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BMP SIGNALING AND HOX TRANSCRIPTION FACTORS IN LIMB DEVELOPMENT

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1. ABSTRACT

Limb development, a complicated biological event that includes diverse processes such as three-dimensional patterning, cartilage and bone differentiation and programmed cell death, or apoptosis, is regulated by a network of signal molecules that work in concert to ensure proper morphogenesis. Bone Morphogenetic Proteins (BMPs), members of the TGFβ superfamily, play a pivotal role in the signaling network and are involved in nearly all processes associated with limb development. While the canonical BMP/Smad signaling cascade has been clarified, the pathway by itself does not explain how BMPs exert such diverse functions. The answer may lie in the crosstalk between BMPs and other signaling pathways, as well as the diverse transcription factors used by BMPs. The major objective of this review is to summarize the main functions of BMP signaling during limb development and to describe the crosstalk between BMPs and other signaling molecules such as Wnts, FGFs and Shh. In addition, distinct transcription factors downstream of BMP signaling will be discussed. Among the various transcription factors, we will focus on the Hox family of proteins, which play an important role in limb patterning.

2. INTRODUCTION

Bone Morphogenetic Proteins (BMPs) are members of the TGFβ superfamily of signaling molecules that regulate diverse biological events including cell growth, differentiation and apoptosis. Thus far, more than 20 members have been identified as belonging to the BMP family. The original three family members – BMP1, BMP2A and BMP3 – were first identified as the active components in bovine bone preparations that induced ectopic cartilage and bone formation in adult rats (1). Growing evidence during the past decade supports the notion that BMPs have many more functions in addition to bone induction. For example, these signaling molecules play essential roles in many morphogenetic events during embryogenesis, including limb patterning, neural development and the development of many other organs such as liver, kidney and teeth (2).

BMPs mediate cell responses through the Smad signaling pathway. Briefly, BMP ligands bind and activate the type II cell surface receptors, which in turn recruit the type I receptors. The type I receptors are then phosphorylated, following which they recruit and activate intracytoplasmic Receptor-regulated Smads (R-Smads, Smad1, 5 and 8). The activated R-Smads form heterodimers with the Common partner Smad (Co-Smad, Smad4), which then translocate into the nucleus. Within the nucleus, the Smad complexes recruit distinct transcription cofactors and mediate gene transcription in a cell type-specific manner (3;4).

Hox transcription factors represent a family of homeodomain-containing proteins whose activities are essential for normal development. In mice and humans there are 39 members within the hox gene family. The homeobox sequence is highly conserved among the members. The 39 genes are arranged into four clusters, HOXA, HOXB, HOXC and HOXD, with each cluster located on a different chromosome. Based on the sequence similarity and position on the chromosomes, genes in the four clusters are divided into 13 paralogs (5-7). Hox transcription factors play essential roles in body organization during embryonic development, including trunk patterning and appendicular skeleton patterning (8;9).

In this review, we will discuss the various roles of BMPs during limb development, including limb spatial patterning, chondrogenesis and apoptosis, and the potential involvement of Hox proteins in BMP-mediated biological processes. We will also overview how BMP signaling interacts with other signals such as Wnt, Shh and FGFs to mediate proper limb morphogenesis.

3. BMPS AND LIMB PATTERNING

Limb development is an intricate process. Limb spatial patterning is specified in a three-dimensional manner along the proximal-distal (PD), dorsal-ventral (DV) and anterior-posterior (AP) axes. Each axis is controlled by

distinct signal centers (10;11): the PD axis is controlled by the apical ectodermal ridge (AER), composed of epithelial-like cells located at the distal DV border; the DV axis is controlled by dorsal and ventral ectoderm; and the AP axis is specified by a zone of polarizing activity (ZPA), which is located in the posterior mesenchyme. The key molecules secreted by these three signal centers are FGFs, Wnt7a and Shh, respectively (12-17). It is now well known that a network of these molecules, as well as other signaling molecules, function in concert to ensure proper morphological development. BMPs are the crucial proteins involved in this network and participate in nearly all of the processes during the limb patterning.

According to dorsal-ventral (DV) patterning, BMP-2, 4 and 7 are expressed in the ventral ectoderm of early limb buds, in a pattern similar to that of Engrailed 1 (EN1) (18), the homeodomain transcription factor that is essential for ventral patterning (19). These BMPs act upstream of Engrailed 1 (En1) to restrict the expression of Wnt7a to dorsal ectoderm, since loss of BMP signaling results in absence of EN1 and ectopic Wnt7a in ventral ectoderm (18). BMPs and Wnt7a are expressed in complementary patterns and thus cooperate with each other to specify the DV axis. BMPs control proximal-distal (PD) patterning by inducing AER formation and FGF8 expression. Misexpression of either Noggin or constitutively activated (ca) BMPR leads to AER disruption. BMP-induced AER formation is through Msx and is En1-independent (18). On the other hand, BMPs mediate AER regression at later stages to ensure the proper length of the limb (20). Consistently, conditional knockout of BMP receptor-1A in limb ectoderm leads to AER disruption and loss of FGF8 expression (21). BMPs are also involved in AP patterning. BMP-2 expression is limited in the posterior mesenchyme during early development, with the expression domain largely overlapping with that of Hoxd13 (22). Hoxd13 is the homeodomain-containing transcription factor that acts as a downstream component of polarizing signaling to control AP patterning (23). Shh is the key molecule secreted by ZPA to mediate AP patterning. ZPA implantation (22) or Shh overexpression (24;25) can induce ectopic BMP-2 expression. However, introducing BMP-2 alone into anterior mesenchyme does disrupt the AP axis (22), indicating that BMP-2 acts as a downstream component of Shh signaling rather than as a polarizing signal itself. Thus, BMPs are involved in all three axes of patterning via their interaction with other signaling pathways.

4. BMPS AND CHONDROGENESIS

BMPs were first identified by their ability to induce ectopic cartilage and bone formation in adult rats. Loss- or gain-of-function studies suggest that BMPs also play crucial roles in chondrogenesis and skeletogenesis during embryoic development. Individual BMPs exhibit distinct expression patterns. BMP-2 is expressed in areas surrounding the initial cartilage condensation, while BMP-4 is expressed in perichondrium. (26). BMP-2 is also expressed in periosteal and osteogenic zones (27). BMP-5 is expressed in initial cartilage condensation as well as in perichondrium and periosteum at later stages of development (28). BMP-6 is expressed in hypertrophic chondrocytes (27). BMP-7 is highly expressed in the perichondrium, but and its expression is absent in the zones of joint formation (29). Distinct temporal and spatial expression patterns of individual BMPs indicate that different members mediate specific events of chondrogenesis. For example, BMP-2 recruits the mesenchymal cells surrounding the initial cartilage condensation into chondrogenic fate, while BMP-4 recruits perichondrial cells (26). BMP-6 may be essential for terminal differentiation of chondrocytes (27). In contrast to BMP-2 (29), which plays a positive role in joint formation, the absence of BMP-7 expression in the zone of joint formation indicates its negative role in joint formation.

Mutation studies have provided direct evidence for specific functions of distinct BMPs in cartilage and bone formation. These studies have been reviewed elsewhere (30). Briefly, the *Short ear* mice phenotype has been attributed to a BMP5 mutation (28;31). A BMP7 mutation results in defects in multiple skeletal elements (32;33), while BMP6 mutants show only minor defects (34). Interestingly, a BMP3 mutation causes increased bone formation instead of bone loss (35). It has been suggested that BMP3 may activate the TGFβ pathway and antagonize the BMP pathway (35;36). Unfortunately, there is very little genetic evidence regarding the role of BMP2 and BMP4 in skeletogenesis. Both BMP2 and BMP4 knockout mice die during early development because of the failure of mesoderm induction (37-39). Generation of conditional knockout mice for BMP2 and BMP4 may provide important clues for their functional roles in skeletogenesis (40).

BMPs are potent inducers of cartilage and bone formation *in vitro*. BMP2 induces cartilage nodule formation in chick limb bud mesenchyme cultures (41). BMP-6 accelerates hypertrophic chondrocyte differentiation and mineral accretion (42). In multipotential mesenchymal cells (MMCs) isolated from human bone marrow, BMP2 and BMP9 promote chondrogenic differentiation, possibly through activation of Sox9, a chondrogenic-related transcription factor. BMP9 appears to have stronger effects than BMP2 (43). BMP2 and BMP7 have been shown to induce both chondrocyte and osteoblast differentiation in C3H10T1/2 cells (44;45). Although the mechanisms are not fully understood, it has been proposed that BMPs can induce both undifferentiated stem cells and more differentiated multipotent cells into chondrogenic or osteogenic pathways (2;41;46-48).

Two types of BMP type I receptors are found in mammals, BMPR-IA and BMPR-IB. They are also known as ALK3 and ALK6, respectively (49). These two receptors exhibit different expression patterns during limb development and, thus, play distinct roles in cartilage differentiation (25;50). BMPR-IB is expressed in prechondrogenic cells and is required to initiate cartilage formation. After the initiation of chondrocyte differentiation, its expression decreases. In contrast, BMPR-IA is expressed at a later stage and regulates

chondrocyte differentiation. Dominant-negative BMPR-IB blocks cartilage formation *in vitro* while dominant-negative BMPR-IA does not (25;50). In addition, BMPR-IB$^{-/-}$ mice show appendicular skeletal defects (51). Thus, BMPR-IB seems to play a major role in mediating BMP-induced chondrogenesis.

It has been shown that BMP signaling coordinates with other signaling pathways to regulate cartilage differentiation, one of which is Ihh/PTHrP signaling. It has been proposed that Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP) interact in a negative feedback loop to regulate the onset of hypertrophy. Ihh is expressed in prehypertrophic chondrocytes and signals to induce PTHrP expression in the periarticular region. PTHrP in turn prevents hypertrophic differentiation (52). Expression of various BMPs, including BMP2/4 and BMP7, is induced in the perichondrium by overexpression of Ihh (53;54). However, BMPs seem not to act as downstream regulators of Ihh since blocking BMP signaling by Noggin has no effect on Ihh-mediated onset of hypertrophic differentiation and induction of PTHrP expression (54). Other studies, however, showed that overexpression of constitutively active BMPR-IA in limb buds during early development results in upregulation of PTHrP and blocking of hypertrophic differentiation (50). This could be due to a different role of BMP signaling during early stages of development. These results suggest that BMPs and Ihh induce their expression reciprocally and that the two separate pathways act in parallel to regulate different steps of chondrocyte differentiation (54).

FGFs are another group of molecules that play a role in chondrogenesis (55). In chick limb buds, FGF-4 antagonizes BMP-4-induced chondrocyte differentiation, resulting in reduced bone size (56). Further studies show that FGF-2 inhibits Ihh expression, promotes hypertrophic differentiation and reduces chondrocyte proliferation. Therefore, FGFs and BMPs mediate the same stages of cartilage differentiation, but with opposite effects (57). The balance between BMP and FGF signals is crucial for normal cartilage differentiation.

The signaling molecules of Wnt family have been shown to be involved in cartilage formation. Wnt-3A acts as a chondro-enhancing factor (58-60), while Wnt-7A has chondro-inhibitory effects (60-62). *In vitro* studies indicate that Wnt-3A and Wnt-7A participate in BMP signaling. In C3H10T1/2 cells, treatment with BMP-2 results in up-regulation of Wnt-3A and down-regulation of Wnt-7A. Treatment with lithium, a Wnt-7A mimetic, inhibits BMP-2-induced chondrogenesis. Overexpression of Wnt-3A accelerates the BMP-2-induced chondrogenesis. In the absence of BMP-2, Wnt-3A overexpression alone has no effect on chondrogenesis. At the molecular level, BMP-2 induces the nuclear translocation of β-catenin, the major transduction molecule in canonical Wnt pathway, and enhances its interaction with Smad4 (60;63). Further studies in embryo limb buds will be necessary to confirm the crosstalk between the Wnt and BMP pathways in regulating chondrogenesis.

5. TRANSCRIPTION FACTORS IN CHONDROGENESIS AND SKELETOGENESIS

While the BMP/TGFβ pathway mediates diverse cellular responses by regulating gene transcription, the number of Smads is quite limited. In addition, although Smads alone can bind to specific DNA elements, the binding affinity seems to be too weak for effective mediation of transcriptional activity (64). One of the possible mechanisms responsible for BMP/TGFβ-mediated cellular responses is the recruitment of various transcription factors by Smads and regulation of gene transcription by Smad-transcription factor complexes. FAST, AP-1, SP-1, TFE3, Mixer, Runx2, LEF1/TCF and Miz1 have been described as DNA binding partners in the TGFβ pathway (65-68). While less is known about the partners involved in BMP signaling, Hox proteins, Sox9, Runx2/Cbfa1 and AP1 are the major transcription factors involved in cartilage and bone formation. The roles of Runx2/Cbfa1 and AP1 in BMP-mediated cartilage and osteoblast differentiation have been described elsewhere (69). Here we will focus on Hox and Sox transcription factors.

The homeodomain (Hox) proteins are among the major factors that control vertebrate skeletal element patterning. 13 Hox proteins are expressed in osteoblast-like cell lines (70), indicating their roles beyond skeleton pattern information. Hoxc8 has been shown to regulate cartilage and bone differentiation. Hoxc8 transgenic mice show cartilage defects with an accumulation of proliferating chondrocytes and reduced maturation (71). Consistent with this observation, the direct interactions between Hox and Smads provide a novel mechanism for osteopontin (OPN) and osteoprotegerin (OPG) gene activation induced by BMP (72-74). The model demonstrates that Hoxc8 binds to the DNA elements upstream of OPN and OPG genes, thereby repressing their transcription. Upon BMP stimulation, Smad1 and Smad4 translocate into the nucleus where they interact with Hoxc8 at the homeodomain and remove it from the DNA elements. This results in activation of OPN and OPG transcription. As a negative regulation mechanism, Smad6, the inhibitory Smad, forms a heterodimer with Hoxc8 within the DNA elements and prevents Smad1 and Smad4 from binding to Hoxc8, thereby repressing BMP-induced gene transcription (75). Overexpression of the interaction domain of Smad1 with Hoxc8 in osteoblast precursors stimulates osteoblast differentiation-related gene expression and results in mineralized bone matrix formation. Smad1 and Smad4 have also been shown to interact with Hoxa9 (76). Since the domain of Hox interacting with Smads is the homeodomain, which is highly conserved within the Hox family, it will be interesting to determine whether Smads interact with other Hox proteins that are normally expressed in chondrocytes and osteoblasts.

Sox9, a member of Sox family of transcription factors, has been proposed to have two major functions during chondrocyte differentiation. Initially, it is required for mesenchymal condensation. At later stage it inhibits

hypertrophic chondrocyte differentiation (55). Recent studies have demonstrated that Sox9 is an important downstream component involved in mediation of the BMP pathway. In C3H10T1/2 cells, BMP-2 upregulates Sox9 expression in a dose-dependent manner, which in turn increases the expression level of Col2a1, the marker gene for chondrocyte differentiation. Treatment of Sox9 antisense oligonucleotides blocks the up-regulation of Col2a1 by BMP-2, confirming the role of Sox9 in BMP-induced chondrogenesis (77). Similar results were obtained in other studies (43;78). *In vivo* studies revealed that Sox9 is normally expressed in chondrogenic areas of the developing limb. BMP-2 induces ectopic Sox9 expression, while overexpression of Noggin leads to severe reduction of Sox9 expression and cartilage defects. These results suggest that Sox9 expression during limb development is BMP-dependent (79). Studies on the interactions between Sox9 and Smads may reveal detailed mechanisms for the role of Sox9 in BMP-mediated cartilage and bone formation.

6. BMPS AND PROGRAMMED CELL DEATH

It is now well established that BMPs mediate programmed cell death (PCD) during limb development, a process essential for elimination of unnecessary tissue and proper morphogenesis. BMP-2 and BMP-4 are expressed in the anterior necrotic zone (ANZ), the posterior necrotic zone (PNZ) and the interdigital necrotic zone (INZ) before PCD occurs, and their expression continues throughout the process. The expression domains are closely related with the distribution of apoptotic cells. The level of BMP-4 expression is higher than that of BMP-2, indicating that BMP-4 is the primary factor in this process (80). Overexpression of either dominant-negative BMPR-IA (80) or dnBMPR-IB (81) suppresses apoptosis in limb buds, leading to the webbing phenotype. Conversely, constitutively active BMPR-IB overexpression increases apoptosis (50). BMP-2 and BMP-4 also induce apoptosis in mesenchymal cells isolated from the interdigital region of chick limb buds, while TGF-β1 and activin do not have this effect (80). In Noggin transgenic mice, the interdigital tissue shows incomplete regression (82). Thus, BMPs play crucial roles in apoptosis during limb development.

Several lines of evidence suggest that Msx2 and Dickkopf-1 (DKK-1) are the downstream factors that mediate BMP-induced apoptosis in limb buds. DnBMPR-IB overexpression leads to downregulation of Msx2 and suppression of apoptosis *in vivo* (81). In P19 cells, an embryonal carcinoma cell line that gives rise to ectoderm and mesoderm, Msx2 expression is induced by BMP-4, and the expression pattern is similar to the distribution of apoptosis. Ectopic Msx2 expression results in increased apoptosis (83). Dickkopf-1 (Dkk-1), a potent inhibitor of the Wnt/β-catenin pathway, has recently been shown to be another factor that mediates BMP-induced apoptosis (84). In the developing limb, the expression domain of Dkk-1 overlaps largely with the sites of apoptosis. Its expression is upregulated by BMP-4. Overexpression of Dkk-1 enhances BMP-induced apoptosis in developing limbs. More importantly, the mouse mutant Fused toes (Ft) embryo, which has ectopic BMP signaling activation, shows similar ectopic Dkk-1 expression, giving physiological evidence for a correlation between BMP signaling and Dkk-1 expression (84). It has been proposed that BMPs mediate cellular responses through two alternative pathways, the Smad pathway and the MAPK pathway (3). It appears that BMP-mediated Dkk-1 activation is mainly via the MAPK pathway since BMP-4 fails to upregulate Dkk-1 expression in c-jun$^{-/-}$ embryonic fibroblasts, which already show a lowered basal level of Dkk-1 when compared to wild type fibroblasts. In addition, Dkk-1 transcripts cannot be detected in Jnk$^{-/-}$ fibroblasts (84). In view of the complicated events associated with programmed cell death, it is not surprising that additional factors that mediate this process will be discovered.

7. HOX AND LIMB DEVELOPMENT

As mentioned above, a network of signaling pathways work in concert to specify three-dimensional limb patterning. To accomplish this task, the signals must use specific transcription factors. Strong evidence indicates that the 5' HOXA and HOXD transcription factors (paralog 9-13) are the crucial molecules involved in this process. A comparison of the expression patterns of Hoxd9-13 genes during limb development reveals spatiotemporal colinearity, i.e., the Hox genes are activated sequentially according to their physical positions along the Hox cluster. The more 5' Hox genes are expressed later and more distally during limb development. In addition, each Hox gene exhibits a graded expression along the anterior-posterior axis, with maximum levels at the posterior margin (85). The 5' Hoxa gene expression patterns show similar colinearity except that individual Hoxa genes have unique expression domains and rarely overlap with one another. In addition, Hoxa genes are initially expressed at the posterior end portion but extend towards the anterior end during later stages of development (86;87). Based on the characteristic expression patterns and evidence from mutation studies, it has been proposed that HoxA and HoxD genes from paralog9 and 10 specify stylopodium (upper arm), paralog10-12 specifies zeugopodium (lower arm) and paralog11-13 specifies autopodium (digits) (9).

Due to the lack of natural mutations, most evidence for Hox gene function in limb patterning comes from experiments with targeted gene disruption. These studies demonstrate that each Hox gene acts to identify distinct regions along the limb, and that the genetic interactions between paralogous and nonparalogous genes are necessary for this process. Hoxd9$^{-/-}$ mice exhibited mild defects in forelimb development at the stylopodal level, with a slight shortening of the humerus and malformation of the deltoid crest. No hindlimb defect was found in these knockout mice. Hoxa9$^{-/-}$ mice had no limb defect. However, inactivation of both Hoxa9 and Hoxd9 led to a severe reduction in humeral length and deltoid crest alteration as compared to Hoxd9$^{-/-}$ mice. These results indicate that Hoxa9 and Hoxd9 act together to mediate stylopod patterning (88). Hoxa10 disruption results in proximal hindlimb defects, while forelimb development is

normal. Hindlimb defects include enlargement of the third trochanter, reduction in size of the medial sesamoid bone, and malformation of the lateral sesamoid bone (89). Hoxd10$^{-/-}$ mice also showed hindlimb alteration at the proximal level, with a shift in the position of the patella and occasional production of ectopic sesamoid bone. Alteration was also detected in articulation between femur and tibia (90). A Hoxa10 and Hoxd10 double mutation resulted in stronger defects in hindlimbs, indicating a synergistic functional relationship between Hoxa10 and Hoxd10 (91). Hoxd11 inactivation mainly affected autopod and distal zeugopod of the forelimb. Metacarpal length reduction, fusion of carpal bones and a gap between radius and ulna at the distal end were the obvious defects seen in mutant mice (92;93). Hoxa11$^{-/-}$ mice had both forelimb and hindlimb malformations. In forelimbs, fusion of carpal bones and misshaped radii and ulnas were observed. In hindlimbs, fibula and tibia were fused incompletely and were malformed at their distal ends (94). When compared to Hoxa11$^{-/-}$ and Hoxd11$^{-/-}$ knockouts, disruption of both Hoxa11 and Hoxd11 genes resulted in a much more severe phenotype. In Hoxa11$^{-/-}$/Hoxd11$^{-/-}$ double mutant mice, the radius and ulna were almost missing. Hoxa11$^{+/-}$/Hoxd11$^{-/-}$ and Hoxa11$^{-/-}$/Hoxd11$^{+/-}$ mice had an intermediate phenotype with reduced length of radii and ulnas. This phenotype demonstrates a dose-dependent synergy between Hoxa11 and Hoxd11 in specifying forelimb zeugopod. Hoxa11$^{-/-}$/Hoxd11$^{-/-}$ double mutants also exhibited hindlimb defects that were not observed in single mutants, such as the absence of proximal tarsal bones (95). There is also evidence for genetic interaction between nonparalogous genes. Hoxa10$^{-/-}$/Hoxd11$^{-/-}$ mice had truncated radii and ulnas, which was not seen in either Hoxa10$^{-/-}$ or Hoxd11$^{-/-}$ single mutants. In addition, the carpal and digital defects seen in Hoxd11$^{-/-}$ mutants were exacerbated in double mutants (89).

Consistent with their position along the gene cluster, Hoxd12 and Hoxd13 gene disruption affects autopod patterning. Hoxd12$^{-/-}$ mice have mild defects in digit II and V. The metacarpals and phalanges of the digits are shortened (96). When compared with the Hoxd12 mutation, Hoxd13$^{-/-}$ mice have –more severe defects. All five digits are affected. Many metacarpal and phalangeal bones are strongly shortened. Some phalanges are absent. Extra rudimentary digits were detected in some mice (96;97). In extreme cases, mice were generated with simultaneous inactivation of Hoxd11, Hoxd12 and Hoxd13. This multiple deficiency in Hox function resulted in a dramatic size reduction (ectrodactyly), extra digits (polydactyly), and improper digit fusion (98). Other studies (99;100) provide evidence for involvement of Hoxa13 in the genetic interactions that mediate autopod patterning. In addition to the experimental loss-of-function studies, there are naturally occurring Hox mutations. For example, *Hypodactyly* (*Hd*) in mice has a deletion mutation of Hoxa13 (101), and human Synpolydactyly (SPD) is due to an in-frame insertion mutation of HOXD13 (102).

Taken together, the 5' HoxA and HoxD genes play crucial roles during limb patterning, with the more 3' Hox genes specifying proximal parts and the more 5' Hox genes specifying distal parts. As the number of Hox genes is limited, the interactions between paralogous as well as nonparalogous genes are important for the tissue diversity seen in vertebrate limb development.

8. CONCLUSION

BMPs are among the signal molecules that play crucial roles in a wide range of biological processes including patterning, cartilage differentiation and programmed cell death during limb development. At different stages of embryonic development, BMP members show distinct expression patterns that are tightly regulated. Therefore, BMP-mediated tissue responses vary according to different biological contexts. Due to the limited number of receptors and Smads within the BMP pathway, the crosstalk between BMP and other signaling pathways appears to be very important for the diverse functions associated with BMPs. Although the detailed mechanisms are not fully understood, interactions between different signaling pathways have been demonstrated to be essential for proper morphogenesis. The recruitment of distinct intracellular factors adds another level of specificity and diversity. Among these factors are the Hox proteins, which have distinct functions during limb development. Further studies on Hox involvement in the BMP signaling pathway will provide additional information on mechanisms governing limb development.

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