β-Catenin Signaling Pathway Is Crucial for Bone Morphogenetic Protein 2 to Induce New Bone Formation*

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Endochondral ossification is recapitulated during bone morphogenetic protein (BMP)-induced ectopic bone formation. Although BMP and β-catenin have been investigated in bone development and in mesenchymal cells, how they interact in this process is not clear. We implanted recombinant BMP-2 into the muscle of mice to investigate the effect of β-catenin signaling on BMP-induced in vivo endochondral bone formation. BMP-2 induced expression of several Wnt ligands and their receptors and also activated β-catenin-mediated T cell factor-dependent transcriptional activity. An adenovirus expressing Dickkopf-1 (Dkk-1, an inhibitor of canonical Wnt pathway) inhibited β-catenin signaling and endochondral bone formation. Interestingly, Dkk-1 inhibited both chondrogenesis and osteogenesis. Likewise, mice expressing conditional β-catenin null alleles also displayed an inhibition of BMP-induced chondrogenesis and osteogenesis. This is in contrast to studies of embryonic skeletogenesis, which demonstrate that β-catenin is required for osteogenesis but is dispensable for chondrogenesis. These findings suggest that embryonic development pathways are not always recapitulated during post-natal regenerative processes, and the biochemical pathways utilized to regulate cell differentiation may be different. During in vivo ectopic bone formation, BMP-2 induces β-catenin-mediated signaling through Wnt ligands, and β-catenin is required for both chondrogenesis and osteogenesis.

Bone arises from condensation of undifferentiated mesenchymal stem cells during embryogenesis. Bone develops through two different mechanisms, endochondral and intramembranous ossification (1). Endochondral bone develops from a cartilaginous template forming by the recruitment and condensation of mesenchymal cells, which differentiate into chondrocytes. Cartilage is then replaced with osteoblasts and bone matrix. Endochondral bone formation gives rise to the axial and appendicular portions of the skeleton and is recapitulated during fracture repair (2, 3). Intramembranous ossification occurs by direct formation of bone, bypassing the cartilaginous template. A variety of signaling pathways have been investigated in endochondral bone development, including Wnt signaling and bone morphogenetic protein (BMP) signaling (4–6).

The Wnt signaling pathway plays a critical role in processes such as body-axis formation, central nervous system development and axial specification during limb development (6–7). This pathway consists of secreted ligands (Wnts) and various secreted and membrane-bound antagonists of Wnt signaling. These components activate transmembrane receptors such as low density lipoprotein receptor related protein receptor related protein-5/6 (Lrp-5/6) and Frizzleds (Fzs). There are at least three different Wnt signaling pathways, including the canonical Wnt pathway, which regulates β-catenin, the planar cell polarity pathway, and the Wnt/Ca2+ pathway.

Signaling through the canonical Wnt pathway is initiated by Wnt ligands activating Fzs and Lrp-5/6. In the absence of appropriate Wnt ligands, β-catenin is targeted for phosphorylation, ubiquitination, and proteosomal degradation by a multiprotein complex comprising glycogen synthase kinase-3β (GSK-3β), APC, and Axin (8–10). In the presence of an appropriate Wnt ligand, this multiprotein complex does not target β-catenin for degradation, and β-catenin can translocate to the nucleus, where in concert with members of the T cell factor/lymphoid-enhancer-factor (Tcf/Lef) family, activates the transcription of a wide range of genes.

Studies on embryonic skeletogenesis have demonstrated that Wnt/β-catenin signaling represents a mechanism in mesenchymal precursor cells for selecting between chondrocytic and osteoblastic fates. For example, based on experiments with loss- and gain-of-function alleles of β-catenin, Hill et al. (11) demonstrated that β-catenin is required to repress chondrocytic differentiation. In the absence of β-catenin, progenitor cells differentiated into chondrocytes instead of osteoblasts (11, 12). In addition, Wnt/β-catenin signaling regulates bone density by activating osteoblasts and inhibiting osteoclasts (13, 14).

BMPs are members of transforming growth factor β gene superfamily, which signal through Smad transcription factors.

The abbreviations used are: BMP, bone morphogenetic protein; ALP, alkaline phosphatase; Dkk, dickkopf; Fz, Frizzled; HE, hematoyalin-eosin; GSK-3β, glycogen synthase kinase 3β; Lrp, low density lipoprotein receptor-related protein; SO, Safranin O; Tcf, T cell factor; Lef, lymphoid enhancer factor; GFP, green fluorescent protein; RT, reverse transcription; PKC, protein kinase C.

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Some BMPs have demonstrated their ability to induce ectopic bone formation, such as BMP-2. When administered in skeletal muscle, BMP-2 induces bone formation via an endochondral mechanism (15–17). Although previous studies have investigated BMP, Wnt, β-catenin, and their interactions in mesenchymal cells, the role of these factors, and their interactions in endochondral bone formation is not completely elucidated. Furthermore, the role of Wnt and β-catenin in ectopic ossification in mature tissues in vivo is unknown. In this study, we aim at investigating the role of β-catenin signaling pathway in BMP-2-induced in vivo ectopic endochondral bone formation.

**EXPERIMENTAL PROCEDURES**

**BMP-2 and Dkk-1—**Human recombinant BMP-2 (rhBMP-2) was obtained from ProSpec-Tany TechnoGene (Rehovot, Israel). Adenovirus harboring murine Dickkopf-1 (Dkk-1) cDNA and C-terminal His6 and Flag epitope tags (Ad-Dkk1) was generated as previously described (18). Dkk-1 is a secreted protein that binds to Lrp-5/6 and Kremen proteins, thus inducing Lrp endocytosis, which prevents the formation of Wnt-Fz-Lrp endocytosis, which prevents the formation of Wnt-Fz-Lrp-5/6 receptor complexes and blocks canonical Wnt signaling (19–21). As a control, the same adenovirus expressing GFP was utilized.

Transgenic Mice—Generation of transgenic mice expressing homozygous β-catenin floxed alleles was previously described (22). These mice possess loxP sites located in introns 1 and 6 of the β-catenin (Catnbfloxed/floxed) gene, resulting into conditional Catnb deletion when treated with a Cre recombinase. Recombination of the loxP sites was accomplished as previously reported using infection with an Ad-Cre (23). This results in recombination in 75% of cells. Tcf reporter mice, as described previously (24, 25), contain a LacZ gene downstream of a c-fos minimal promoter and three consensus Tcf-binding motifs. Binding of a β-catenin-TCF complex activates the expression of LacZ.

Cell Culture—Primary calvarial osteoblasts were prepared from calvaria of 2- or 3-day-old Tcf reporter mice by a sequential enzymatic digestion method as described previously (26). Cells were plated in multiple wells and were exposed to rhBMP-2 at 0–400 ng/ml for 24 h. Cells were then harvested for mRNA and protein analysis. β-Galactosidase activity of cell lysate was conducted as described previously (25).

Reverse Transcription-PCR (RT-PCR) and Western Blot—Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. Quantitative RT-PCR was performed as described previously (25), with primers listed in Table 1. Expression was compared with β2-microglobulin as a housekeeping control. Our previous work shows excellent correlation between this quantitative PCR and real-time PCR under these conditions (27). All experiments were performed in triplicate. Western blot analysis was performed to determine protein expression (25). Nitrocellulose membranes (Amersham Biosciences) were probed with an antibody against gene of interest. Target protein bands were detected using horseradish peroxidase-conjugated secondary antibody and the ECL chemoluminescence detection system (Amersham Biosciences).

In Vivo BMP-2 Treatment—All animal procedures were reviewed and approved by our Laboratory Animal Care Committee. 12–14-Week-old male mice were utilized for this study. A 5-mm skin incision was made on the dorsal surface of the right lower limb, and a muscle pouch was produced in the gluteus of mice. Freeze-dried collagen type I sponges (Helisit, 5 × 5 × 5 mm3 in size, were loaded with 30 µg of rhBMP-2 or with same volume of phosphate-buffered saline as a control were placed into the muscle pouch. For adenovirus treatment, animals were injected with Ad-Dkk1, Ad-Cre, and Ad-GFP (control), respectively, at a dosage of 109 plaque-forming units per mouse 2 days before BMP-2 implantation.

At 3 days, 1, 2, and 3 weeks after operation, mice were sacrificed. Tissues from the site of BMP implantation were harvested, fixed in 4% paraformaldehyde, decalcified in 20% EDTA (pH 7.4), and embedded in paraffin. Sections were prepared stained using hematoxylin-eosin (HE) and Safranin O (SO). For RNA analysis, RNA was isolated using TRIzol Reagent (Invitrogen). Proteins were extracted using Tissue PE LB buffer (Bio-Rad) according to the manufacturer’s protocol.

**Immunohistochemistry and β-Galactosidase Assay—**For immunostaining, tissue sections were de-paraffinized, dehydrated, rinsed, and incubated with 0.3% H2O2 for 20 min, 3% bovine serum albumin in phosphate-buffered saline for 10 min to block nonspecific staining. A goat anti-mouse collagen type II or human anti-mouse collagen type X polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:50 was added to each section and incubated at 4 °C overnight. LacZ staining was performed as described previously (25) on tissues harvested from Tcf reporter mice fixed in 4% paraformaldehyde. Sections were counterstained with Neutral Red.

**TABLE 1**

PCR primer sequences used

| Oligonucleotide name | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|----------------------|--------------------------|-------------------------|
| β-Actin              | TTTTTTGGGCTACCTCTCTGTCG  | TGGATGCGCTACATGGCTCGG   |
| β2-Macroglobulin     | AAATCTTGTAAGACCTGAAA     | GATGGTACGTAAGACCTCGG   |
| ALP                  | CGGCGAGATGCTCCTCCCCCCC  | TGTTACCCGAGATCCCTTCT   |
| Osteocalcin          | GCAGCTGGTGCACAGCTTAG    | ACCCTTTGACCCCGCTGGTT   |
| β-Catenin            | GCCGCTGAACATGCTACTGAAG  | TATTAACTACGCCAGCTGGCTCT |
| Fz-1                 | TGGGCGCTCATCAAAACTATACAC| AGAGGAGACTGGTCAAGTC    |
| Fz-3                 | CTATTTGGTTCCTCCGCCC    | CATGTTTTTTGCTCAGCACCA  |
| Lrp-6                | GGTCGCAAAAGCCCTGGC     | GGCTGCAAGACTGGTCAAATTC |
| Wnt-7α               | GCTACTGCCACCCACCTCTTT  | GCCTCCACACACTGCTCCC   |
| Wnt-10b              | GATACCCACAACCCACACTC   | GGCTCCACCCACACTCTCCC  |
| Wnt-11               | CAGTACCGACTGACCTTTGCT  | TGTTTCCGACCTGTCGAC    |

**Figure 2**

Comparison of LacZ expression profile between a) control and Dkk-1 treatment, and b) control and Cre treatment. LacZ expression was examined at 3, 7, and 14 days after operation. LacZ expression was quantified using digital microscopy and Adobe Photoshop software. LacZ expression is shown in red. The red areas in the sections correspond to LacZ expression.
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**Antibodies**—The rabbit polyclonal antibody to β-catenin was purchased from Upstate (catalog number 06-734). The monoclonal antibody to Runx-2 was purchased from MBL (catalog number D130-3). The polyclonal antibodies to GS3β and phosphor-GSK-3β (Ser-9) were purchased from Cell Signaling Technology (catalog numbers 9332 and 9336). The monoclonal antibody to β-tubulin was purchased from Sigma (catalog number T0198). The monoclonal antibody to HA-probe (F-7) was purchased from Santa Cruz Biotechnology (catalog number sc-7392). The monoclonal antibody to Wnt-5α was purchased from R&D Systems (catalog number AF645). The monoclonal antibody to PKC-α, the horseradish peroxidase-conjugated antibody to mouse or rabbit IgG were purchased from BD Pharmingen (catalog numbers 554207, 554002, and 554021). The monoclonal antibody to β-actin was purchased from Calbiochem (catalog number CP01). The polyclonal antibody to Sox-9 was purchased from Abcam Inc. (catalog number ab3697).

**RESULTS**

**β-Catenin-mediated Signaling Is Activated during BMP-2-induced in Vivo Bone Formation**—Cell culture data show that BMP can induce or inhibit Wnt signaling in mesenchymal cells. To address the role of β-catenin in BMP induced in vivo bone formation, we implanted rhBMP-2/collagen complex into the hindlimb muscle of Tcf reporter mice and observed changes in Tcf-transcriptional activity using LacZ staining.

Three days after implantation, undifferentiated fibroblast-like mesenchymal cells were present on the surface of the BMP/ rhBMP-2 implantation, only slight LacZ staining was detected in osteoblasts lining the trabeculae and cortical matrix. The staining was also greatly decreased in adipocytes (data not shown). Implantation of the collagen sponge alone resulted in limited accumulation of fibroblast-like cells at the implantation site, and with only very mild LacZ staining in these cells. These results suggested that β-catenin-mediated Tcf-dependent transcription is activated during BMP-2 induction of endochondral bone formation, in particular, during early chondrogenesis (but not late stage of chondrogenesis) and throughout osteogenesis.

To determine the level of expression of β-catenin during these processes, we analyzed protein extracts from tissues harvested at different time period using Western analysis. As shown in Fig. 1I, β-catenin protein level increased starting following implantation of BMP-2, peaking at 1 week after implantation. The expression of β-catenin was also maintained at high level at 2 and 3 weeks after surgery, the time point when osteogenesis proceeds. These data suggested that the expression of β-catenin peaks at the stage of chondrogenesis, during endochondral ossification.

**BMP-2 Induces Expression of Wnt Ligands and Receptors**—To determine whether BMP regulates β-catenin by altering the expression of Wnt ligands, we examined the expression of Wnt ligands and frizzled or Lrp receptors using RT-PCR in primary calvarial cultures from the Tcf reporter mice. BMP-2 causes osteoblast differentiation, as confirmed by increased mRNA for both alkaline phosphatase (ALP) and osteocalcin, genes which are marker of osteoblast differentiation (Fig. 2A). Using semi-

**FIGURE 1. β-Catenin-mediated Tcf-dependent transcription is activated during BMP-2-induced ectopic bone formation**. rhBMP-2 at a dosage of 30 μg was implanted into the muscle of Tcf reporter mice. Samples were harvested, and Tcf-dependent transcriptional activity was determined using LacZ staining at different time points. A and E, 3 days after BMP-2 implantation; B and F, 1 week after BMP-2 implantation; C and G, 2 weeks after BMP-2 implantation; D and H, 3 weeks after BMP-2 implantation (A–D, original magnification (×50); E–H, higher magnification (×100)). There was no staining at day 3 after BMP implantation. One week following implantation, staining was detected in prechondrogenic mesenchymal cells and premature chondrocytes, although staining was absent in chondrocytes displaying a mature and hypertrophic phenotype. Osteoblasts at 2 and 3 weeks following BMP-2 implantation also showed positive staining. Cell lysates were examined using Western analysis, and a high level of β-catenin was observed during ectopic bone formation, peaking at 1 week after implantation (I).
quantitative RT-PCR, in which expression was compared with the housekeeping gene, β2-macroglobulin, we found that the expression of Wnt signaling family members was up-regulated by BMP-2. BMP-2 treatment increased expression of Wnt-7a, Wnt-10b, Wnt-11, and Wnt-13 mRNA in a dose-dependent manner. In a similar manner, expression of the Wnt receptors, Fz-1, Fz-3, Fz-10, and Lrp-6, was also increased with BMP-2 treatment (Fig. 2B). The protein level of β-catenin, as measured using Western analysis, showed a dose-dependent increase after BMP-2 treatment (Fig. 2C). Accordingly, β-catenin-mediated Tcf-dependent transcription was also activated after BMP-2 treatment, as detected using the β-galactosidase activity assay (Fig. 2D). Taken together, these data suggested an activation of Wnt/β-catenin signaling during BMP-2-mediated osteoblast differentiation.

Canonical Wnt activation is associated with the regulation of osteoblast differentiation. Primary calvarial osteoblast cells were treated with rhBMP-2 at different dosages (0, 100, and 400 ng/ml) for 24 h. Expression was examined using semiquantitative RT-PCR analysis, and protein levels were determined using Western analysis. β-Galactosidase activity was used to determine β-catenin-mediated Tcf-dependent transcriptional activity. A, induction of ALP and osteocalcin mRNA level by BMP-2, confirming osteoblastic differentiation. B, expression of several Wnt ligands and receptors during BMP-2-induced osteoblast differentiation. C, up-regulation of β-catenin protein by BMP-2 treatment. D, activation of β-catenin-mediated Tcf-dependent transcription by BMP-2 treatment. Error bars are 95% confidence intervals. BMP treatment induced expression of Wnts, Wnt receptors, and activated β-catenin-mediated Tcf-dependent transcription.

Since Wnt can act through canonical and non-canonical pathways, we also investigated expression of Wnt-5a and PKC-α from bone samples. Wnt-5a mediates calcium-dependent non-canonical pathway through PKC-α (28–30). As shown in Fig. 3B, significantly higher expression of Wnt-5a and PKC-α were detected after BMP implantation during the period in which primarily cartilage is formed and that the expression level gradually decreased as cartilage replaced by bone starting from 2 weeks time point. Thus, it is possible that non-canonical Wnt signaling plays a role during chondrogenesis.

Conditional Inactivation of β-Catenin Inhibits in Vivo Chondrogenesis and Osteogenesis Induced by BMP-2—To determine the function of β-catenin in BMP-2-induced endochondral bone formation, we conditionally inactivated the β-catenin gene using injection of Ad-Cre into the muscle of Catnb<sup>flox/flox</sup> transgenic mice 2 days before BMP implantation. β-Catenin level was reduced to roughly half of control animals with expression of the Ad-Cre (data not shown). These animals exhibited a substantially reduced chondrogenic differentiation at 1 week after BMP-2 treatment, characterized by significantly less cartilaginous matrix (Fig. 4, A–C and G–I). Immunostaining demonstrated that mice expressing the conditional β-catenin null alleles showed low levels of expression of type II (Fig. 5, A, C, D, and F) and type X collagen (Fig. 6, A, C, D, and F). Although some bone tissue and bone marrow were present 3 weeks following implantation, the bone volume was dramatically reduced as compared with mice expressing wild-type β-catenin alleles (Fig. 7, A–C and G–I). In contrast to previous studies of the role of β-catenin during embryonic bone development, these data show that β-catenin is required for osteogenesis as well as chondrogenesis during BMP-2-induced bone formation.
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Dkk-1 Inhibits in Vivo Chondrogenesis and Osteogenesis Induced by BMP-2—To determine whether the results we observed are mediated by the regulation of Wnt ligands by BMP, we used Ad-Dkk1 to block Wnt ligand-mediated signal-

FIGURE 4. Chondrogenesis induced by BMP-2 is inhibited by Dkk-1 or by expression of conditional null alleles of β-catenin. Ad-Dkk1, Ad-Cre, or control viruses were injected 2 days before BMP implantation. Samples were harvested and examined using SO staining. A–C, SO staining from control vector injected mice. D–F, SO staining from Ad-Dkk1-injected mice. G–I, SO staining from Ad-Cre-injected mice, which express β-catenin null allele (A, D, and G, original magnification (×25); B, E, and H, higher magnification (×50); C, F, and I, higher magnification (×100)). At 1 week following BMP implantation, treatment with either Ad-Dkk1 or Ad-Cre inhibited chondrogenic differentiation. An obvious lack of SO-stained cells is apparent. Cell lysate were examined by Western analysis. Expression of Dkk-1 was detected at this time point, and there was a lower level of β-catenin expression. J, detection of Dkk-1 expression using anti-His antibody at 1 week after BMP-2 implantation; K, detection of β-catenin expression at 3 weeks after BMP-2 implantation.

FIGURE 5. Dkk-1 or conditional null alleles of β-catenin expression by Cre-inhibited collagen type II expression. Ad-Dkk1, Ad-Cre, or control vectors were injected into mice expressing β-catenin conditional null alleles 2 days before BMP implantation. Samples were harvested and analyzed using immunostaining with a collagen type II antibody 1 week after BMP-2 treatment. A and D, immunostaining from control vector-injected mice. B and E, immunostaining from Ad-Dkk1-injected mice. C and F, immunostaining from Ad-Cre-injected mice (A–C, staining without collagen type II antibody; D–F, staining with collagen type II antibody; original magnification (×50)). Ad-Dkk1 or Ad-Cre treatment resulted into a substantially reduced level and area of collagen type II expression, as compared with control group. Because less cartilage formed, the area of cartilage did not fill the entire microscopic field in samples treated with Ad-Dkk1 or Ad-Cre, compared with controls.

FIGURE 6. Dkk-1 or conditional null alleles of β-catenin expression by Cre inhibited collagen type X expression. Samples as in Fig. 5 underwent immunostaining with a collagen type X antibody at 1 week after operation. A and D, immunostaining from control vector-injected mice. B and E, immunostaining from Ad-Dkk1-injected mice. C and F, immunostaining from Ad-Cre-injected mice (A–C, staining without collagen type X antibody; D–F, staining with collagen type X antibody; original magnification (×50)). Treatment either by Ad-Dkk1 or Ad-Cre resulted into a greatly inhibited collagen type X expression, as compared with control group.

FIGURE 7. Dkk-1- or Cre-mediated inactivation of β-catenin signaling inhibits osteogenesis induced by BMP-2. Ad-Dkk1, Ad-Cre, or control vectors were injected 2 days before BMP implantation. Samples were harvested and analyzed using hematoxylin-eosin staining 3 weeks after implantation. A–C, HE staining from control vector-injected mice. D–F, HE staining from Ad-Dkk1-injected mice. G–I, HE staining from Ad-Cre-injected mice (A, D, and G, original magnification (×25); B, E, and H, higher magnification (×50); C, F, and I, higher magnification (×100)). Treatment either by Ad-Dkk1 or Ad-Cre resulted into an inhibition of osteogenesis characterized by reduced number of trabeculae and osteoblasts, as compared with control group.
was no difference between Ad-Dkk1- and Ad-GFP-treated animals at this stage.

One week following BMP-2 implantation, SO staining displayed only limited areas with chondrogenic differentiation (Fig. 4, A–F). Immunohistochemistry showed only a small number of chondrocytes expressing type II and type X collagen, as compared with control animals (Fig. 5, A, B, D, and E and Fig. 6, A, B, D, and E). At 3 weeks after implantation, Ad-Dkk1-treated animals exhibited substantially less bone and nearly no bone marrow cavity. The implant was filled with large amount of undifferentiated mesenchymal-like tissues (Fig. 7, A–F). These results are nearly identical to the results in the mice expressing the conditional β-catenin null alleles, suggesting that the effect of BMP-2 on β-catenin signaling is mediated through a Wnt-dependent mechanism. Like the data from the mice expressing the β-catenin null alleles, these data also indicated that Wnt signaling is required for BMP to induce both chondrogenesis and osteogenesis.

Inhibition of Wnt/β-Catenin Signaling Suppresses Expression of Markers of Cartilage and Bone Formation—To further confirm that Dkk-1 inhibited both chondrogenesis and osteogenesis, we examined the protein level of Sox-9, a transcription factor important in chondrogenic differentiation (32, 33), and Runx-2, a transcription factor up-regulated during osteoblast differentiation (34). We found that both Sox-9 and Runx-2 were expressed at substantially lower levels in mice expressing Dkk-1 (Fig. 8, A and B).

**DISCUSSION**

In the present study, we demonstrated that BMP-2 induces expression of Wnt ligands and receptors, resulting in β-catenin-mediated Tcf-dependent transcriptional activity. Surprisingly, we found that Wnt/β-catenin signaling is required not only for osteoblast differentiation but also for cartilaginous differentiation during BMP-2-induced in vivo bone formation. When the β-catenin pathway was inactivated by treatment of Dkk-1 or by activating conditional null alleles of β-catenin, not only did we observe an inhibition of chondrocytes but also we also found a significant down-regulation of Sox-9, a marker of chondrocyte differentiation.

Although there are a variety of studies on BMP, Wnt, β-catenin, and bone (4–6), there is conflicting data on the role and interaction of these factors, especially in the early chondrogenic phases of endochondral ossification. As the exogenous application of BMP-2 in mouse muscle causes a reproducible, robust, and easily observable process of ectopic endochondral ossification in vivo (16, 17), it is an excellent system in which to study factors important in this process. Furthermore, ossification progresses through the same stages of endochondral development during the ectopic induction of bone by BMP-2 in mature animals as during fetal long bone development. Despite this similarity, in our case we are studying a reparative process in mature animals, rather than a developmental process in fetal progenitor cells, and as such our results need to be interpreted in this context.

Several studies examined the role of β-catenin during embryonic bone formation (11, 12, 35, and 36). These studies reported that β-catenin is crucial for precursor cells to differentiate to osteoblasts but not to chondrocytes. Indeed, β-catenin deficiency tilted differentiation away from an osteoblastic toward a chondrocytic phenotype (11, 12). In stark contrast to these studies, we observed that during BMP-2-induced ectopic endochondral bone formation, β-catenin deficiency inhibited cartilage formation as well. Although there are a number of potential explanations for the unanticipated finding, one possibility is that the difference is due to the different cell populations present between fetal and mature animals. As such, β-catenin could play a different role regulating differentiation between fetal and mature precursors.

There are a number of potential mechanisms by which Wnt signaling, β-catenin-mediated Tcf/Lef-dependent transcriptional activity, and BMP signaling can interact in mesenchymal cells (4–6). Previous studies in cell cultures showed a variety of such interactions. For instance, Zhang and Stott (37) reported that BMP-2 stimulation of micromass cultures down-regulated β-catenin-mediated signaling. In contrast, Dong et al. (38) suggested that in chick sternal chondrocytes, BMP activated Wnt signaling. Both Wnt and BMP play roles regulating osteoblastic differentiation in cell cultures, and these two pathways could interact with each other. Mbalavie et al. (39) showed that constitutively active β-catenin protein synergized with BMP-2 to increase osteoblast differentiation. In addition, the ability of BMP to induce alkaline phosphatase was found to require canonical Wnt signaling in a variety of mesenchymal cell lines, suggesting that a Wnt autocrine/paracrine loop is active during BMP-2-induced osteoblast matrix mineralization (40).

Furthermore, there are a number of other pathways with which BMP and Wnt interact in an intracellular context. Nakashima et al. (41) reported that canonical Wnt signaling inhibited the ability of BMP-2 to induce myotubes in the C2C12 cell line and that this was mediated by inhibitor of DNA binding/differentiation-1 (Id-1). They also found that BMP-2 induced β-catenin-mediated Tcf-dependent transcription in this same cell line. Liu et al. (42) recently demonstrated that Wnt and BMP signals were coupled through intracellular interactions of disheveled and Smad1. Our studies indicated that BMP-2-induced expres-
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Section of Wnts and that blockade of Wnt signaling using Dkk-1 not only inhibited β-catenin-mediated signaling in response to BMP-2 but also the ability of BMP-2 to induce chondrocyte and osteoblast differentiation. Thus, although various interactions between the pathway members may occur in an intracellular context, and previous cell culture studies found that BMP stimulation could up- or down-regulate β-catenin, we suggest that the overriding action of BMP-2 in vivo is up-regulation of Wnt ligands and activation of β-catenin signaling. This is similar to our previous findings in cutaneous wound healing, in which β-catenin is activated through growth factors expressed during the initiation of the reparative process (23). In BMP-2-stimulated ectopic bone formation, β-catenin is induced by the BMP-2 through a Wnt ligand-dependent mechanism.

Interestingly, during cartilage formation induced by BMP-2, we detected a high level expression of Wnt-5a and PKC-α, which decreased as the process progressed to bone formation. These results are consistent with findings that non-canonical Wnt pathway plays a role at early stage of endochondral ossification during embryonic limb development (28–30). These data suggest that both canonical and non-canonical Wnt signaling plays a role in chondrogenesis.

Although findings from fetal bone development showed that β-catenin is dispensable for chondrocytic differentiation, studies in cell culture have examined the role of Wnt signaling and β-catenin in chondrocyte behavior. The disparate results from these studies have confounded the understanding of the role of β-catenin-mediated signaling in chondrocytes. Some studies reported a role for Wnt signaling in regulating hypertrophic differentiation. For instance, Dong et al. (38) observed that Wnt signaling induced Runx-2 expression and hypertrophic differentiation in sternal chick chondrocytes. Yano et al. (43) found that Lef-1 and LiCl could induce chondrocyte differentiation in a Sox-9-dependent manner. Other investigations suggested that Wnt signaling has a negative effect on chondrocyte differentiation or that β-catenin did not play an important role in Wnt regulation of chondrocyte behavior. For instance, Reinhold et al. (44) demonstrated that the canonical Wnt target gene. Twist1, inhibited chondrogenesis and chondrocyte gene expression in chondrocyte cell cultures. Ryu and Chun (45) suggested that Wnt-5a and Wnt-11 regulate chondrocyte differentiation but through a non-canonical c-Jun- or PKC-mediated mechanism. Studies of overexpression of activated β-catenin-mediated signaling in mice indicated that activation of β-catenin-mediated signaling in immature cells blocked maturation, while activation in mature cells stimulated hypertrophy (36, 46). Recently, Gaur et al. (47) reported on studies of micromass cultures from mice lacking the Wnt antagonist, secreted frizzled related protein-1 (SFRP-1), and observed increased chondrogenesis and accelerated differentiation. Our results are in agreement with the data of Gaur et al. (47), in which activation of β-catenin-mediated signaling caused increased chondrogenesis. In our work, deficiency in β-catenin-mediated signaling inhibits chondrogenic differentiation.

During osteogenesis, the later phase of in vivo endochondral bone formation, we continued to observe β-catenin-mediated Tcf-dependent transcriptional activity. We also found a substantial inhibition of osteogenesis with β-catenin deficiency or inactivation of β-catenin signaling. Furthermore, there was also a dramatic down-regulation of Runx-2, a transcription factor expressed by osteoblasts. This is in agreement with studies in developmental processes showing that β-catenin plays a crucial role in osteoblast function (13, 47, and 48).

In this study, we found that BMP-2 induces Wnt and activates β-catenin-mediated signaling during ectopic endochondral ossification and that β-catenin-mediated signaling is active in early phases of chondrogenesis and osteogenesis. In our in vivo system, β-catenin is clearly induced by BMP-2, and BMP-2 imparts its activity via a Wnt ligand-dependent process. In stark contrast to studies showing that β-catenin is required for osteogenesis but dispensable for chondrogenesis during embryonic skeletogenesis, we found that β-catenin is crucial to not only osteogenesis but also chondrogenesis. These findings suggest that embryonic development pathways are not always recapitulated during post-natal regenerative processes, and the biochemical pathways utilized to regulate cell differentiation may be different.

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