (11, 13). Recently, it has been reported that BMP-3 and 3b are antagonists of BMP-2 (14, 15). Furthermore, we have

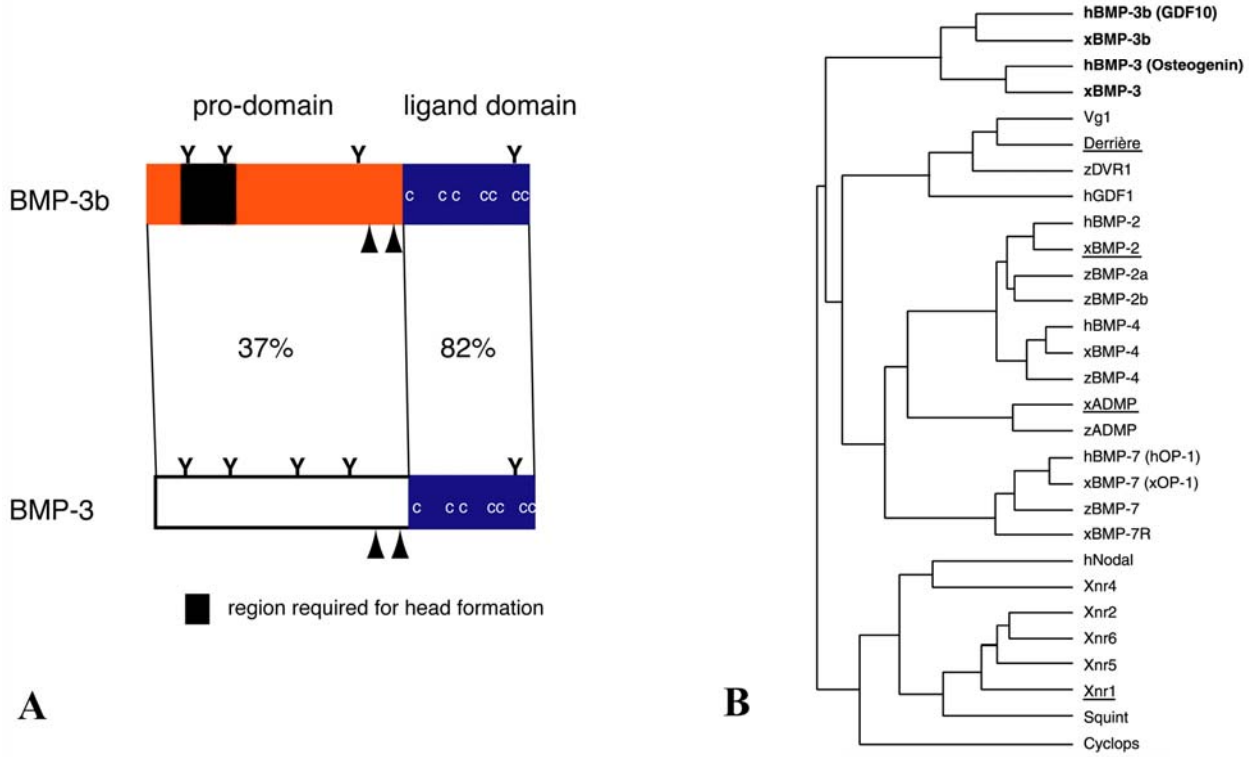

Figure 1. Structural characterization of BMP-3 and 3b. (A) Schematic representation of human BMP-3 and 3b. The amino-acid sequences are represented by bars. Percent identity in each domain is indicated. Potential glycosylation sites (Y) and proteolytic cleavage sites (triangle) are indicated. The seven cysteine residues conserved within the TGF-ß superfamily are indicated by (c). (B) BMP-3 and 3b in the BMP family (adapted from 15). The complete precursor amino-acid sequences were aligned by the UPGMA method (GENETYX program, SDC Ltd, Japan). The underlined proteins were used in this paper. h, human; x, *Xenopus*; z, zebrafish.

demonstrated that BMP-3b, but not BMP-3, is essential for head formation in *Xenopus* embryos, and that regulation of the processing of BMP-3/3b precursor and assembly plays an important role in embryonic patterning (15) In this review, we will discuss the unique functions, characteristics, and regulation of BMP-3 and 3b.

3. IDENTIFICATION AND STRUCTURAL FEATURES OF BMP-3 AND BMP-3B

3.1. Comparison at the protein level

Bone morphogenetic protein-3b (BMP-3b) was isolated from rat and human femur by RT-PCR (11, 16). While purifying and identifying known BMPs (BMP-2, 3, 6 and 7) from demineralized bovine bone matrix, we noticed the presence of a novel BMP-3-related protein (unpublished data). Cunningham *et al.* have also cloned by RT-PCR the murine orthologue of BMP-3b (which they named GDF-10) from brain and lung (12).

BMP-3 has been co-purified with BMP-1 and BMP-2 from bovine bone using the rat ectopic bone formation assay (1, 13). After that, orthologues of both molecules were identified in other species (15, 17). Like other TGF-ß family members, both proteins are biologically and structurally well conserved throughout vertebrate evolution (18, 19); for example, rat BMP-3 and 3b have the same activities as their *Xenopus* orthologues (15).

BMP-3b shares 82% amino-acid sequence identity in the mature region with BMP-3, with which it constitutes a unique subgroup in TGF-ß superfamily (Figure 1). However, the proteins are only 37% similar in their pro-regions, a unique feature among TGF-ß superfamily members. Most TGF-ß subgroups are much more highly homologous; for example, BMP-2, 4, 5, 6, and 7 share 62% homology in their pro-regions (18). Importantly, the pro-region of BMP-3b contains a segment which is required for head-forming activity (Figure 1A). This segment is relatively conserved between *Xenopus* and rat, and is divergent from that of BMP-3. This feature is unique for BMP-3b since the activity of other BMP family members is normally determined by the mature region of the protein.

Both BMP-3 and 3b have two cleavage sites. One is a consensus tetrabasic (RXXR) site, and the other is an alternative tribasic site that was discovered by protein purification and N-terminal sequence analysis (Figure 1A); these sites give rise to two different sizes of ligand protein upon over-expression of the precursor in CHO cells (15). Some other TGF-ß family members have multiple cleavage sites (20-22) that are known to be important for their activities (23, 24). Further analysis is required to examine how these two cleavage sites control the activity of BMP-3 and 3b.

	neonatal		adult		costa	costicartilage	brain	cerebellum	aorta	lung	heart	liver	kidney	ovary	testis
	femur	calvaria	femur	calvaria											
BMP-3b	++	+++	+	+	+	++	+	+++++	++	-	-	-	-	+	+
BMP-3	+	+++	++	+	+	-	-	-	+	++	-	-	+	++	-

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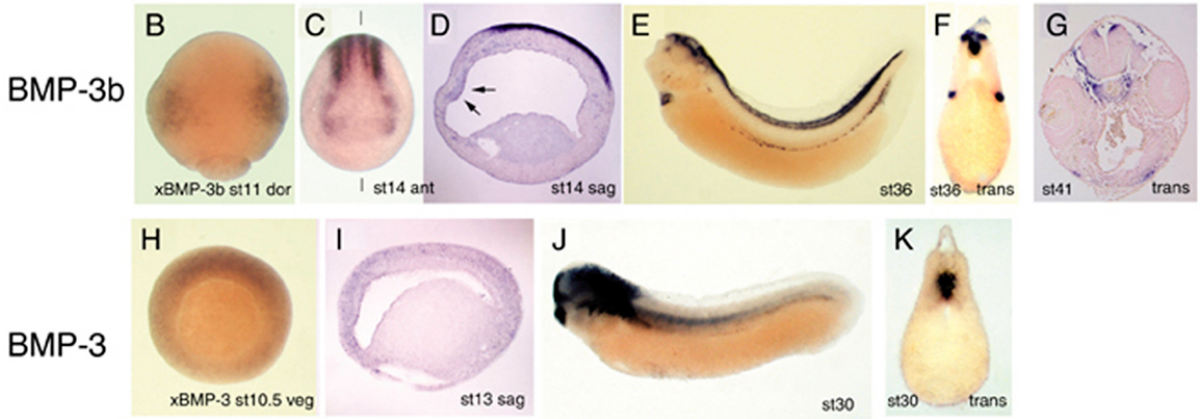

Figure 2. Expression patterns of *BMP-3* and *3b* in various tissues, and developmental embryos. (A) Expression levels of both genes in rat tissues were analysed by Northern blot (adapted from 11). Expression level was quantified and ranked from + to +++++. -, no signal (B-K) Embryonic expression of *Xenopus BMP-3b* and *BMP-3* by whole-mount *in situ* hybridization (adapted from 15). (B) Dorsal view of stage 11. Anterior region faces top. (C) Solid line indicates level of section. (D) Sagittal section of embryo. Anterior faces left. *BMP-3b* expression is intense in ectoderm and faint in mesoderm and endoderm. Arrows highlight *BMP-3b* expression in the prechordal plate. (H) Dorsal faces top. (I) Anterior faces left.

3.2. Comparison at the genomic level

The human BMP-3 and 3b genes are composed of three exons, and, for both genes, each splice junction is located at the same position (16, unpublished data); this genomic structure is well conserved between humans and mice. In the human TGF-ß superfamily, the genes for the TGF-ß subfamily (-ß1, -ß2 and -ß3) and BMP-7 subfamily (-5, -6, -7/OP-1 and -8/OP-2) contain seven exons and similar splice junction locations (21, 25 and unpublished data). On the other hand, the genes for BMP-2 and BMP-4 contain only two exons that cover the entire coding region, with each splice junction located in an identical position (26, 27). These findings suggest that both genes are derived from a common ancestor. The human BMP-3 and 3b genes mapped to chromosome 4p14-q21 and 10q11.2-q21.1, respectively (data not shown and ref. 28). At present, these chromosomal regions have not been directly linked to any other genetic disorders.

4. EXPRESSION PATTERNS OF *BMP-3* AND *BMP-3B*

4.1. Mammalian tissues

Several studies have described the mRNA expression of BMP-3 and 3b (11, 16, 12, 29-33). By Northern blotting analysis in rat and human tissues, both *BMP-3* and *3b* were shown to be expressed in bone (Figure 2A, ref. 16). The expression level of *BMP-3b* in the neonatal femur and calvaria is higher than in the adult, whereas the *BMP-3* mRNA level in the femur is lower during neonatal stages. *BMP-3b*, but not *BMP-3*, is expressed highly in costicartilage. In other tissues, BMP-3b mRNA is most abundant in the cerebellum, followed by the aorta, and is expressed at a lower level in the ovary and testis. In the embryonic cerebellum (18 dpc), expression of *BMP-3b* is weak, but it increases immediately after birth and remains high up to and throughout adulthood (11). In contrast, *BMP-3* is expressed at a high level in the ovaries and lungs, but at a low level in the kidneys and aorta. Cunningham *et al.* reported that *BMP-3b* (*GDF-10*) is highly expressed in the mouse uterus and adipose tissue (12). They subsequently examined *BMP-3b* expression during mouse embryogenesis, showing that *BMP-3b* mRNA can be detected at 8.5 dpc, and is expressed in developing skeletal structures at 12.5 and 14.5 dpc (31). Examination of BMP-3 mRNA in rat embryos revealed wide-spread expression in regions including the hair, teeth, and kidneys (29). In the rat ovary, strong expression of *BMP-3b* was observed during folliculogenesis, but *BMP-3* expression was relatively weak (32). In the human

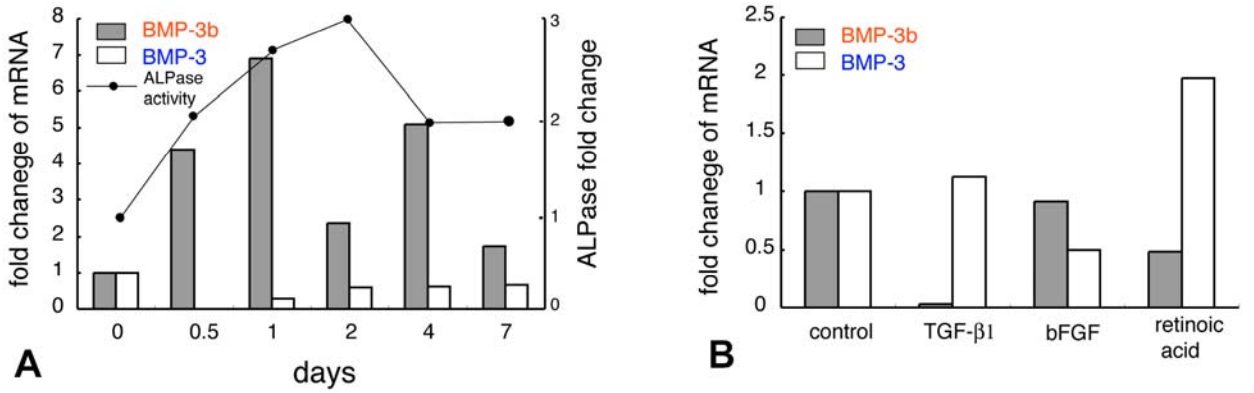

Figure 3. Expression of *BMP-3* and *3b* in primary osteoblasts (adapted from 35). Expression level was analysed by Northern blotting and quantified using BAS 5000 (Fuji Film, Japan). (A) Time courses of BMP-2 effects on *BMP-3* and *3b* mRNA levels and ALPase activity. Day 0: Start of the treatment with BMP-2 (100 ng/ml). The day-0 level was set at 1.0 (left ordinate). Cellular ALPase activity was measured with p-nitrophenyl phosphate as the substrate (right ordinate). The day-0 level was set at 1.0. (B) Effects of TGF-ß, bFGF, and retinoic acid on expression of *BMP-3* and *3b*. These cells were treated with these various substances for 24 h. Control level was set at 1.0.

ovary, BMP-3 mRNA is expressed in granulosa cells (33). Taken together, expression of BMP-3 and 3b in mammalian tissues is distinct; this temporal expression pattern of BMP-3 and 3b may be important for their functions.

4.2. *Xenopus* embryos

Figures 2B-K show whole-mount *in situ* analysis of *BMP-3* and *3b* in *Xenopus* embryos (adapted from 15). Expression of *xBMP-3b* is initially detected on the dorsal-lateral ectoderm (Figure 2B) and is enhanced in the neural fold from the rostral to the caudal regions (Figure 2C). *XBMP-3b* mRNA is detected in the prechordal plate and endoderm (Figure 2D). At the hatching stage, *xBMP-3b* is enriched in the region from the dorsal mesenchyme to the optic cup as well as posterior to the stomodeum (Figure 2E). *BMP-3b* expression is transient in the ventricular chamber (Figure 2E). *XBMP-3b* is also detected in the spinal cord and the somite (Figure 2F). At the swimming stage, *BMP-3b* is expressed in the head mesoderm (Figure 2G). *XBMP-3* is initially detected in the ectoderm and mesoderm (Figure 2H), but its expression is eventually restricted to the prechordal and chordal mesoderm (Figure 2I). At hatching, *xBMP-3* is expressed in cranial neural-crest derivatives and in the cement gland (Figure 2J), whereas expression of *xBMP-3* is restricted to the notochord (Figure 2K).

In summary, *BMP-3* and *3b* are expressed in a variety of embryonic tissues of *Xenopus*, but tend to mark the dorsal ectoderm and mesodermal cells.

5. REGULATION OF *BMP-3* AND *BMP-3B* EXPRESSION IN OSTEOBLASTS

Expression of *BMP-3* and *3b* was analyzed in primary calvarial osteoblasts, in which both genes are highly expressed (Figure 3). BMP-2, which induces ectopic bone formation, increases alkaline phosphatase (ALPase) activity and regulates expression of *BMP-3* and *3b* in these cells, as shown by the time courses in Figure 3A. There was a dramatic, time-dependent increase in *BMP-3b* transcription beginning within 6 h after treatment and exhibiting a maximum 6.9-fold increase. In contrast, the *BMP-3* mRNA level was decreased by BMP-2, especially during the early phase. Chen *et al.* (34) reported that *BMP-3* expression in a similar osteoblastic cell system was enhanced by long-term treatment with BMP-2, for 5-to-17 days after treatment. Figure 3B shows the effects of TGF-ß, bFGF and retinoic acid on expression of *BMP-3* and *3b* in these cells. Among these growth factors, TGF-ß completely and retinoic acid partially inhibited BMP-3b expression and bFGF had weak suppressive effect. Further analysis of the effect of TGF-ß on *BMP-3b* expression in these cells showed that TGF-ß causes a dramatic, rapid decrease in *BMP-3b* transcription, by nearly 50% after 3 h of incubation and almost 100% after 6 h (35). TGF-ß acts as an inhibitor of osteoblastic differentiation in these cells; therefore, these data suggest that *BMP-3b* mRNA level decreases with dedifferentiation of osteoblasts. On the other hand, *BMP-3* expression was inhibited by bFGF and enhanced by retinoic acid.

These results demonstrate that the regulation of *BMP-3b* differs from that of *BMP-3,* and that *BMP-3b* expression is regulated during osteoblast differentiation, indicating that BMP-3b functions in highly differentiated osteoblasts.

6. BIOLOGICAL ACTIVITY OF BMP-3 AND BMP-3B IN OSTEOBLASTS

BMP-3b was discovered in bone, and BMP-3 was isolated from a highly purified osteoinductive fraction of bone extracts (11, 1, 13). Native BMP-3 (osteogenin) purified from bone is osteoinductive (13). Furthermore, several groups found that a large amount of BMP-3 exists in bone (1, 13, 36 and unpublished data) and that *BMP-3,* as well as *BMP-3b,* is highly expressed in bone (11, 12, 16). Therefore, BMP-3 and 3b were thought to have osteogenic activity, like BMP-2. However, recombinant BMP-3 and 3b produced by CHO cells did not induce ectopic bone formation (11). To resolve these conflicting functions of BMP-3 and 3b in osteoblasts, we transfected

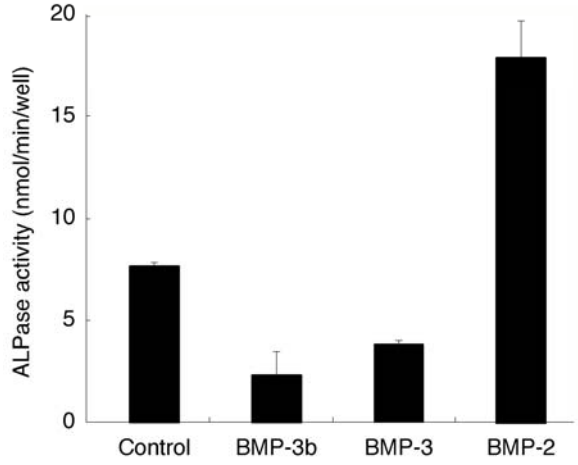

Figure 4. BMP-3 and 3b activities in osteoblast MC3T3-E1 cells. BMP-3 and 3b expression plasmids were transfected into MC3T3-E1 cells. ALPase activities were determined as described (Figure 3, ref. 35)

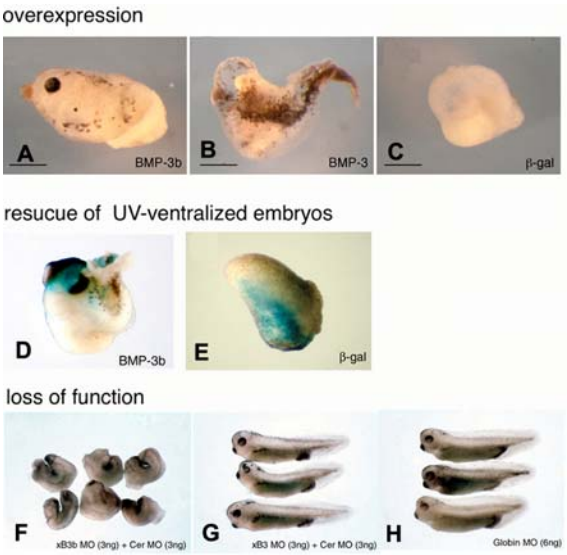

Figure 5. Functions of BMP-3 and 3b during embryonic development (adapted from 15). (A-C) Differentiation of ventral marginal zone explants (VMZs) injected with BMP-3 or 3b mRNA. (A) VMZs injected with BMP-3b generated head-like structures consisting of an eye and cement glands. (B) VMZs injected with BMP-3 elongated and formed melanocytes. (C) Control VMZs developed into a cylindrical structure. (D-E) Rescue of UV-ventralized embryos by BMP-3b. (D) Injection of BMP-3b rescued the anterior structure of ventralized embryos. Cells expressing BMP-3b were localized in the head region. (E) Control embryos. (F-H) Loss-of-function analyses using morpholino antisense oligonucleotides (MO). (F) Simultaneous injection of xBMP-3b and cerberus MOs led to loss of head and dorsal structures, (G) whereas injection of xBMP-3 and cerberus MOs did not. (H) Control embryos.

BMP-3 and 3b expression plasmids in osteoblasts and found that BMP-3 and 3b inhibit ALPase activity over 50%, whereas BMP-2 increased ALPase activity more than two-fold. These results indicate that BMP-3 and 3b have opposite effect to BMP-2 (Figure 4). This finding agrees with that of Daluiski *et al*., who reported that BMP-3 antagonizes BMP-2 signaling (14). Collectively, these results confirmed the role of BMP-3 and 3b as negative regulators of osteogenesis.

7. FUNCTIONS OF BMP-3 AND BMP-3B IN *XENOPUS* EMBRYONIC DEVELOPMENT

7.1. Dorsalizing activities of BMP-3 and BMP-3b in *Xenopus* embryos

BMPs and their antagonists are involved in the axial patterning of vertebrate embryos (10). Moreover, we showed that BMP-3 and 3b are antagonists of BMP-2 in osteoblasts (Figure 4 and ref.14). Therefore, we examined the biological activity of BMP-3 and 3b in *Xenopus* embryos. BMP-3b injected into *Xenopus* embryos triggered secondary head formation, whereas BMP-3 induced aberrant tail formation (15). When injected into the ventral marginal zone (VMZ), BMP-3b generated head-like structures, leading to secondary head formation (Figure 5A). These effects of BMP-3b in embryos are comparable to those of cerberus, a known head inducer (8). In contrast, when the VMZ was injected with BMP-3, it elongated and formed melanocytes (Figure 5B). The effect of BMP-3 is similar to that of the dominant-negative forms of BMP-2, 4, and 7 (23). These results reveal that while both BMP-3 and 3b are dorsalizing factors, they act distinctly in *Xenopus* embryos.

7.2. The involvement of BMP-3b in head formation

To further examine the effect of BMP-3b on head formation, we first performed rescue and lineage tracing of UV-ventralized embryos (Figure 5D). Injection of BMP-3b mRNA generated a head structure with cyclopia and a cement gland; cells expressing *BMP-3b* were found in the head region. These observations indicate that BMP-3b triggers head formation through an intrinsic developmental pathway. Next, we performed loss-of-function analyses in *Xenopus* embryos using antisense morpholino oligonucleotides (MO) against BMP-3, 3b, and cerberus. MO injection alone did not obviously perturb the *Xenopus* embryos, suggesting that the molecules they inhibit have some functional redundancies (15). Although BMP-3b and cerberus are structurally different, they both trigger secondary head formation in *Xenopus* embryos. Moreover, both proteins are expressed concurrently with the differentiation of the organizer cells (Figure 2, ref.15). We therefore co-injected either BMP-3 or BMP-3b MO together with cerberus MO into embryos. Figures 5F-G show that co-injection of cerberus MO with BMP-3b MO, but not BMP-3 MO, generated headless and malformed dorsal axial structures. Histological analysis revealed that depletion of BMP-3 and cerberus affects the size of the somite (15). These results were confirmed by a rescue experiment (15), suggesting the BMP-3b cooperates with cerberus in *Xenopus* larval head formation.

7.3. The divergent activity of BMP-3 and BMP-3b

The molecular characteristics of BMP-3 and 3b were analyzed by Western blotting and by interference assay (15). These results are summarized in Figure 6. First,

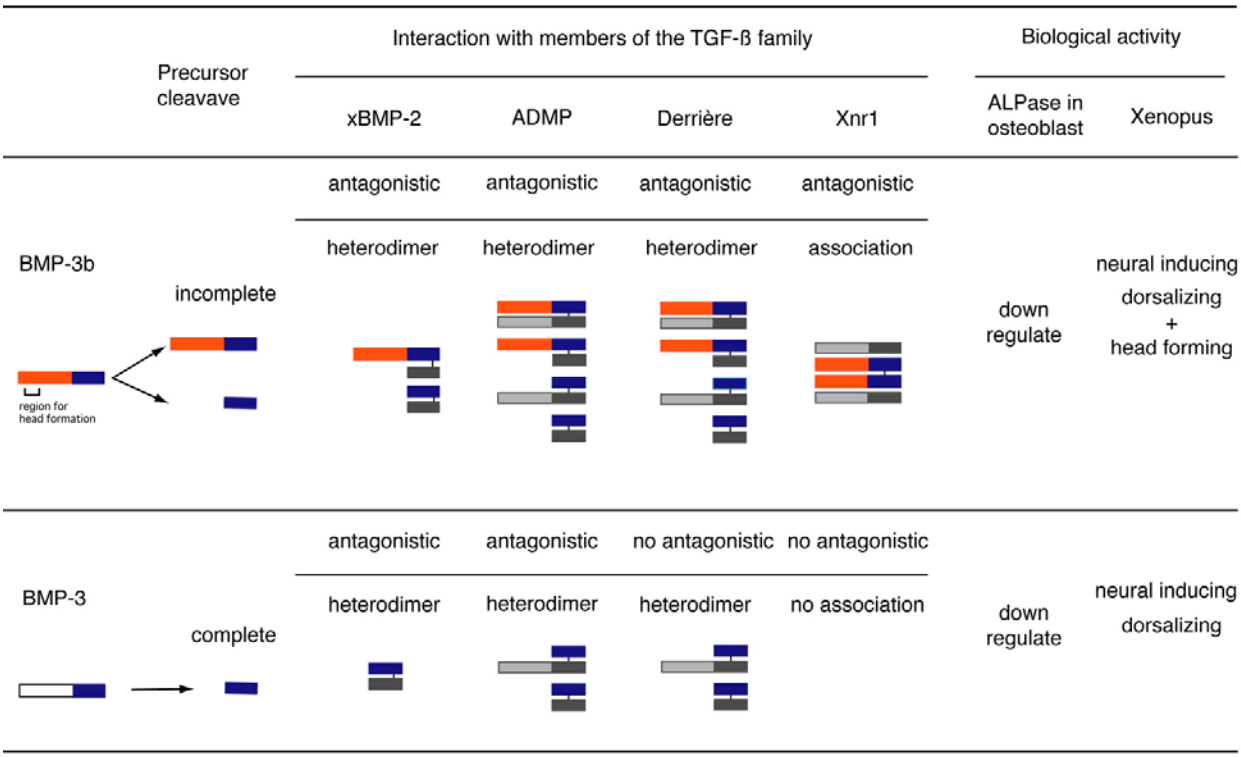

Figure 6. Molecular characteristics and biological activities of BMP-3 and 3b (adapted from 15). The results of biochemical analyses and interference assay between both proteins and TGF-ß members are summarised with their activities. Incomplete processing of BMP-3b, controlled by the pro-domain, generates precursor BMP-3b, which might be responsible for its unique activity. Both BMP-3 and 3b inhibit BMP-2 and ADMP. BMP-3b also blocks derrière and Xnr1. Heterodimers of precursor BMP-3b might reduce the activity of derrière and complex formation between BMP-3b and Xnr1 might alter Xnr1 function. These different types of BMP-3b antagonism result in ectopic head formation

we analyzed the biosynthesis of BMP-3 and 3b. BMP-3b precursor is processed incompletely in *Xenopous* embryos and in CHO cells, whereas the BMP-3 precursor is processed completely. These results are consistent with chimeric protein analyses, showing that the BMP-3b pro-region interferes with the maturation of the mature region, whereas the BMP-3 pro-region promotes the maturation of the mature region (15). Furthermore, these analyses showed that the region required for head formation for BMP-3b is located in the pro-region, and that the BMP-3b mature region has the same activity as that of BMP-3.

Next, we carried out an interference assay in an animal cap, because the inhibition of BMP-2, nodal and ADMP activities are involved in head formation in *Xenopus* embryos. We found that BMP-3b antagonizes ventralizing BMPs (BMP-2 and ADMP) and nodal-like proteins (derrière and Xnr1), whereas BMP-3 antagonizes only ventralizing BMPs (Figure 6, ref.15). These patterns of inhibition are in agreement with the finding that BMP-3b, but not BMP-3, causes head formation.

Finally, we showed that the interaction between BMP-3 or BMP-3b and other TGF-ß family members is direct. BMP-3b forms heterodimers with BMP-2, ADMP, and derrière and forms non-covalent complexes with monomeric Xnr1, whereas BMP-3 only forms heterodimers with BMP-2, ADMP, and derrière. These features are essentially consistent with a suppresive effect of BMP-3 or 3b on other TGF-ß family members. Heterodimers of precursor BMP-3b and derrière might inhibit derrière activity and BMP-3b/Xnr1complex formation might change Xnr1 function. In this regard, Xnr1 may interact with the region of BMP-3b that is required for head formation.

7.4. The role of BMP-3b in head formation

We showed that *Xenopus* BMP-3b indeed participates in embryonic head formation by coordinating with cerberus. Figure 7 shows a model of head formation by BMP-3b and cerberus. Initially, cerberus secreted from anterior endo-mesodermal cells in the organizer protects neighboring cells against nodal and ventralizing factors such as BMP-2, ADMP, and Wnt proteins, although nodal is essential for induction of mesodermal cells and for *cerberus* expression. As gastrulation proceeds, BMP-3b accumulates in the anterior mesodermal cells and forms a non-covalent complex with nodal, altering its function. When cerberus and BMP-3b successively block nodal and ventralizing factors from interacting with the anterior mesodermal cells, transcription factors such as Otx2 in the cells could proceed with expression of downstream genes and complete their self-differentiation as “head-organizing cells”.

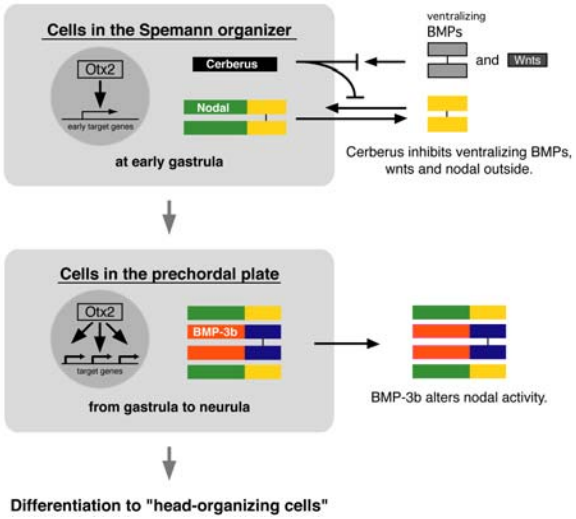

Figure 7. Model of head formation by BMP-3b and cerberus (adapted from 15). At the early gastrula stage, cells in the Spemann organizer express cerberus, nodal, and Otx2. Cerberus inhibits ventralizing BMPs, Wnts, and nodal, although nodal is required for organizer-cell induction and differentiation. As development proceeds, BMP-3b is expressed in the prechordal plate and could alter nodal function by forming non-covalent complexes. While cerberus and BMP-3b block both ventralizing factors and nodal, transcription factors such as Otx2 could induce downstream gene expression and complete differentiation of "head-organizing cells"

8. BMP-3 AND BMP-3B ARE ANTAGONISTS OF BMP-2, DORSALIZING BMPS, NEURAL INDUCERS, AND NEGATIVE REGULATORS OF BONE FORMATION

Both BMP-3 and 3b antagonize ventralizing BMPs such as BMP-2 and ADMP; thus, they act as dorsalizing BMPs (15, 14). Additionally, our data imply that BMP-3 and BMP-3b function as potent neural inducers (15). These biological functions are consistent with their expression pattern (15). Our biochemical analyses demonstrate that BMP-3 and 3b form heterodimers with BMP-2. The biological activity of such heterodimers remains unclear, but interference assays suggests that BMP-3/3b heterodimers with BMP-2 might abolish the activity induced by BMP-2 and thus trigger NCAM expression (15).

In support of this hypothesis, we have showed that BMP-3 and BMP-3b inhibit ALPase activity in MC3T3-E1 cells (Figure 4). This effect could be due to the formation of heterodimers of BMP-3 and 3b with other BMP family members such as BMP-4 which is produced in MC3T3-E1 cells. Further analyses using recombinant proteins are required to define the dynamics of the BMP heterodimers as well as of BMP homodimers.

It is not known whether BMP-3 and 3b (that is, dorsalizing BMPs) bind receptors and mediate signal transduction through Smad proteins. Daluiski *et al.* have reported that recombinant BMP-3 antagonizes BMP-2 signaling through an activin receptor pathway (ALK-4 and ActRII) (14). However, we show that dorsalizing BMPs, unlike activin, do not induce mesoderm in animal caps (15). Therefore, BMP-3 binds activin receptors, but the downstream signaling molecules may be different from those used by activin. Alternatively, dorsalizing BMPs may also bind a specific receptor in *Xenopus* embryos and transduce their signals through unknown molecules or inhibitory Smads (Smad 6, 7 and 8).

9. THE PRO-REGION OF BMP-3B DETERMINES ITS ACTIVITY

In TGF-ß family members, the pro-region simply modifies the activity of the mature region through control of its assembly, secretion, and turnover (37-41). Precursors of TGF-ßs, synthesized in the cells, are cleaved by proteolytic processing, generating pro-regions and mature regions. Then, both regions are secreted as inactive high-molecular-weight complexes (42-44). In these complexes, the mature region of TGF-ß is non-covalently associated with a pro-region of TGF-ß, which covalently binds to the TGF-ß binding protein. The TGF-ß pro-region itself can act as a functional binding domain (45). Similar function of pro-region for Myostatin/GDF-8 has been described and GDF-8 also belongs to the TGF-ß superfamily (46). Typically, the function of the TGF-ß pro-region is simply to inhibit the activity of the mature region. In contrast, the BMP-3b pro-region alters the activity of the mature region through control of its precursor processing. In other words, the BMP-3b precursor has an additional activity different from that of its mature region, while the TGF-ß precursor is an inactive form.

Our chimeric protein analyses demonstrate that the pro-region of BMP-3b contains the region that is required for head formation, and interferes with maturation of its mature region (15). In practice, BMP-3b precursor is secreted and forms heterodimers or complexes with other TGF-ß family members. These unique characteristics of BMP-3b could result in autonomous head formation in *Xenopus* embryos. Our results show that the mature region of BMP-3b is secreted and has the same activity of BMP-3, namely dorsalizing activity and antagonism of ventralizing BMPs.

The activity of BMP-3 and 3b may be regulated at different levels: transcriptional and post-translational levels. In the case of transcription, we demonstrated the regulation of BMP-3 and 3b in osteoblasts (Figure 3) and their distribution in various tissues (Figure 2), indicating that regulation and distribution of *BMP-3b* are different from those of *BMP-3*. In the case of post-translation, BMP-3b has two different activities regulated by precursor processing and assembly in *Xenopus* embryos: dorsalizing activity identical to that of BMP-3, and head-forming activity. These regulatory mechanisms might determine correct spatial and temporal gradients of BMP-3 and 3b activity in various physiological systems. In embryonic development, processing and assembly of the BMP-3 and 3b precursors are crucial to anterior-posterior patterning, and the pro-region of BMP-3b is essential for the regulation of its activity. This post-translational

regulation is unique among TGF-ß family members, and further analysis of it may provide a clue to other salient, yet unidentified regulatory systems mediated by TGF-ß superfamily members in other tissues.

10. PERSPECTIVE

We have focused here on recent findings regarding the structure and function of two TGF-ß superfamily members, BMP-3 and 3b. The most striking feature is that despite their close relatedness, BMP-3 and 3b have apparently different activities in *Xenopus* embryos. These differences appear to be due to their respective pro-regions, which affect their precursor processing and assembly. Understanding the biological activities of BMP-3 and 3b is just beginning. Although BMP-3 and 3b are highly expressed in several adult tissues including brain, aorta, and ovary, their functions in these tissues are still unknown. In these tissues, other TGF-ß family members may interact with BMP-3 and 3b and BMP-3 and 3b may act as antagonists of these TGF-ß family members as they do in *Xenopus* embryos. It will be exciting to see how BMP-3 and 3b function and interact with other TGF-ß family members or BMP-binding proteins in the nervous, cardiovascular, and reproductory systems.

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12. REFERENCES

1. Wozney, J.M., V. Rosen, A. J. Celeste, L. M. Mitsock, M. J. Whitters, R. W. Kriz, R. M. Hewick & E. A. Wang: Novel regulator of bone formation. *Science* 242, 1528-1534 (1988)

2. Reddi, A.H: Role of morphogenetic proteins in skeltal tissue engineering and regeneration. *Nature Biotech* 16, 247-252 (1998)

3. Hogan, B.L.M: Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes & Dev* 10, 1580-1594 (1996)

4. Reddi, A.H: Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogenisis. *Cytokine & Growth Factor Reviews* 8, 11-20 (1997)

5. Rosen, V. & J.M. Wozeny: Bone morphogenetic proteins. In*:* Principles of Bone Biology. Eds: Bilezikian J P, Raisz L G, Rodan G A, Academic Press, SD 919-928 (2002)

6. Massagué, J: How cells read TGFßs. *Nature Rev Med* 1, 169-178 (2000)

7. Harland, R: Neural induction. *Curr Opin Genet Dev* 10, 357-362 (2000)

8. Bouwmeester, T., S.-H. Kim, Y. Sasai, B. Lu & E. M. De Robertis: Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* 382, 595-601 (1996)

9. Piccolo, S., E. Agius, L. Leyns, S. Bhattacharyya, H. Grunz, T. Bouwmeester & E. M. De Robertis: The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP, and Wnt signals. *Nature* 397, 707-710 (1999)

10. Thomsen, G.H: Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning. *Trends Genet* 13, 209-211 (1997)

11. Takao, M., J. Hino, N. Takeshita, Y. Konno, T. Nishizawa, H. Matsuo & K. Kangawa: Identification of rat bone morphogenetic protein-3b (BMP-3b), a new member of BMP-3. *Biochem Biophys Res Commun* 219, 656-662 (1996)

12. Cunningham, N.S., N.A. Jenkins, D.J. Gilbert, N.G. Copeland, A.H. Reddi & S-J. Lee: Growth/Differentiation factor-10: A new member of the transforming growth facotor-ß superfamily related to bone morphogenetic protein-3. *Growth Factors* 12, 99-109 (1995)

13. Luyten, F.P., N.S. Cunningham, S. Ma, N. Muthukumaran, R. G. Hammonds, W. B. Nevins, W. I Wood & A. H. Reddi: Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. *J Biol Chem* 264, 1337-13380 (1989)

14. Daluiski, A., T. Engstrand, M. E. Bahamonde, L. W. Gamer, E. Agius, S. L. Stevenson, K. Cox, V. Rosen & K. M. Lyons: Bone morphogenetic protein-3 is a negative regulator of bone density. *Nat Genet* 27, 84-88 (2001)

15. Hino, J., S. Nishimatsu, T. Nagai, H. Matsuo, K. Kangawa & T. Nohno: Coordination of BMP-3b and cerberus is required for head formation of *Xenopus* embryos. *Dev Biol* 260, 138-157 (2003)

16. Hino, J., M. Takao, N. Takeshita, Y. Konno, T. Nishizawa, H. Matsuo & K. Kangawa: cDNA cloning and genomic structure of human bone morphogenetic protein-3b (BMP-3b). *Biochem Biophys Res Commun* 223, 304-10 (1996)

17. Kapanen, A., J. Ryhanen, E. Birr, H. K. Vaananen & J. Tuukkanen: Bone morphogenetic protein 3b

expressing reindeer antler. *J Biomed Mater Res* 59, 78-83 (2002)

18. Burt, D.W. & A. S. Law: Evolution of the transforming growth factor-beta superfamily. *Prog Growth Factor Res* 5, 99-118 (1994)

19. Nishimatsu, S., A. Suzuki, A. Shoda, K. Murakami & N. Ueno: Genes for bone morphogenetic proteins are differentially transcribed in early amphibian embryos. *Biochem Biophys Res Commun* 186, 1487-1495 (1992)

20. Celeste, A.J., J. A. Iannzzi, R. C. Taylor, R. M. Hewick, V. Rosen, E. A. Wang & J. M. Wozney: Identification of transforming growth factor ß family members present in bone-inductive protein purifed from bovine bone. *Proc Natl Acad Sci USA*, 87, 9843-9847 (1990)

21. Özkaynak, E., P. N. J. Schnegelsberg, D. F. Jin, G. M. Clifford, F. D. Warren, E. A. Drier & H. Oppermann: Osteogenic protein-2 *J Biol Chem* 267, 25220-25227 (1992)

22. Meno, C., Y. Saijoh, H. Fujii, M. Ikeda, T. Yokoyama, M. Yokoyama Y. Toyoda & H. Hamada: Left-right asymmetric expression of the TGFß-family member *lefty* in mouse embryos. *Nature* 381, 151-155 (1996)

23. Nishimatsu, S. & G. H. Thomsen: Ventral mesoderm induction and patterning by bone morphogenetic protein heterodimers in *Xenopus* embryos. *Mech Dev* 74, 75-88 (1998)

24. Cui, Y., R. Hackenmiller, L. Berg, F. Jean, T. Nakayama, G. Thomas, & J. L. Christian: The activity and signaling range of mature BMP-4 is regulated by sequential cleavage at two sites within the prodomain of the precursor. *Genes Dev* 15, 2797-2802 (2001)

25. Derynck, R., L. Rhee, E.Y. Chen & A.Van Tilburg: Intron-exon structure of the human transforming growth factor-beta precursor gene. *Nucleic Acids Res* 15, 3188-3189 (1987)

26. Feng, J.Q., D. Chen, A. J. Cooney, M. J. Tsai, M. A. Harris, S. Y. Tsai, M. Feng, G. R. Mundy & S. E. Harris: The mouse bone morphogenetic protein-4 gene. Analysis of promoter utilization in fetal rat calvarial osteoblasts and regulation by COUP-TFI orphan receptor. *J Biol Chem* 270, 28364-73 (1995)

27. van den Wijngaard, A., van Kraay, E. J. van Zoelen, W. Olijve & C. J. Boersma: Genomic organization of the human bone morphogenetic protein-4 gene: molecular basis for multiple transcripts. *Biochem Biophys Res Commun* 219, 789-94 (1996)

28. Tabas, J.A., M. Zasloff, J. J. Wasmuth, B. S. Emanuel, M. R. Altherr, J. D. McPherson, J. M. Wozney & F. S. Kaplan: Bone morphogenetic protein: chromosomal localization of human gnenes for BMP1, BMP2A, and BMP3. *Genomics* 9, 283-289 (1991)

29. Takahashi, H. & T. Ikeda: Transcripts for two members of the transforming growth factor-beta superfamily BMP-3 and BMP-7 are expressed in developing rat embryos. *Dev Dyn* 207, 439-49 (1996)

30. Thomadakis, G., L. N. Ramoshebi, J. Crooks, D. C. Rueger & U. Ripamonti: Immunolocalization of Bone Morphogenetic Protein-2 and -3 and Osteogenic Protein-1 during murine tooth root morphogenesis and in other craniofacial structures. *Eur J Oral Sci* 107, 368-77 (1999)

31. Zhao, R., A. M. Lawlerand & S. -J. Lee: Characterization of GDF-10 expression patterns and null mice. *Dev Biol* 212, 68-79 (1999)

32. Erickson, G.F., S. Shimasaki: The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. *Reprod Biol Endocrinol* 1, 9 (2003)

33. Jaatinen, R., V. Rosen, T. Tuuri & O. Ritvos: Identification of ovarian granulosa cells as a novel site of expression for bone morphogenetic protein-3 (BMP-3/osteogenin) and regulation of BMP-3 messenger ribonucleic acids by chorionic gonadotropin in cultured human granulosa-luteal cells. *J Clin Endocrinol Metab* 81, 3877-3882 (1996)

34. Chen, D., M. A. Harris, G. Rossini, C. R. Dunstan, S. L. Dallas, J. Q. Feng, G. R. Mundy & S. E. Harris: Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. *Calcif Tissue Int* 60, 283-90 (1997)

35. Hino, J., H. Matsuo & K. Kangawa: Bone morphogenetic protein-3b (BMP-3b) gene expression is correlated with differentiation in rat calvarial osteoblasts. *Biochem Biophys Res Commun* 256, 419-424(1999)

36. Sampath, T.K., J. E. Coughlin, R. M. Whetstone, D. Banach, C. Corbett, R. J. Ridge, E. Ozkaynak, H. Oppermann & D. C. Rueger: Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-beta superfamily. *J Biol Chem* 265, 13198-13205 (1990)

37. Gray, A.M. & A. J. Mason: Requirement for activin A and transforming growth factor-ß1 pro-regions in homodimer assembly. *Science* 247, 1328-1330 (1990)

38. Thomsen, G.H. & D. A. Melton: Processed Vg1 protein is an axial mesoderm inducer in *Xenopus Cell* 74, 433-441(1993)

39. Constam, D.B. & E. J. Robertson: Regulation of bone morphogenetic protein activity by pro domains

and proprotein convertases. *J Cell Biol* 144, 139-149 (1999)

40. Pepinsky, R.B., L. K. Sinclair, E. P. Chow, R. J. Mattaliano, T. F. Manganaro, P. K. Donahoe & R. L. Cate: Proteolytic processing of mullerian inhibiting substance produces a transforming growth factor-ß-like fragment. *J Biol Chem* 263, 18961-18964 (1988)

41. Wilson, C.A., N. di Clemente, C. Ehrenfels, R. B. Pepinsky, N. Josso, B. Vigier & R. L. Cate: Mullerian inhibiting substance requires its N-terminal domain for maintenance of biological activity, a novel finding within the transforming growth factor-beta superfamily. *Mol Endocrinol* 7, 247-57 (1993)

42. Lawrence, D.A., R. Pircher, C. Kryceve-Martinerie & P. Jullien: Normal embryo fibroblasts release transforming growth factors in a latent form. *J Cell Physiol* 121, 184-188 (1984)

43. Miyazono, K., U. Hellman, C. Wernstedt & C. H. Heldin: Latent high molecular weight complex of transforming growth factor ß1. Purification from human platelets and structural characterization. *J Biol Chem* 263, 6407-6415 (1988)

44. Kanzaki, T., A. Olofsson, A. Moren, C. Wernstedt, U. Hellman, K. Miyazono, L. Claesson-Welsh & C. H. Heldin: TGF-ß1 binding protein: a component of the large latent complex of TGF-ß1 with multiple repeat sequences. *Cell* 61, 1051-1061 (1990)

45. Gentry, L.E. & B. W. Nash: The pro domain of pre-pro-transforming growth factor ß1 when independently expressed is a functional binding protein for the mature growth factor. *Biochemistry* 29, 6851-6857 (1990)

46. Zimmers, T.A., M. V. Davies, L. G. Koniaris, P. Haynes, A. F. Esquela, K. N. Tomkinson, A. C. McPherron, N. M. Wolfman & S. J. Lee: Induction of cachexia in mice by systemically administered myostatin. *Science* 296, 1486-1488 (2002)

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Figure 3 from Takao, M., J. Hino, N. Takeshita, Y. Konno, T. Nishizawa, H. Matsuo & K. Kangawa: Identification of rat bone morphogenetic protein-3b (BMP-3b), a new member of BMP-3. *Biochem Biophys Res Commun* 219, 656-662 (1996)

Figures 1B, 2B, 2D, 2E, 2G, 2K, 2O, 2P, 2I, 2R, 2T, 2X, 3D, 3E, 3F, 4A, 4D, 7E, 8F, 8G and 8H from Hino, J., S. Nishimatsu, T. Nagai, H. Matsuo, K. Kangawa & T. Nohno: Coordination of BMP-3b and cerberus is required for head formation of Xenopus embryos. *Dev Biol* 260, 138-157 (2003)

Figures 3 and 4 from Hino, J., H. Matsuo & K. Kangawa: Bone morphogenetic protein-3b (BMP-3b) gene expression is correlated with differentiation in rat calvarial osteoblasts. *Biochem Biophys Res Commun 256*, 419-424(1999)

Key Words: BMP-3, Osteogenin, BMP-3b, GDF-10, TGF-ß, Osteogenic activity, Osteoblast, ALPase, dorsalizing activity, Cerberus, Head formation, Neural inducer, Precursor processing, Embryos, Review

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