Sequential roles of Hedgehog and Wnt signaling in osteoblast development

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Accepted 3 November 2004
Development 132, 49-60
Published by The Company of Biologists 2005
doi:10.1242/dev.01564

Summary
Signals that govern development of the osteoblast lineage are not well understood. Indian hedgehog (Ihh), a member of the hedgehog (Hh) family of proteins, is essential for osteogenesis in the endochondral skeleton during embryogenesis. The canonical pathway of Wnt signaling has been implicated by studies of Lrp5, a co-receptor for Wnt proteins, in postnatal bone mass homeostasis. In the present study we demonstrate that β-catenin, a central player in the canonical Wnt pathway, is indispensable for osteoblast differentiation in the mouse embryo. Moreover, we present evidence that Wnt signaling functions downstream of Ihh in development of the osteoblast lineage. Finally Wnt7b is identified as a potential endogenous ligand regulating osteogenesis. These data support a model that integrates Hh and Wnt signaling in the regulation of osteoblast development.

Key words: Hh, Wnt, Osteoblast, Mouse

Introduction
The molecular basis underlying osteoblast development is poorly understood. Collagen I and alkaline phosphatase (Ap) are among the earliest markers expressed by the osteoblast lineage (Aubin, 1998). Runx2, a runt-domain transcription factor, is essential for osteoblast differentiation as Runx2−/− mice possess a completely cartilaginous skeleton due to the lack of osteoblasts (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Similarly, mice lacking the osterix (Osx; Sp7 – Mouse Genome Informatics) gene, which encodes a zinc-finger transcription factor, do not develop bone, indicating an essential role of this factor in osteoblast differentiation (Nakashima et al., 2002). Osx is believed to function downstream of Runx2 as Runx2 is expressed normally in Osx−/− mice but Osx is not expressed in the Runx2−/− animals (Nakashima et al., 2002).

Indian hedgehog (Ihh) signaling is indispensable for osteoblast development in the endochondral skeleton. In developing cartilage, Ihh is primarily expressed by prehypertrophic chondrocytes (chondrocytes immediately prior to hypertrophy) as well as in early hypertrophic chondrocytes, and Ihh signals to both immature chondrocytes and the overlying perichondrial cells (St-Jacques et al., 1999; Vortkamp et al., 1996). Ihh−/− mice completely lack osteoblasts in the endochondral skeleton, at least in part because of the failure to express Runx2 (St-Jacques et al., 1999). Furthermore, Ihh is required for the formation of ectopic bone collar in growth plates containing both wild-type and PTH/PTHrP receptor null chondrocytes (Chung et al., 2001). Genetic manipulation of Smoothened (Smo), which encodes an obligatory component of the Hh signaling pathway, has revealed that cells devoid of Smo, hence Hh signaling, fail to undergo osteoblast differentiation (Long et al., 2004). Therefore, direct Ihh input in early osteogenic progenitors is necessary for osteoblast differentiation. However, it is not known whether Ihh interacts with other osteogenic signals in this regulation, or whether Ihh also functions at later stages of osteoblast development.

Wnt signaling has been implicated in postnatal bone mass homeostasis. Wnt molecules exert their functions by activating several distinct intracellular pathways, including that mediated by β-catenin, known as the canonical pathway (Huelsken and Birchmeier, 2001; Wodarz and Nusse, 1998). In this pathway, Wnt proteins signal through the Frizzled family of receptors and the low-density lipoprotein receptor-related proteins (Lrp5 and Lrp6, Arrow in Drosophila) as co-receptors, resulting in stabilization of β-catenin (Mao et al., 2001b; Pinson et al., 2000; Tamai et al., 2000; Wahrli et al., 2000). The stabilized β-catenin transports to and accumulates in the nucleus where it interacts with transcription regulators including the lymphoid enhancer-binding factor 1 (Lef1) and T-cell factors (Tcf1, Tcf3 and Tcf4), leading to transcriptional activation of downstream target genes (Eastman and Grosschedl, 1999). Recently, Lrp5 was found to be inactivated in patients with the osteoporosis-pseudoglioma syndrome (Gong et al., 2001). Conversely, an activating mutation in Lrp5 has been linked in two separate cases to individuals with a high bone density syndrome (Boyden et al., 2002; Little et al., 2002). Furthermore, mice deficient in Lrp5 were viable but postnatally developed a low bone mass phenotype because of reduced osteoblast proliferation and function (Kato et al., 2002). The endogenous Wnt ligand(s) that signal through Lrp5 have not been identified. Moreover, the role of the Wnt/β-catenin pathway in...
the development of the osteoblast lineage remains unclear as mice deficient in β-catenin die because of gastrulation defects by E8.5 before skeletal development occurs (Haegele et al., 1995; Huelsken et al., 2000). Interestingly, several studies have suggested that activation of canonical Wnt signaling can induce osteoblast differentiation in cell culture models (Bain et al., 2003; Gong et al., 2001; Rawadi et al., 2003).

Here, we examine the role of β-catenin in osteoblast development by removing it from early osteogenic tissues using the Cre/LoxP-mediated gene inactivation. We demonstrate that Wnt signaling is essential for osteoblast development and acts downstream of Hh. We also identify Wnt7b as a potential ligand controlling osteogenesis in vivo.

Materials and methods

Mouse strains

All mouse strains, including Ihh+/−, β-catenin+/− and Dermo1-Cre are as previously described (Brault et al., 2001; St-Jacques et al., 1999; Yu et al., 2003). The β-catenin+/− strain was acquired through the Jackson Laboratory (Bar Harbor, ME).

Analyses of mouse embryos

Embryonic tissues were collected in PBS, fixed in 10% formalin overnight at room temperature, then processed and embedded in paraffin prior to sectioning at 6 μm. For detection of mineralization, sections were stained with 1% silver nitrate (von Kossa method) and counterstained with Methyl Green. In situ hybridization was performed as described previously, using 35S-labeled riboprobes (Long et al., 2001) or using digoxigenin-labeled riboprobes (Brent et al., 2003). For BrdU analysis, pregnant females were injected with BrdU at 0.1 mg/g body weight at 2 hours prior to harvest. Embryos were collected in ice-cold PBS, processed and sectioned as above. BrdU detection was performed using a kit from Zymed Laboratories (South San Francisco, CA) as per instructions. Labeling index was scored for a least four sections from various planes of section through the cartilage. Multiple wild-type and mutant pairs of littersmates were analyzed and results from a representative pair are reported here. For immunohistochemistry, sections were prepared as above and stained with a monoclonal antibody against β-catenin (BD Biosciences/Pharmingen, San Diego, CA) following antigen retrieval using SDS (BD Biosciences/Pharmingen protocol). Signal was detected using the Vectorstain Elite ABC kit (Vector Laboratories, Burlingame, CA) and DAB as chromagen (Zymed Laboratories).

Cell lines and osteogenesis assays

The C3H10T1/2 cells, the Wnt3a-conditioned medium and the control conditioned medium were harvested as per instructions. The recombinant N-terminal fragment of Shh (N-Shh) was purchased from R&D Systems (Minneapolis, MN) and used at 1 μg/ml. C3H10T1/2 cells were cultured to confluence prior to N-Shh or Wnt3a stimulation. AP expression was detected either by substrate staining or a chemical assay as previously described (Katagiri et al., 1994). In experiments where both GFP fluorescence and AP staining were visualized, BCIP and NBT were used as substrates for AP (Roche Diagnostics Cooperation, Indianapolis, IN). To assess the effects of cycloheximide on Hh-induced gene expression, C3H10T1/2 cells were treated with N-Shh for 24 hours with the addition of either DMSO or 10 μg/ml cycloheximide (Sigma, dissolved in DMSO) before harvested for real-time PCR (see below).

Expression constructs and retroviral infections

Retroviral expression constructs were generated by a two-step procedure. The various cDNA fragments were first cloned into the vector pCIG which contains the GFP sequence following the internal ribosomal entry site (IRES) and the nuclear localization signal (NLS) (Megasan and McMahon, 2002). The fragment containing the cloned cDNA along with the IRES-NLS-GFP sequence was subsequently cloned into the retroviral vector pSGF (Ory et al., 1996). The Smo* cDNA was as previously described (Long et al., 2001). Dkk1 was donated by Dr Christopher Niehrs (Glinka et al., 1998). The dominant-negative form of Tcf4 (dnTcf4) was originally from Dr McCormick (Tetsu and McCormick, 1999). A control virus was also generated by cloning the NLS-GFP sequence into pSGF. Viruses were packaged as previously described (Ory et al., 1996). Viral titers were determined by counting the GFP-positive cells under a fluorescence microscope. For infections, cells ~50% confluent were incubated with the virus-containing medium for 24 hours, and then cultured in regular complete medium for an additional 4 days before harvest for either AP assay or RNA extraction. For bone nodule formation assays, C3H10T1/2 cells were infected with either the Smo* or the control virus for 24 hours and then cultured in regular complete medium in the presence of ascorbic acid and β-glycerophosphate for 2 weeks with medium changed as needed. The cells were finally fixed in 10% formalin and stained with Alizarin Red. For transient transfections of Wnt genes, full-length sequences of Wnt5a, Wnt7b or Wnt9a (ATCC) were cloned into pCIG and then transfected with Lipofectamine (GibcoBRL, Gaithersburg, MD).

Real-time PCR

Total cellular RNA was isolated using Trizol reagent (GibcoBRL). The cDNA was prepared using reagents from Roche Diagnostics Cooperation. Real-time PCR was performed on GeneAmp5700 (Applied Biosystems, Foster City, CA) as per instructions. All primers were designed using the ABI software Primer Express and sequences are available upon request. Three independent samples were analyzed for each condition and results were normalized to GAPDH in each sample. For analyses of cartilage samples, cartilage elements were isolated from the hindlimbs of E14.5 embryos and collected in liquid nitrogen. The cartilage was pulverized in liquid nitrogen with a small pestle fit in 1.5 ml tubes, and then extracted for RNA using the Trizol reagent.

Results

β-Catenin is critical for osteoblast development

To determine the role of canonical Wnt signaling in osteoblast development, we generated Dermo1-Cre; β-catenin<sup>Cre</sup> conditional knockout (CKO) embryos in which β-catenin was specifically removed from the progenitor cells giving rise to the skeleton. The Dermo1-Cre line directs Cre expression in the mesenchymal precursors of both chondrocytes and osteoblasts (Yu et al., 2003). The T-cell factor 1 (Tcf1) and dickkopf 1 (Dkk1) have been shown to be transcriptional targets of the Wnt canonical signaling pathway (Cong et al., 2003; Roose et al., 1999). At E14.5 both molecules are expressed in the perichondrium flanking the prehypertrophic and the hypertrophic regions in wild-type embryos (Fig. 1A, B, arrows), indicating active Wnt signaling within this domain. In the CKO littersmates, however, Tcf1 and Dkk1 are no longer detectable in the perichordium (Fig. 1A′, B′, arrows). These results confirm that removal of β-catenin abolishes canonical Wnt signaling in the perichondrial cells of Dermo1-Cre; β-catenin<sup>Cre</sup> embryos.

At E18.5 the mutant embryos exhibited shortened limbs and a twisted body axis. Whole-mount skeletal preparations revealed that the mutant skeleton lacked bone although
cartilage was present (data not shown). The von Kossa staining confirmed that bone was not present in either the endochondral or the intramembranous skeleton. Specifically, in the humerus of E18.5 wild-type embryos, a bone collar surrounding the cartilage characteristically extends from the diaphysis to the perichondrial region flanking the hypertrophic cartilage (Fig. 1C,C′, arrow), which also undergoes mineralization (Fig. 1C′, asterisk). In the humerus of E18.5 CKO embryos, no bone collar was detected on the cartilage surface (Figures 1D,D′, arrow) even though mineralization of hypertrophic cartilage occurred (Fig. 1D′, asterisk). Similarly, in the skull of wild-type embryos, the parietal bone is well ossified by E18.5 (Fig. 1E, arrow). However, no ossification was detected in a similar region in CKO embryos (Fig. 1E′, arrow).

Consistent with the lack of bone at the histological level, molecular analyses by in situ hybridization demonstrated that mature osteoblasts failed to develop in Dermo1-Cre; β-catenin<sup>−/−</sup> embryos. Collagen type I (Col1a1) was expressed at a lower level in early osteoblast progenitors but was subsequently upregulated with further differentiation. In the humerus of E18.5 wild-type embryos, robust expression of Col1a1 was detected in the perichondrium surrounding the prehypertrophic and hypertrophic cartilage (Fig. 1F, arrow), in the bone collar flanking the marrow cavity (Fig. 1F, ‘M′), as
Development

Expression of Col1a1 detected in the perichondrium (Fig. 1F, Col1a1; Bsp, bone sialoprotein (Bsp), ligament; L, ligament; M, marrow cavity. Asterisks indicate expression in chondrocytes. Arrowheads and arrows indicate various regions of the perichondrium (see text).

well as in the primary spongiosa (Fig. 1F, asterisk). In CKO littermates however, only a low level of Collal expression was detected in the perichondrium (Fig. 1F', arrow), with no Collal expression in the marrow cavity (Fig. 1F', ‘M’). Like Collal, bone sialoprotein (Bsp; Spp1 – Mouse Genome Informatics) was normally expressed in the perichondrium surrounding the prehypertrophic and hypertrophic cartilage (Fig. 1G, arrow), as well as in the primary spongiosa (Fig. 1G, asterisk). In CKO embryos however, Bsp was not detectable in either the perichondrium or the marrow cavity (Fig. 1G', arrow, ‘M’). Finally, osteocalcin (OC; Bglap1 – Mouse Genome Informatics) was normally expressed by mature osteoblasts present either in the bone collar (Fig. 1H, arrowhead), or in the primary spongiosa (Fig. 1H, arrowhead), but no OC was detectable in CKO embryos (Fig. 1H', arrow). Similarly in the skull, whereas Collal, Bsp and OC are normally all expressed in the frontal bone at E18.5 (Fig. 1I-K, arrows), none of these markers was detected in the equivalent regions of CKO littermates (Fig. 1I'-K', arrows). Thus, removal of β-catenin by Dermo1-Cre disrupted osteoblast differentiation.

Histological analyses of the developing long bones also revealed a significant delay in chondrocyte maturation in CKO embryos. At E14.5, chondrocytes at the diaphysis of a normal humerus became hypertrophic (Fig. 1L); this, however, was not the case in the CKO embryo where all chondrocytes remained small (Fig. 1L'). The delay in hypertrophy in the CKO mutant continued through E15.5, when only a small cluster of chondrocytes at the core of the humerus initiated hypertrophy (Fig. 1M', magenta arrow), whereas the vast majority of cells remained immature (Fig. 1M', orange arrows). By contrast, in E15.5 wild-type littermates, blood vessels invaded the hypertrophic cartilage of the humerus (Fig. 1M, inset). Extensive hypertrophy did eventually occur in the CKO mutant by E17.5 and this was accompanied by vascular invasion as evidenced by red blood cells in the core of the humerus (Fig. 1N'). However, no bone was detected in the mutant humerus at either E17.5 (Fig. 1N') or E18.5 (Fig. 1O') even though the primary spongiosa (Fig. 1N, arrows) and a bone collar (Fig. 1O, arrows) were evident in the wild-type littermate. Instead, the humerus cartilage in CKO mutants was surrounded by thin layers of perichondrial cells at E18.5 (Fig. 1O', arrows). Thus, β-catenin is required for both the normal schedule of chondrocyte hypertrophy, as well as bone formation.

**Molecular basis for osteoblast defect in Dermo1-Cre; β-catenin<sup>CKO</sup> embryo**

To characterize further the arrest of osteoblast development in Dermo1-Cre; β-catenin<sup>CKO</sup> (CKO) embryos, additional molecular analyses were performed on the humerus of E14.5 embryos. Consistent with the analyses at E18.5, Collal was normally expressed at a low level in the perichondrium towards the epiphysis (Fig. 2A, arrowhead) but was strongly upregulated in regions surrounding the hypertrophic cartilage (Fig. 2A, arrow). In CKO embryos however, a low level of Collal was maintained throughout the perichondrium without any increase at the diaphysis (Fig. 1A', arrowhead). Alkaline phosphatase (AP) was expressed in a similar pattern as Collal in the wild-type embryo (compare Fig. 2A with 2B, arrowheads and arrows), except that AP was also expressed by early hypertrophic chondrocytes (Fig. 2B, ‘H’). In the CKO embryo, only a low level of AP was detected in the perichondrium (Fig. 2B', arrowhead). No AP expression was evident in the chondrocytes of the E14.5 humerus, consistent with a delay in chondrocyte maturation with the removal of β-catenin (Fig. 2B'). The failure to upregulate Collal and AP in the perichondrium of the CKO mutant was not secondary to a delay in chondrocyte hypertrophy; as both markers remained expressed at low levels even at E18.5 after...
hypertrophy had occurred (Fig. 1F’; data not shown). These data indicate that in the absence of β-catenin, osteoblast development was arrested after low levels of Col1a1 and AP were expressed in the early progenitors.

Additional analyses were performed to define the arrest in osteoblast development. At E14.5 in the wild-type embryo, Runx2 was most robustly expressed in the perichondrium and in the hypertrophic chondrocytes (Fig. 2C, arrow and ‘H’, respectively), although it was also expressed at a low level in immature chondrocytes (Fig. 2C, asterisk). In the CKO embryo, Runx2 expression was maintained in both the perichondrium and immature chondrocytes (Fig. 2C’, arrow and asterisk respectively) although chondrocyte hypertrophy had not yet occurred. Osx was normally expressed at high levels in the perichondrium flanking the prehypertrophic and hypertrophic chondrocytes (Fig. 2D, arrow) in addition to a lower level in the prehypertrophic cells (Fig. 2D’, asterisk). However, it was not detectable in the CKO embryo (Fig. 2D’, arrow). The lack of Osx expression in the perichondrium was not secondary to the delay in chondrocyte maturation, as it was not detected there in these embryos at either E15.5 (Fig. 2E’) or E18.5 (Fig. 2F’) after hypertrophy had occurred. Therefore, osteoblast development was arrested after Runx2 but prior to Osx expression in the Dermo1-Cre; β-catenin/cembryo.

Compared with the Dermo1-Cre; β-catenin/c mutant, Ihh–/–embryos exhibited an earlier defect in osteoblast development. Specifically, at E14.5 Col1a1 was not detectable in the perichondrium of long bones of the Ihh–/–mutant (Fig. 2A’’, arrowhead), although Col1a1 was expressed in the ligament (Fig. 2A’’, ‘L’). Similarly, no AP-expressing cells were present...
in the perichondrium of the Ihh\textsuperscript{−/−} mutant (Fig. 2B, arrowhead). Moreover, Runx2 was not expressed in the perichondrium of the Ihh\textsuperscript{−/−} mutant (Fig. 2C, arrow) although scattered expression was detected in chondrocytes (Fig. 2C', asterisk). Similarly, the Ihh\textsuperscript{−/−} mutant did not exhibit any expression of Osx in the perichondrium (Fig. 2D, arrow), although a low level of expression in chondrocytes was detectable (Fig. 2D', asterisk). These results demonstrate that Ihh signaling is required for the initiation of the osteogenic program.

\textbf{β-Catenin promotes chondrocyte maturation and proliferation}

Consistent with observations at the histological level as described earlier, molecular analyses confirmed that β-catenin was necessary for the proper maturation of chondrocytes. At E15.5 in the wild-type humerus, strong expression of collagen type X Col10a1, a specific marker for hypertrophic chondrocytes (Linsenmayer et al., 1991), characteristically defines two hypertrophic zones (Fig. 3A, ‘H’) separated by a nascent marrow cavity (Fig. 3A, ‘M’). In the CKO embryos, however, expression of Col10a1 had just begun in a small cluster of cells in the center of the humerus (Fig. 3A', arrow), indicating a marked delay in chondrocyte maturation. Consistent with the delay, Ihh expression, which delineates two discreet prehypertrophic domains in the wild-type humerus at E15.5 (Fig. 3B), remained in a contiguous region of the diaphysis in a CKO littermate (Fig. 3B'). Patched 1 (Ptc1h), which encodes the receptor for Hh proteins, is also a transcriptional target of the Hh pathway (McMahon et al., 2003). In the wild-type embryo, Ptc1h was characteristically upregulated in the immature proliferating chondrocytes (Fig. 3C, ‘P’) adjacent to the Ihh-expressing domain, at the primary spongiosa within the marrow cavity (Fig. 3C, ‘M’), as well as in the perichondrium flanking the Ihh-expressing chondrocytes (Fig. 3C, arrow). Remarkably, in the CKO embryo, although Ptc1h expression in the chondrocytes was similar to the wild-type level, expression in the perichondrium was much reduced (Fig. 3C', arrow). Thus, the osteoblast defect in the CKO embryo correlates with a suppressed response of the perichondrial cells to Ihh signaling.

Chondrocyte proliferation was also impaired in the Dermo1-Cre; β-catenin\textsuperscript{−/−} mutant. The BrdU labeling experiments showed that at E15.5 in CKO embryos the proliferation rate of chondrocytes was reduced by ~40% compared with that of wild-type littermates (Fig. 3D). The defects in chondrocyte maturation and proliferation described here are similar to that observed in the Col2a1-Cre; β-catenin\textsuperscript{−/−} mutant (Akiyama et al., 2004).

\textbf{Canonical Wnt signaling is disrupted in Ihh\textsuperscript{−/−} embryos}

We next examined the potential relationship between Ihh and canonical Wnt signaling in osteoblast development. Immunohistochemistry was performed on sections of long bones to determine the distribution of β-catenin. At E14.5, β-catenin was normally detected in both prehypertrophic and early hypertrophic chondrocytes (Fig. 4A, ‘PH’ and ‘EH’ respectively) as well as in the perichondrium (Fig. 4A, box). Immature and proliferating chondrocytes, on the other hand, were largely negative for the β-catenin signal (Fig. 4A, ‘P’). At higher magnification, β-catenin clearly accumulated in the nucleus of the perichondrial cells flanking the prehypertrophic chondrocytes (Fig. 4A, arrows). Some staining was also observed at the boundaries of the cells (Fig. 4A, arrowheads), probably reflecting β-catenin in association with the adherens junction. Remarkably, in the Ihh\textsuperscript{−/−} mutant, no nuclear β-catenin was detectable in perichondrial cells although low levels of β-catenin were detected at the cell boundaries (Fig. 4B, arrowheads). In addition, in Ihh\textsuperscript{−/−} embryos, no signal was detected in chondrocytes (Fig. 4B, asterisk), consistent with the absence of hypertrophic chondrocytes at this stage.

To discern whether the lack of nuclear β-catenin in the perichondrial cells was due to the delay in chondrocyte hypertrophy in Ihh\textsuperscript{−/−} embryos, we examined β-catenin localization at E16.5 when hypertrophy has occurred in the mutant animals. In E16.5 wild-type embryos, β-catenin continued to be present in the prehypertrophic and early
hypertrophic chondrocytes, but was not found in late hypertrophic chondrocytes (Fig. 4C, ‘PH’, ‘EH’ and ‘LH’ respectively). In addition β-catenin also accumulated in the ‘columnar chondrocytes’ (chondrocytes organized in columns in the growth plate, Fig. 4C, ‘C’), as well as in chondrocytes at the periphery abutting the perichondrium (Fig. 4C, arrow). Importantly, the perichondrial cells flanking the prehypertrophic chondrocytes accumulated a high level of β-catenin in the nucleus (Fig. 4C, box; Fig. 4C, red arrows). The high level of nuclear accumulation appeared to be restricted to a small population of cells in the perichondrium, as the neighboring perichondrial cells closer either to the epiphysis or to the diaphysis did not exhibit such a significant level of β-catenin (Fig. 4C, box; Fig. 4C, red arrows). The high level of nuclear accumulation appeared to be restricted to a small population of cells in the perichondrium, as the neighboring perichondrial cells closer either to the epiphysis or to the diaphysis did not exhibit such a significant level of β-catenin (Fig. 4C, box; Fig. 4C, red arrows).

In E16.5 Ihh−/− embryos, no significant nuclear staining for β-catenin was observed (Fig. 4D, asterisks), probably reflecting a dysregulated process of chondrocyte maturation in the Ihh−/− mutant. Finally, chondrocytes adjacent to the perichondrium maintained a wild-type level of β-catenin (Fig. 4D, arrows). In situ hybridization for Col10a1 confirmed that a majority of chondrocytes became hypertrophic at E16.5 in the Ihh−/− embryo (Fig. 4E). Therefore, β-catenin normally accumulates in the nucleus of perichondrial cells within the osteogenic region; however, this accumulation is disrupted in the absence of Hh signaling.

We next examined canonical Wnt signaling in the long bones of wild type versus Ihh−/− embryos by assaying the expression of target genes Tcf1 and Dkk1. As described earlier, in E14.5 wild-type embryos, both Tcf1 and Dkk1 were expressed in the perichondrium flanking the prehypertrophic and the hypertrophic regions (Fig. 5A,B, arrows). Tcf1 was also expressed at a lower level in prehypertrophic and proliferating chondrocytes (Fig. 5A, ‘PH’ and ‘P’ respectively). In the Ihh−/− littermate, however, no expression was detected in the perichondrium for either Tcf1 or Dkk1 (Fig. 5A′,B′, arrows), although expression of Tcf1 in the chondrocytes was maintained (Fig. 5A′,C′). Other members of the Lef/Tcf family were not detectable in the perichondrium of the E14.5 wild-type tibia (Fig. 5C,E, arrows), but were expressed in chondrocytes in distinct patterns. In particular, Lef1 expression was largely restricted to proliferating chondrocytes (Fig. 5D); Tcf4 was expressed both in the proliferating chondrocytes and at a higher level in hypertrophic chondrocytes (Fig. 5E, ‘P’ and ‘H’ respectively). In the Ihh−/− littermate, whereas Tcf3 and Tcf4 remained expressed in the chondrocytes (Fig. 5D’,E’, ‘C’), Lef1 was no longer detectable (Fig. 5C’,C’). Thus, Tcf1 is the primary member of the Lef/Tcf family expressed in the perichondrium. Moreover, the canonical Wnt signaling pathway is impaired in the Ihh−/− mutant.

**Hh-induced osteogenesis requires Wnt signaling.**

To gain insight in the functional relationship between Hh and Wnt signaling, we investigated osteogenesis induced by Hh and Wnt in C3H10T1/2 cells. The pluripotent mouse embryonic mesenchymal cell line C3H10T1/2 gives rise to mesodermal cell types, including osteoblasts, chondrocytes and adipocytes in response to external stimuli (Taylor and Jones, 1979). Consistent with previous reports (Kinto et al., 1997; Nakamura et al., 1997; Spinella-Jaegle et al., 2001; Yuasa et al., 2002), recombinant Hh protein (N-Shh) induced alkaline phosphatase (AP) expression in these cells as early as...
day 2 of Hh stimulation (Fig. 6A). AP expression reached very high levels by day 4 and continued to rise at day 6 (Fig. 6A). To examine whether Hh signaling induced osteogenesis in C3H10T1/2 cells in a cell-autonomous manner, a constitutively active allele of Smo (Smo*) that cell-autonomously activates Hh signaling (Long et al., 2001; Xie et al., 1998) was expressed in the cells via a retroviral vector. The green fluorescent protein (GFP) with a nuclear localization signal (NLS) was co-expressed with Smo* via an internal ribosome entry site (IRES) to monitor expression. The cells were infected at a low titer of virus to ensure a low percentage of infected cells and the cells were eventually stained for AP activity. This experiment demonstrated that AP expression was activated exclusively in the cells expressing nuclear GFP hence Smo* (Fig. 6A, compare B with B′, where the same number represents the same cell). Cells that were in direct contact with the GFP-positive cells, but were themselves negative, were never found to express AP. Interestingly, all GFP-positive cells did not express AP (Fig. 6A, compare B with B′, asterisks), indicating heterogeneity of the culture such that not all cells responded to Hh to undergo osteoblast differentiation. Furthermore, with ascorbic acid and β-glycerophosphate, constitutive Hh signaling by virally expressed Smo* in C3H10T1/2 cells was sufficient to induce bone nodules stained by Alizarin Red (Fig. 6C, arrows). Thus, consistent with the previous finding that Hh signaling is directly required for osteoblast development in vivo (Long et al., 2004), Hh signaling cell-autonomously stimulates osteogenesis in C3H10T1/2 cells.

To gain insight to the osteogenic process induced by Hh signaling, expression of osteoblast markers were examined by real-time PCR following stimulation with N-Shh. By day 3 of Hh treatment, the mRNA levels for Coll1a1 and Bsp were both induced significantly more than controls (Fig. 6D,E). Interestingly, Runx2 was only modestly induced by Hh during 3 days of treatment (Fig. 6F), whereas expression of Osx was increased approximately threefold over the control at all time points (Fig. 6G). Moreover, stimulation of Osx by Hh did not require de novo protein synthesis, as cycloheximide was unable to inhibit its induction (Fig. 6I), although it did repress the induction of Runx2 (Fig. 6H). Thus, Hh-induced osteogenesis in C3H10T1/2 cells correlates with a modest increase of Runx2 and a marked induction of Osx expression. These results demonstrate dual roles for Hh signaling in osteoblast development, which were not possible to discern in the Ihh−/− mutant owing to the early block in osteogenesis.

We next investigated osteogenesis induced by Wnt signaling in C3H10T1/2 cells. Wnt3a-conditioned medium induced AP as early as day 2 of treatment, and AP expression reached a maximum after 4 days of stimulation (Fig. 7A). Like AP, other osteoblast markers, including Coll1a1, osteopontin (OP; Spp1 – Mouse Genome Informatics) and OC were all significantly induced within 48 hours of Wnt3a stimulation (Fig. 7D-F, respectively). However, neither Runx2 nor Osx was induced during this time (Fig. 6B,C, respectively). Thus, in C3H10T1/2 cells Wnt3a can induce osteogenesis without changes in either Runx2 or Osx. These results therefore reveal a second role for Wnt signaling that is downstream of Runx2 and Osx, and is in addition to its requirement for Osx expression as described earlier.

We next determined whether Wnt signaling was required for Hh-induced osteogenesis. To this end, we generated retroviruses that express either Dkk1, a secreted antagonist that prevents Wnt ligands from binding to the LRPS/6 co-receptor (Mao et al., 2001a), or a dominant-negative form of Tcf4 (dnTcf4) that prevents transcriptional activation by canonical Wnt signaling (Tetsu and McCormick, 1999). Consistent with the results from the staining experiment described earlier (Fig. 6B and 6B′), virally expressed Smo* induced AP expression in C3H10T1/2 cells (Fig. 7G, red bar). Importantly, when either the Dkk1 or the dnTcf4 virus was co-infected at similar titers with the Smo* virus, AP expression was reduced by ∼50% (Fig. 7G). Similar inhibition was detected at the mRNA level by real-time PCR for both AP and Bsp (Fig. 7H,I,
Hh and Wnt in osteoblast development

Thus, Hh-induced osteogenesis is at least in part mediated by canonical Wnt signaling.

**Wnt7b as a potential endogenous signal controlling osteogenesis**

As an initial step to identify endogenous Wnt ligands that mediate Hh-induced osteogenesis, we examined the expression of Wnt genes in relation to Hh signaling. We first surveyed all 19 murine Wnt molecules by real-time PCR in C3H10T1/2 cells following Hh stimulation. These experiments revealed that Wnt5a, Wnt7b and Wnt9a were consistently induced over control levels following 24 or 48 hours of Hh treatment (Fig. 8A,B, respectively). Moreover, induction of Wnt7b and Wnt9a did not require de novo protein synthesis, as cycloheximide did not inhibit it although it did repress Wnt5a induction. The mechanism for the induction of Wnt7b and Wnt9a expression by cycloheximide is not understood but a similar stimulatory effect has been previously reported (Mullor et al., 2001).

We next examined expression of Wnt5a, Wnt7b and Wnt9a in the developing long bones of both wild-type and Ihh−/− embryos. At E14.5, Wnt5a was normally expressed by prehypertrophic chondrocytes (Fig. 8F, asterisk) as well as perichondrial cells flanking the prehypertrophic and hypertrophic chondrocytes (Fig. 8F, arrow). This expression pattern was largely maintained in the Ihh−/− embryo, although chondrocyte hypertrophy was delayed at this stage (Fig. 8F′). Wnt9a was normally expressed in the outer layers of the perichondrium surrounding either the shaft or the joint region of the cartilage element (Fig. 8H, red arrow and orange arrow, respectively); the expression was much reduced in the Ihh−/− embryo (Fig. 8H′). Finally, in the wild-type embryo, Wnt7b was strictly expressed by perichondrial cells flanking the prehypertrophic chondrocytes (Fig. 8G, arrow), but the expression was abolished in the Ihh−/− mutant (Fig. 8G′, arrow). Wnt7b downregulation was confirmed by real-time PCR with RNA from limb cartilage elements isolated from E14.5 Ihh−/− versus wild-type embryos (Fig. 8I). Thus, Wnt7b is specifically expressed by potential osteogenic cells in the developing long bone and is regulated by Hh signaling.

We next determined whether Wnt5a, Wnt7b or Wnt9a could...
Development immunostaining with an antibody against catenin did not cause any noticeable changes in the osteoblast development. A comparative analysis with the component of the canonical Wnt pathway is required for osteoblast development.


demonstrates that Wnt signaling at least in part mediates during early phases of osteogenesis. Results from C3H10T1/2

In this study, we provide genetic evidence that Runx2 initiate the osteogenic program by activating expression of signals control osteoblast development in a sequential manner (Fig. 9). On the other hand, Hh acts on very early progenitors to initiate the osteogenic program by activating expression of Runx2, low levels of Coll1 and AP (Fig. 9, ‘1’). This phase of signaling appears to require only a weak Hh response, as indicated by low levels of Ptc1 expression. On the other hand, Hh induces expression of Wnt ligands that signal through β-catenin, which is in turn required for Osa expression and further osteoblast differentiation (Fig. 9, ‘2’). Whereas Wnt/β-catenin signaling is not sufficient to induce Osa, Hh can directly stimulate Osa expression (Fig. 9, ‘3’), and this phase of signaling correlates with a strong Hh response, as reflected by robust Ptc1 expression. Finally, Wnt signaling can promote osteoblast differentiation without inducing Osa expression (Fig. 9, ‘4’). In summary, Hh and Wnt signals orchestrate the progression of osteoblast development.

**Discussion**

In this study, we provide genetic evidence that β-catenin, a key component of the canonical Wnt pathway is required for osteoblast development. A comparative analysis with the Ihh-/- mutant places Wnt signaling downstream of Ihh signaling during early phases of osteogenesis. Results from C3H10T1/2 cells demonstrate that Wnt signaling at least in part mediates osteogenesis induced by Hh proteins. Finally, Wnt7b is identified as a potential endogenous Wnt signal important for osteoblast development.

Despite its role in the adherens junctions, removal of β-catenin did not cause any noticeable changes in the morphology and organization of cells. Moreover, immunostaining with an antibody against α-catenin, which normally interacts with β-catenin, did not show any changes in the distribution in either chondrocytes or the perichondrial cells of the Dermo1Cre; βcatc/c mutant (data not shown). It is likely that plakoglobin, which is closely related to β-catenin substitutes for β-catenin to maintain the integrity of adherens junctions, as previously reported in the β-catenin-/- embryo (Huelsken et al., 2000). Similarly, deletion of β-catenin from the apical ectodermal ridge (AER) of the limb bud did not change the distribution of E-cadherin in the ectoderm (Barrow et al., 2003). Thus, the osteoblast defect observed in the present study is most likely due to disruption of canonical Wnt signaling. In keeping with this notion, β-catenin normally accumulates at high levels in the nucleus of potential osteogenic cells in the developing long bone, and expression of the Wnt target genes Tcf1 and Dkk1 was abolished in the Dermo1Cre; βcatc/c mutant.

Although bone was largely absent throughout the Dermo1Cre; βcatc/c embryo, some bone formation was observed in the scapula of the mutant. Interestingly, expression of Tcf1 and Dkk1 was detected in the scapula of the mutant embryo at E15.5 (data not shown). This most probably reflects incomplete removal of β-catenin in the scapula by Dermo1-Cre, resulting in residual Wnt signaling in this tissue. Consistent with the crucial role of β-catenin in osteogenesis, a previous study showed that removal of β-catenin from the cranial neural crest cells by Wnt1-Cre resulted in agenesis of most craniofacial bones (Brault et al., 2001).

Whereas Wnt/β-catenin is required for osteogenesis throughout the skeleton, Ihh is required only for the endochondral bones. Recently, Smo, which encodes an obligatory component of the Hh pathway, was removed with Wnt1-Cre to eliminate Hh response in cranial neural crest cells (CNCC) that normally give rise to many of the craniofacial bones via intramembranous ossification (Jeong et al., 2004). This resulted in the absence of most CNCC-derived head bones. However, owing to the early growth and patterning defects associated with an increase in apoptosis and a decrease in proliferation, it is difficult to conclude whether osteogenesis per se was impaired by the removal of Hh responsiveness. Thus, it remains to be determined whether Hh or other signals act upstream of Wnt/β-catenin signaling in the head skeleton.

Interestingly, Hh response in the perichondrium is compromised in the Dermo1Cre; βcatc/c embryo. Ptc1 was expressed at a much-reduced level in the perichondrial cells in the absence of β-catenin. It is possible that Wnt/β-catenin signaling directly regulates the amplitude of Hh response in osteogenic cells. Alternatively, strong Hh response may reflect a certain stage of osteoblast development and the loss of a strong response could simply reflect a developmental arrest in the absence of β-catenin. The present study does not discern these possibilities. Regardless of the mechanism, Osa expression requires β-catenin and correlates with robust Hh signaling.

The transcriptional effectors of canonical Wnt signaling in the osteoblast lineage remain to be determined. In situ hybridization experiments identified Tcf1 as the predominant member of the Left/TCf family expressed in the perichondrium of embryonic long bones. Tcf1-/- mice, however, developed a normal skeleton (Verbeek et al., 1995). It is not known whether other family members were upregulated and thus compensated for the loss of Tcf1 in the Tcf1-/- mouse. It is also possible that
other members, although expressed at a much lower level, either alone or in combination are responsible for normal osteogenesis in the Tcf1<sup>–/–</sup> mutant. Tcf1 is unique in the family in the sense that it is itself a target of the canonical Wnt pathway. Its high level of expression in perichondrial cells could simply reflect robust Wnt/β-catenin signaling. Moreover, Tcf1 encodes a number of isoforms including both transcriptional activators and repressors (Van de Wetering et al., 1996). Thus, it is conceivable that deletion of both forms may maintain the overall osteogenic program without causing any obvious phenotype. Further insight on this subject will require analyses of various compound mutants among the Lef/Tcf family members.

Osteogenesis induced by Smo<sup>+</sup> in C3H10T1/2 cells was not completely inhibited by either Dkk1 or dnTcf4. The partial inhibition was not due to lower titers of Dkk1 or dnTcf4 viruses, as deliberately higher titers for these viruses did not change the degree of inhibition (data not shown). This result could indicate that other pathways in addition to canonical Wnt signaling contribute to Hh-induced osteogenesis. Of note, other groups reported that Hh-induced osteogenesis in C3H10T1/2 cells required BMP signaling (Spinella-Jaegle et al., 2001; Yuasa et al., 2002).

The interaction between Hh and Wnt signaling is probably complex. Our studies demonstrate that nuclear localization of β-catenin as well as expression of target genes for the Wnt canonical pathway were abolished in the perichondrium in Ihh<sup>+/–</sup> embryos. This could, among other possibilities, be due to the downregulation of Wnt expression in the absence of Hh signaling. Indeed, expression of Wnt9a and Wnt7b was either reduced or abolished in the perichondrium in Ihh<sup>+/–</sup> embryos. In addition, both genes were induced by Hh signaling in C3H10T1/2 cells. Interestingly, a previous study showed that the Gli family, transcriptional effectors of the Hh pathway, induced expression of several Wnt genes in frog embryos (Mullor et al., 2001). Alternatively, the Hh and Wnt signaling pathways could intersect intracellularly via common regulators such as Suppressor of fused [Su(fu)] (Meng et al., 2001) and Fz coreceptor. Indeed, Ihh signaling could contribute to Ihh-induced osteogenesis. Of note, other groups reported that Hh-induced osteogenesis in C3H10T1/2 cells required BMP signaling (Spinella-Jaegle et al., 2001; Yuasa et al., 2002).

References


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