Inhibition of β-catenin signaling causes defects in postnatal cartilage development

Mo Chen[1,*], Mei Zhu[1,*], Hani Awad[1], Tian-Fang Li[1], Tzong-Jen Sheu[1], Brendan F. Boyce[2], Di Chen[1] and Regis J. O'Keefe[1,‡]

[1]Department of Orthopaedics and [2]Department of Pathology, Center for Musculoskeletal Research, University of Rochester School of Medicine, Rochester, NY 14642, USA

*These authors contributed equally to this work
‡Author for correspondence (e-mail: Regis_okeefe@urmc.rochester.edu)

Accepted 24 January 2008
Journal of Cell Science 121, 1455-1465 Published by The Company of Biologists 2008
doi:10.1242/jcs.020362

Summary

The Wnt/β-catenin signaling pathway is essential for normal skeletal development because conditional gain or loss of function of β-catenin in cartilage results in embryonic or early postnatal death. To address the role of β-catenin in postnatal skeletal growth and development, *Col2a1-ICAT* transgenic mice were generated. Mice were viable and had normal size at birth, but became progressively runted. Transgene expression was limited to the chondrocytes in the growth plate and articular cartilages and was associated with decreased β-catenin signaling. *Col2a1-ICAT* transgenic mice showed reduced chondrocyte proliferation and differentiation, and an increase in chondrocyte apoptosis, leading to decreased widths of the proliferating and hypertrophic zones, delayed formation of the secondary ossification center, and reduced skeletal growth. Isolated primary *Col2a1-ICAT* transgenic chondrocytes showed reduced expression of chondrocyte genes associated with maturation, and demonstrated that VEGF gene expression requires cooperative interactions between BMP2 and β-catenin signaling. Altogether the findings confirm a crucial role for Wnt/β-catenin in postnatal growth.

Key words: Chondrocyte, Endochondral bone formation, Inhibitor of β-catenin and TCF (ICAT), Vascular endothelial growth factor (VEGF), β-catenin

Introduction

Endochondral bone formation is a complex process that is initiated by mesenchymal cell condensation at specific sites and differentiation into chondrocyte precursors. Immature chondrocytes develop in the center of mesenchymal condensation and undergo a highly regulated maturation process that sequentially involves chondrocyte proliferation, hypertrophy and matrix calcification. Calcified cartilage is subsequently invaded by blood vessels that deliver osteoblasts and osteoclasts precursors, in order to begin the primary bone formation and remodeling. Since each step is precisely regulated, mutations of genes involved in these processes frequently result in skeletal malformation or abnormal bone formation (Kronenberg, 2003). Several growth factors and signaling proteins have been reported to be involved in the regulation of chondrocyte differentiation and maturation, including FGF, BMP, Ihh, PTHrP, Runx2, Runx3 and Wnt (Kolpakova and Olsen, 2005; Komori, 2003; Kronenberg, 2003; Ornitz, 2005; Yoon and Lyons, 2004).

β-catenin is a key component of the canonical Wnt signaling pathway and plays a crucial role in multiple steps during chondrogenesis and chondrocyte maturation. Constitutional gene deletion of β-catenin results in lethality at stage E7.5 (Haegel et al., 1995), prior to formation of the skeletal elements. Conditional deletion of the β-catenin gene in early mesenchymal precursors results in enhanced chondrogenesis (Day et al., 2005; Hill et al., 2005), suggesting that β-catenin inhibits early mesenchymal cell differentiation into cartilage. However, conditional deletion of the β-catenin gene in chondrocytes using the *Col2a1-Cre* transgene causes decreased chondrocyte proliferation and delayed chondrocyte maturation (Akiyama et al., 2004). Thus, whereas β-catenin inhibits chondrogenesis, once cartilage has formed, it promotes the maturation of growth plate chondrocytes. Whereas conditional gene deletions targeting cartilage have provided insight into the role of β-catenin in the regulation of cartilage, these mutants perish before or shortly after birth, and have skeletal dysplasia as well as altered cartilage and limb morphologies that limit the understanding of the role of β-catenin as a regulator of endochondral bone formation (Akiyama et al., 2004). Thus, the role of β-catenin in postnatal development remains unknown.

Inhibitor of β-catenin and TCF (ICAT) is an 82-amino-acid peptide that was first identified using the yeast two-hybrid assay (Tago et al., 2000). Its crystal structure reveals that ICAT binds to the armadillo repeats of β-catenin and disrupts the ability of β-catenin and TCF/LEF to form a complex (Daniels and Weis, 2002; Graham et al., 2002). In vitro studies show that ICAT inhibits β-catenin signaling but not cell adhesion (Daniels and Weis, 2002). *ICAT* knockout (KO) mice exhibit malformation of the forebrain and craniofacial bones, and lack of kidney formation (Satoh et al., 2004). These abnormalities are due to the activation of the canonical Wnt/β-catenin signaling in specific tissues where *ICAT* is typically highly expressed. The role of ICAT in chondrocyte function has not been reported.

In order to specifically inhibit β-catenin signaling without disturbing cell adhesion in chondrocytes, we generated *Col2a1-ICAT* transgenic mice. Mice are viable after birth, which allowed us to investigate the functional role of Wnt/β-catenin signaling in chondrocytes during postnatal growth and development. We found that chondrocyte proliferation and differentiation were inhibited in *Col2a1-ICAT* transgenic mice. Defects in chondrocyte maturation and formation of the secondary ossification center were observed. *VegfA* (*Vegf*) is a downstream target gene of the β-catenin signaling

and β-catenin directly activates *Vegf* gene transcription with BMP2 in chondrocytes. These findings provide new insights into the mechanism of β-catenin signaling in chondrocyte maturation.

Results

Wnt/β-catenin signaling is active in chondrocytes

To investigate whether β-catenin signaling is active in chondrocytes during cartilage development, we examined the X-Gal staining pattern in chondrocytes in tissue sections obtained from TOP-gal transgenic embryos (DasGupta and Fuchs, 1999) in which the *lacZ* reporter gene is under the control of 3×TCF responsive elements. We performed X-Gal staining using E14.5 and E16.5 mouse embryos. Extensive X-Gal staining was noted throughout the entire cartilage elements, including the skull, vertebral column, ribs and long bones in E14.5 TOP-gal embryos (Fig. 1A-D). In E16.5 embryos, X-Gal staining was present in proliferating (Fig. 1E), and hypertrophic chondrocytes (Fig. 1F), and was also observed in osteoblasts (Fig. 1F). These results demonstrate that β-catenin signaling is active in chondrocytes during embryonic cartilage development.

Overexpression of *ICAT* delays skeletal growth in transgenic mice and inhibits β-catenin signaling

To investigate the function of β-catenin signaling in cartilage, we generated *Col2a1-ICAT* transgenic mice in which expression of the *ICAT* transgene was targeted to chondrocytes using the 1.0 kb type II collagen promoter (*Col2a1*) followed by the col2 enhancer (Fig. 2A) (Krebsbach et al., 1996; Metsaranta et al., 1991). *Col2a1-ICAT* transgenic mice are viable and fertile. We established three independent lines of *Col2a1-ICAT* transgenic mice, all of which displayed similar phenotypes. The transgenic mice were initially identified by Southern blot analysis and then genotyped using PCR of the DNA extracted from the tail tissues after mice were weaned (Fig. 2B). Body-weight measurements demonstrated that the transgenic mice exhibit an ~30%-40% reduction in body weight compared with wild-type (WT) littermates (Fig. 2C). X-ray radiographic analysis showed that bone growth is delayed in 1-week-old, 2-week-old and 4-week-old *Col2a1-ICAT* transgenic mice (Fig. 2D). The size of newborn *Col2a1-ICAT* transgenic mice is similar to their WT littermates (Fig. 2E), suggesting that embryonic development is relatively normal in *Col2a1-ICAT* transgenic mice. However, *Col2a1-ICAT* transgenic mice are significantly smaller than WT littermates at 2 weeks of age (Fig. 2F), suggesting that the postnatal cartilage development is impaired in *Col2a1-ICAT* transgenic mice.

Expression of the Flag-ICAT protein was analyzed by western blotting and immunostaining using an anti-Flag antibody in primary sternal chondrocytes derived from WT and *Col2a1-ICAT* transgenic mice. Expression of the Flag-ICAT protein was detected by western blotting (Fig. 3A) and immunostaining (Fig. 3B) in chondrocytes isolated from *Col2a1-ICAT* transgenic mice but not in those from WT mice. Immunostaining was also performed using histological sections from 2-week-old mice. Expression of Flag-ICAT was detected in proliferating chondrocytes in growth plate and in articular chondrocytes in *Col2a1-ICAT* transgenic mice (Fig. 3C). To determine whether β-catenin signaling is blocked in *Col2a1-ICAT* transgenic mice, we transfected the TOP-flash reporter construct into primary chondrocytes derived from *Col2a1-ICAT* transgenic mice and WT littermates. The activity of the TOP-flash reporter is about 30% lower in chondrocytes derived from *Col2a1-ICAT* transgenic mice than in those derived from WT mice. Wnt3a-induced reporter activity was completely blocked in chondrocytes derived from *Col2a1-ICAT* transgenic mice (Fig. 3D). These results suggest that β-catenin signaling is inhibited in *Col2a1-ICAT* transgenic chondrocytes. To further determine the specificity of the *ICAT* transgene expression, total RNA was extracted from multiple tissues and the expression of *Flag-ICAT* mRNA was examined. We found that strong expression of *Flag-ICAT* was detected from ribs, weak expression of *Flag-ICAT* was detected from the brain and the transgene was not detected from other tissues, including spleen, kidney, lung and liver (Fig. 3E). Because β-catenin is a bi-functional molecule that also regulates cell adhesion through interaction with the membrane protein, cadherin adhesion assays were performed using primary chondrocytes isolated from *Col2a1-ICAT* transgenic

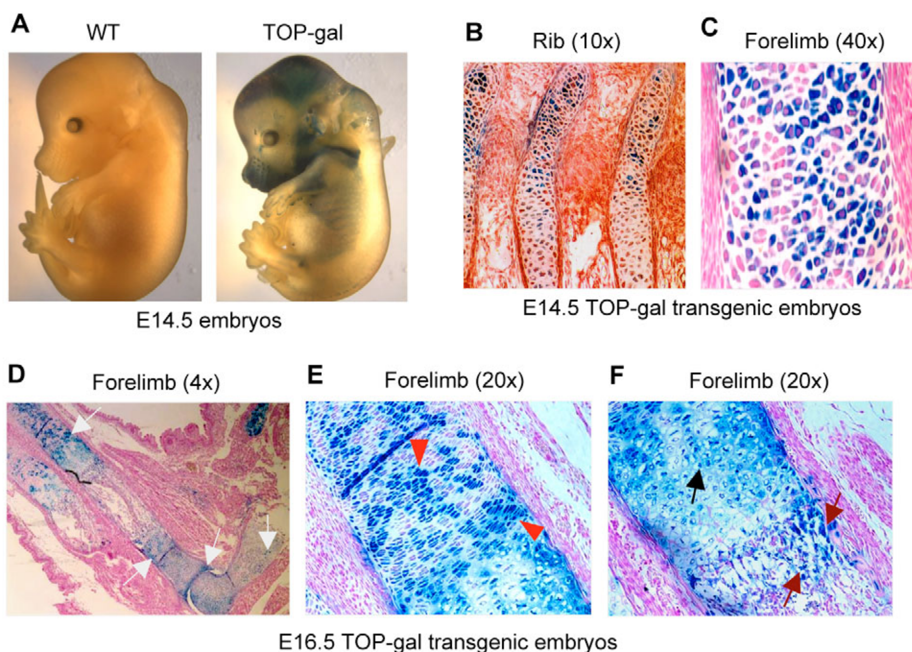

Fig. 1. X-Gal staining in E14.5 and E16.5 TOP-gal transgenic embryos. (A) X-Gal staining of E14.5 whole embryos shows that cartilage, skull, vertebral column, ribs and long bones are stained positive for X-Gal. (B,C) Chondrocytes in the ribs and forelimb of E14.5 transgenic embryos show strong X-Gal-positive staining. (D-F) In E16.5 embryos, (E) proliferating (red arrowhead) and (F) hypertrophic chondrocytes (black arrow) and osteoblasts (red arrows) in the metaphysis show positive X-Gal staining. The results indicate that β-catenin signaling is active in proliferating and hypertrophic chondrocytes during cartilage development.

mice and WT littermates (McCrea et al., 1991; Peifer et al., 1992). Cadherin-mediated cell adhesion is protected by Ca^{2+} (Xue et al., 2005). Therefore, chondrocytes were treated with trypsin-$CaCl_2$ (TC) or trypsin-EDTA (TE) and the relative cell aggregation was determined by the TC:TE ratio (Xue et al., 2005). Protein levels of cadherin and β-catenin were unchanged in *Col2a1-ICAT* transgenic mice (data not shown). Overexpression of *ICAT* did not alter chondrocyte cell adhesion (Fig. 3F). However, as a positive control, deletion of the β-catenin gene – by using Ad-Cre infection – in primary chondrocytes isolated from the β-$catenin^{fx/fx}$ mice (Brault et al., 2001) reduced chondrocyte cell adhesion (Fig. 3F). These results establish that ICAT specifically inhibits β-catenin signaling without affecting cell adhesion in *Col2a1-ICAT* transgenic chondrocytes.

ICAT inhibits chondrocyte proliferation and maturation

Analysis of histological sections showed a dramatic delay in the formation of the secondary ossification center (SOC) in *Col2a1-ICAT* transgenic mice compared with their WT littermates, especially in 2-week-old transgenic mice (Fig. 4A). In WT mice, chondrocyte hypertrophy and vascular invasion were observed in the epiphyseal area in 1-week-old mice. By contrast, vascular invasion was delayed until the age of 2 weeks in *Col2a1-ICAT* transgenic mice (Fig. 4A), indicating defects in chondrocyte maturation and endochondral bone formation. Histomorphometric analyses showed that the width of both the proliferating and the hypertrophic zones in the growth plate of the tibia and the femur were reduced in 2-week-old *Col2a1-ICAT* transgenic mice (Fig. 4B-F), suggesting alterations in both chondrocyte proliferation and maturation in chondrocytes with inhibition of β-catenin signaling.

To evaluate chondrocyte proliferation, we performed Ki-67 and DAPI double immunostaining and counted percentage Ki-67-positive cells by normalizing to DAPI-positive cells. We found that cell proliferation was reduced 24% in growth-plate chondrocytes derived from *Col2a1-ICAT* transgenic mice (Fig. 5A,B). Consistent with this finding, Western blot analysis showed reductions in the expression of cyclin D1, D2 and PCNA proteins in primary chondrocytes isolated from *Col2a1-ICAT* transgenic mice (Fig. 5C). In contrast, the expression of cyclin A was not significantly changed (Fig. 5C). In addition, no significant changes in total and phosphorylated β-catenin and phosphorylated Rb proteins were observed (Fig. 5C). The expression of chondrocyte differentiation marker genes, including collagen type X (*Col10a1*, hereafter referred to as *colX*), alkaline phosphatase liver/bone/kidney (hereafter referred to as *Alp*), vascular endothelial growth factor A (hereafter referred to as *Vegf*) and matrix metalloproteinase 13 (*Mmp13*) were examined by real-time reverse transcriptase (RT)-PCR in primary chondrocytes isolated from sterna of 3-day-old

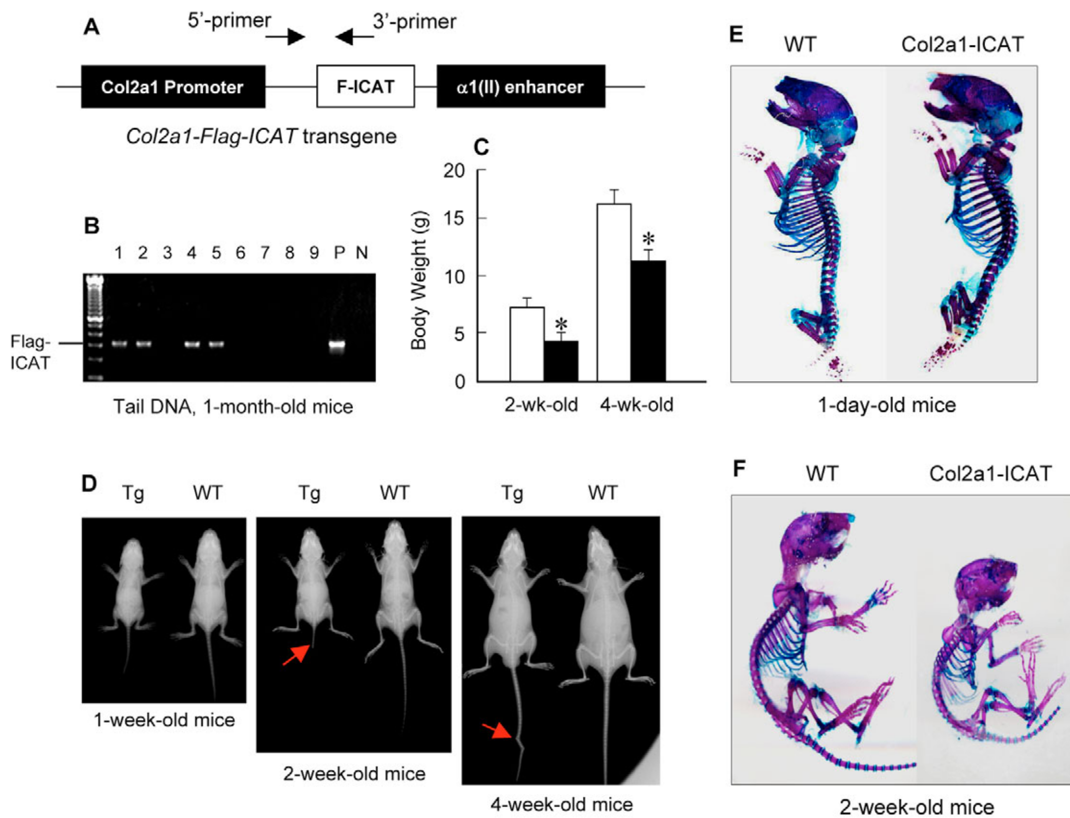

Fig. 2. Skeletal growth is reduced in *Col2a1-ICAT* transgenic mice. (A) Structure of the transgene construct and the priming sites for genotyping the *Col2a1-ICAT* transgenic mice. (B) PCR results; genotyping of *Col2a1-ICAT* transgenic mice. Mice 1, 2, 4 and 5 (lanes 1, 2, 4 and 5) are positive for *Flag-ICAT* transgene expression (P, positive control; N, negative control). (C) Body weight of 2-week-old and 4-week-old transgenic mice and WT littermates. A significant reduction in body weight was observed in *Col2a1-ICAT* transgenic mice. (D) Radiographic analysis showing the delay in the longitudinal growth of 1-week-old, 2-week-old and 4-week-old *Col2a1-ICAT* transgenic mice compared with their WT littermates. (E,F) Alizarin Red and Alcian Blue staining showed that skeletal development was relatively normal in 1-day-old new born *Col2a1-ICAT* transgenic mice. By contrast, a significant reduction in postnatal skeletal growth was observed in 2-week-old *Col2a1-ICAT* transgenic mice.

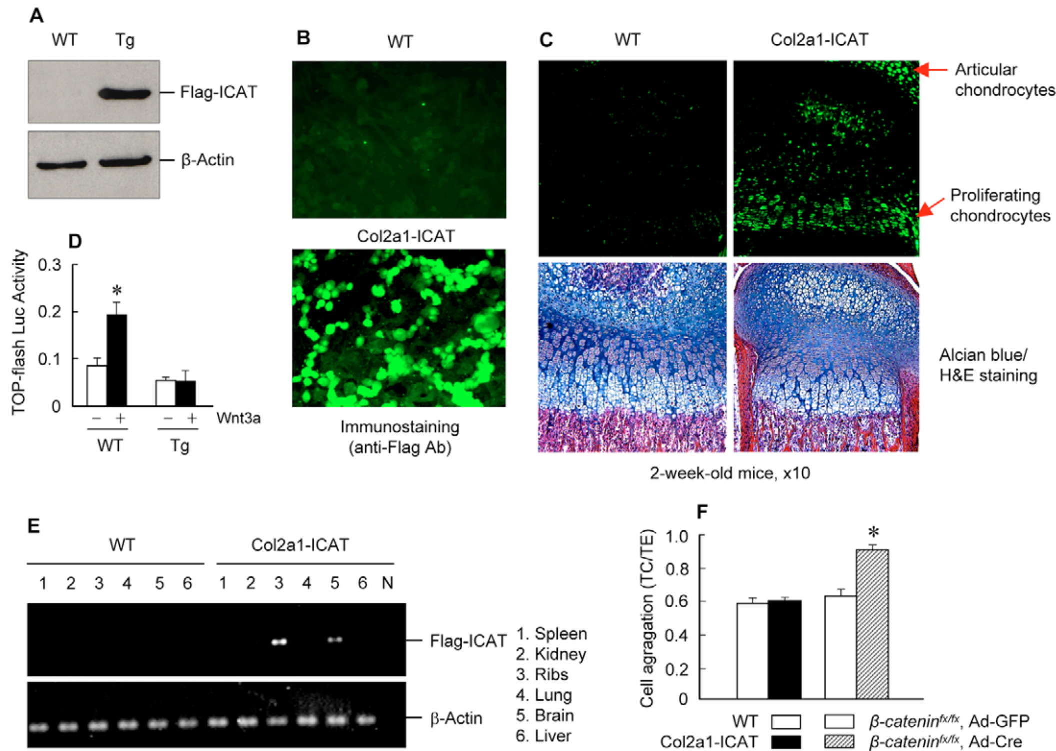

Fig. 3. Expression of the *Flag-ICAT* transgene in *Col2a1-ICAT* transgenic mice. (A,B) Primary sternal chondrocytes were isolated from *Col2a1-ICAT* transgenic mice and their WT littermates. Expression of Flag-ICAT protein was detected in western blot analysis and immunostaining using the anti-Flag M2 antibody in *Col2a1-ICAT* transgenic chondrocytes. (C) Expression of the Flag-ICAT transgene in growth plate chondrocytes was examined by immunostaining using tissue sections from knee joints of 2-week-old WT and *Col2a1-ICAT* transgenic mice. The ICAT transgene was specifically expressed in proliferating chondrocytes in the growth plate of *Col2a1-ICAT* transgenic mice and in articular chondrocytes lining the joint surface. (D) ICAT inhibits canonical Wnt signaling in chondrocytes of *Col2a1-ICAT* transgenic mice. Primary chondrocytes isolated from 3-day-old WT and *Col2a1-ICAT* transgenic mice were transfected with TOP-flash reporter and treated with or without Wnt3a (100 ng/ml) for 24 hours. Wnt3a stimulated the reporter activity in WT but not in *Col2a1-ICAT* transgenic chondrocytes. (E) To determine the specificity of the *Flag-ICAT* transgene expression, total RNA was extracted from multiple tissues and the expression of *Flag-ICAT* was examined by PCR. The expression of *Flag-ICAT* was detected in ribs (strong expression) and brain (weak expression) but not in other tissues. (F) ICAT does not alter cell adhesion. Primary chondrocytes isolated from *Col2a1-ICAT* transgenic mice and WT littermates were placed in 24-well plates and grown to confluence. Cells were then trypsinized with 0.1% trypsin containing either 1 mM $CaCl_2$ (TC) or 1 mM EDTA (TE) and incubated at 37°C for 30 minutes. Cells were pipetted gently five times with 10 ml of PBS and the cell clusters were counted. The degree of adhesion was expressed by determining the ratio of cell clusters in TC and TE containing solutions (TC:TE) for each cell type. A similar experiment was also performed using chondrocytes isolated from β-catenin$^{fx/fx}$ mice and infected with adenovirus expressing Cre recombinase (*Ad-Cre*) or GFP (*Ad-GFP*). Results showed that cell adhesion was not changed in *Col2a1-ICAT* transgenic mice.

neonatal *Col2a1-ICAT* transgenic mice and their WT littermates. The expression of all of the chondrocyte marker genes was significantly decreased in the *Col2a1-ICAT* transgenic mice (Fig. 5D). Apoptosis, as determined by TUNEL staining showed that chondrocyte apoptosis was significantly increased in hypertrophic zone of *Col2a1-ICAT* transgenic mice (Fig. 5E). These results suggest that the reduced width of the hypertrophic zone could be owing to less chondrocytes entering the hypertrophic zone (decreased chondrocyte proliferation) and more cells leaving the hypertrophic zone (increased apoptosis in hypertrophic chondrocytes). Altogether, the findings show a reduction in chondrocyte proliferation and differentiation and an increase in chondrocyte apoptosis in *Col2a1-ICAT* transgenic mice, leading to reduced widths of the proliferating and hypertrophic zones, delayed formation of secondary ossification center, and a reduced rate of skeletal growth.

β-catenin activates BMP signaling in chondrocytes

The observations in the *Col2a1-ICAT* transgenic mice confirm a role of β-catenin in the promotion of chondrocyte maturation in the growth plate. Although the BMP signaling pathway has also been shown to have a role in promoting chondrocyte maturation, interactions between β-catenin and BMP signaling in chondrocytes have not been described previously. Since prior work in our laboratory established that TGFβ stimulates β-catenin signaling (Li et al., 2006a), initial experiments were performed to examine whether BMP2 activates β-catenin signaling in chondrocytes. Primary chondrocytes were isolated from TOP-gal transgenic mice and treated with BMP2 (100 ng/ml) for 24 hours. We found that BMP2 did not stimulate β-catenin signaling in β-Gal assay (data not shown). However, treatment with Wnt3a (100 ng/ml) stimulated *Bmp2* and *Bmp4* mRNA expression in primary chondrocytes (Fig. 6A) and upregulated the BMP signaling reporter (12×SBE) activity (Fig. 6B), providing evidence of cross-talk between these pathways. When Wnt3a was cultured with primary chondrocytes for 4 and 6 days, it induced *colX* mRNA expression. Addition of noggin (400 ng/ml), an antagonist of BMP2 and BMP4 ligands, completely blocked this effect (Fig. 6C). In *Col2a1-ICAT* transgenic chondrocytes, the expression of *Bmp2* and *Bmp4* is reduced (Fig. 6D). These results suggest that BMP signaling is downstream of β-catenin signaling in chondrocytes and that β-catenin stimulates chondrocyte maturation at least in part through activation of BMP signaling.

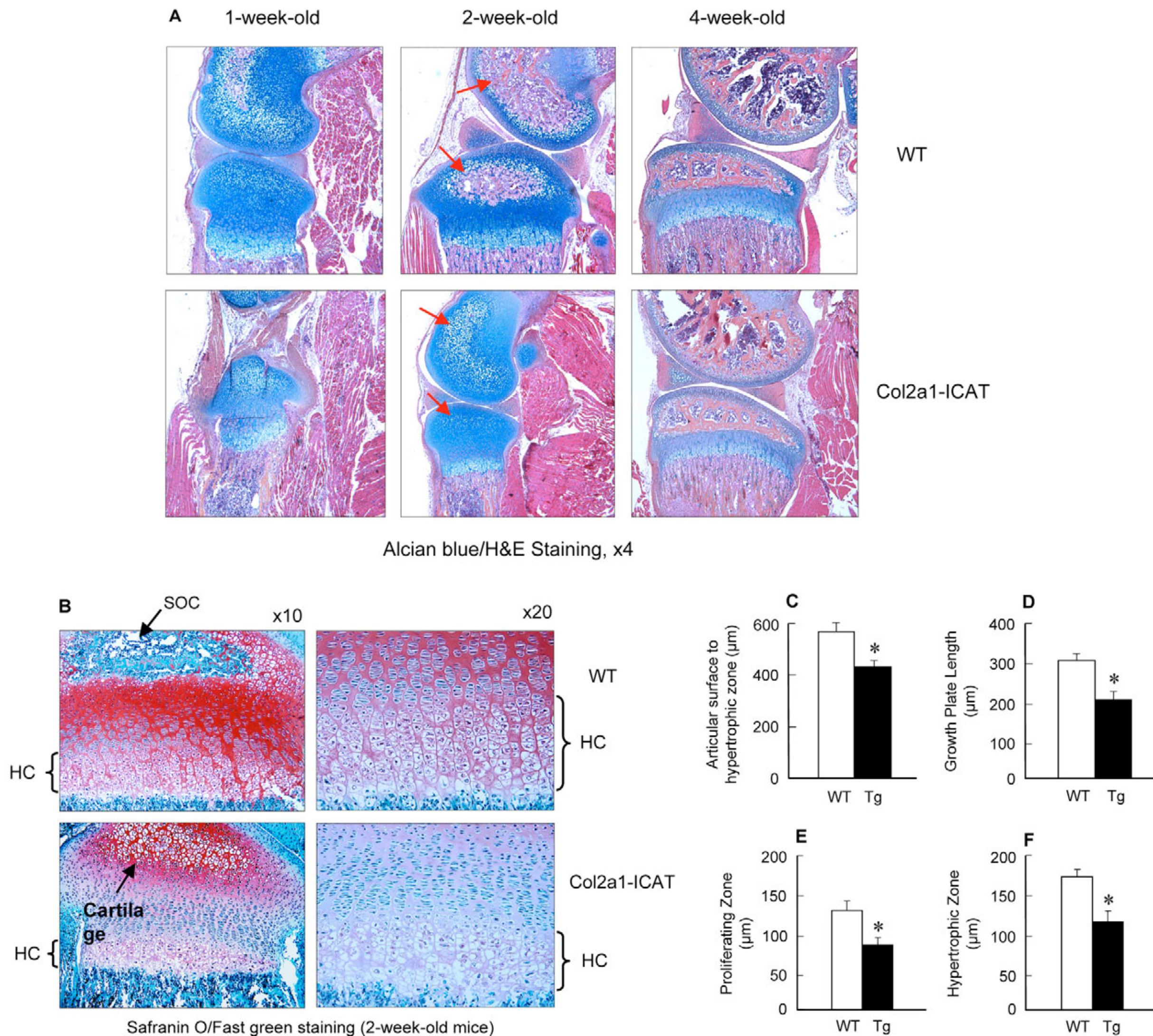

Fig. 4. *Col2a1-ICAT* transgenic mice have delayed appearance of the secondary ossification center (SOC) and altered growth plate morphology. (A) Development of the growth plate in postnatal mice was analyzed by histology staining with Alcian Blue/Hemotoxylin and Orange G. In WT mice, the formation of epiphysal SOC was initiated at 1 week of age and was well developed at age 2 weeks. *Col2a1-ICAT* transgenic mice display an obvious delay in the formation of SOC at 1 and 2 weeks of age. (B) Tissue sections stained with Safranin O and Fast Green show that the length of both the proliferating and hypertrophic zones was reduced in 2-week-old *Col2a1-ICAT* transgenic mice. The hypertrophic chondrocyte columns were disorganized in *Col2a1-ICAT* transgenic mice. (C-F) Histomorphometric measurements show that the (C) distance from articular surface to hypertrophic zone, (D) growth plate length, and the (E,F) lengths of the proliferating (E) and hypertrophic (F) zones are significantly decreased in 2-week-old *Col2a1-ICAT* transgenic mice compared with their WT littermates. $^*P<0.05$, unpaired *t*-test, $n=6$.

β-catenin directly activates *Vegf* gene transcription

To determine whether there is a cooperative role between BMP2 and β-catenin in the induction of chondrocyte maturational markers, primary chondrocytes isolated from WT and *Col2a1-ICAT* transgenic mice were treated with BMP2. Treatment with BMP2 restored *colX* and *Alp* mRNA expression in chondrocytes isolated from *Col2a1-ICAT* transgenic mice and compensated for the loss of β-catenin signaling (Fig. 7A,B). By contrast, BMP2 failed to restore *Vegf* and *Mmp13* expression in *Col2a1-ICAT* transgenic chondrocytes, despite the BMP2 responsiveness of these genes in WT chondrocytes (Fig. 7C,D). These results suggest that BMP2 regulates *Vegf* and *Mmp13* expression in a β-catenin-signaling-dependent manner.

β-catenin has been reported to regulate *VEGF* gene transcription in cancer cells (Easwaran et al., 2003). In the growth plate, VEGF is secreted by mature chondrocytes (Zelzer et al., 2004) and induces the invasion of blood vessels. In *Col2a1-ICAT* transgenic mice, the formation of the secondary ossification center in the epiphysis of the growth plate was substantially delayed. These results suggest that *Vegf* expression is directly controlled by β-catenin in chondrocytes. To further determine the regulatory mechanism of *Vegf* expression by β-catenin and BMP signaling, cells of the the chondrogenic cell line RCJ3.1C5.18 (RCJ3.1) were treated with BMP2 (100 ng/ml) and the GSK-3β inhibitor (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO; 1 μg/ml) in the presence or absence of noggin (400 ng/ml) or Dkk1 (1 μg/ml), which inhibit BMP or Wnt signaling, respectively. BMP2 significantly induced *Vegf* expression within 2 hours. Dkk1 completely inhibited BMP2-induced *Vegf* expression (Fig. 7E). Similar to BMP2, BIO also induced *Vegf* expression at the same time frame in these cells.

Addition of noggin only partially inhibited BIO-induced *Vegf* expression (Fig. 7F). These results suggest that *Vegf* is the direct downstream target gene for Wnt/β-catenin signaling in chondrocytes and that induction by either signaling pathway requires cooperative interactions with the other pathway. To further confirm the regulatory role of β-catenin signaling in the expression of VEGF and MMP13, we performed immunostaining assays to examine changes in the expression of VEGF and MMP13 in *Col2a1-ICAT* transgenic mice. The results demonstrated that the expression of VEGF and MMP13 proteins was significantly reduced in *Col2a1-ICAT* transgenic mice (Fig. 7G,H).

To determine whether β-catenin/TCF directly regulates *VegF* gene transcription, the mouse VEGFa promoter (since *Vegfa* is the *Vegf* isoform expressed in chondrocytes) was cloned and the effect of β-catenin on *Vegf* promoter activity was examined in RCJ3.1 chondrogenic cells. Transfection of constitutively active β-catenin (β-catenin^{S33Y}) stimulated *Vegf* promoter activity more than threefold (Fig. 8A). Subsequent experiments using serial deletion constructs of the *Vegf* promoter show a marked reduction in promoter activity in the –140/+87 reporter (Fig. 8B), while a threefold induction of the promoter is maintained in the –1341/+87 and –940/+87 reporter constructs. Sequence analysis shows multiple TCF-response elements located in the 1-kb region of the *Vegf* proximal promoter and there are five putative TCF/LEF-binding sites within the 1-kb *Vegf* proximal promoter (Fig. 8C). To determine whether β-catenin binds to the *Vegf* promoter, RCJ3.1 cells were treated with BIO, and ChIP assay was performed. Binding of β-catenin to one of these TCF-binding regions located in the proximal promoter was detected by ChIP assay and, as expected, β-catenin addition of BIO significantly enhanced binding of β-catenin to the *Vegf* promoter (Fig. 8D). These results demonstrate that β-catenin directly activates *Vegf* gene transcription in chondrocytes.

Overexpression of *ICAT* in chondrocytes inhibits angiogenesis

Delayed formation of secondary ossification center might be related to the decreased expression of *Vegf* and *Mmp13*. To determine whether there is a defect in the vascularization in *Col2a1-ICAT* transgenic mice, we examined the expression of endothelial cell marker platelet endothelial cell adhesion molecule precursor (PECA1, hereafter referred PECAM-1) in WT and *Col2a1-ICAT* transgenic mice and found that expression of PECAM-1 was significantly decreased in *Col2a1-ICAT* transgenic mice (Fig. 9A). The vascularization was then further examined in 2-week-old WT and *Col2a1-ICAT* transgenic mice by using microtomography (microCT) (Zhang et al., 2005). The results showed that the *Col2a1-ICAT* transgenic mice had less vascular structure than the WT controls (Fig. 9B), demonstrating defects of angiogenesis in cartilage of *Col2a1-ICAT* transgenic mice. To determine the specificity of this defect, we also performed the lead perfusion experiments in livers using WT and transgenic mice of the same

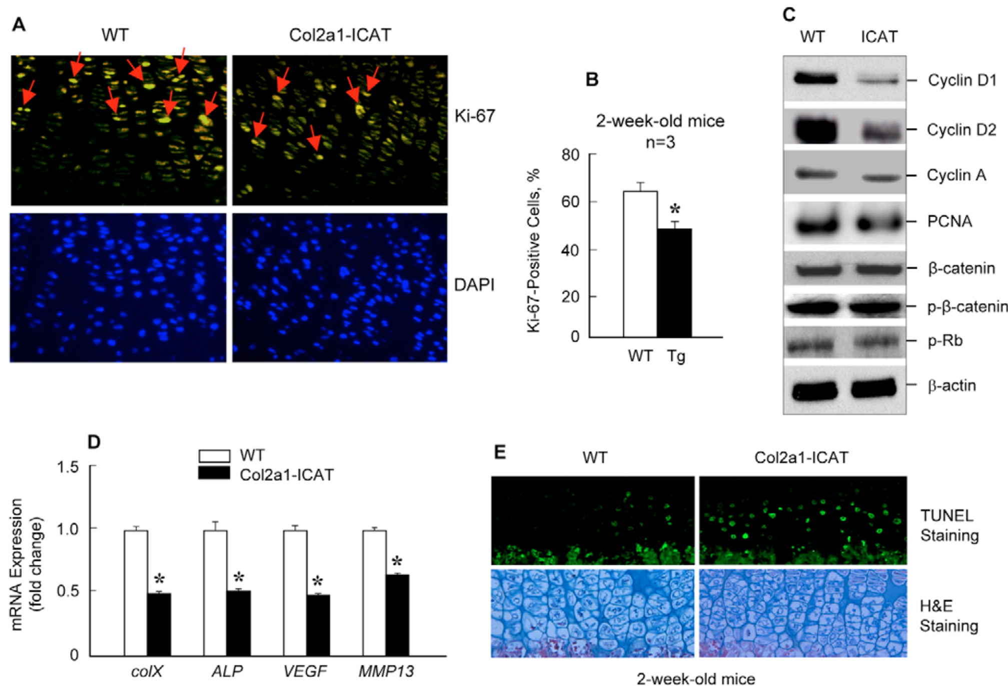

Fig. 5. Chondrocyte proliferation and differentiation are decreased and apoptosis is increased in *Col2a1-ICAT* transgenic mice. (A,B) Ki-67 and DAPI double staining was performed using anti-Ki-67 antibody in tissue sections collected from 2-week-old *Col2a1-ICAT* transgenic mice and their WT littermates. The proliferation rate was determined by counting the numbers of Ki-67-positive cells at the proliferating zone divided by the DAPI-positive cell number. The proliferation of growth plate chondrocytes is decreased by 24% in 2-week-old *Col2a1-ICAT* transgenic mice. **P*<0.05, unpaired *t*-test, *n*=3. (C) The expression of cyclin D1, cyclin D2, cyclin A, PCNA, total and phosphorylated β-catenin and phosphorylated Rb was examined by western blotting. The expression of cyclin D1, cyclin D2 and PCNA was significantly decreased in primary chondrocytes derived from *Col2a1-ICAT* transgenic mice compared with those derived from WT mice. By contrast, the protein levels of cyclin A, total and phosphorylated β-catenin and phosphorylated Rb was not significantly changed. (D) Total RNA was extracted from primary sternal chondrocytes isolated from *Col2a1-ICAT* transgenic mice and WT littermates. The expression of chondrocyte differentiation marker genes, such as collagen type X (*colX*), *Vegf*, *Mmp13* and *Alp* was determined by real-time reverse transcriptase (RT)-PCR and was normalized to β-actin levels. The expression of chondrocyte marker genes was significantly reduced in *Col2a1-ICAT* transgenic mice. **P*<0.05, unpaired *t*-test, *n*=3. (E) The apoptosis of growth-plate chondrocytes was determined by TUNEL staining using 2-week-old WT and *Col2a1-ICAT* transgenic mice. Increased cell apoptosis was observed in the hypertrophic region of the growth plate in *Col2a1-ICAT* transgenic mice.

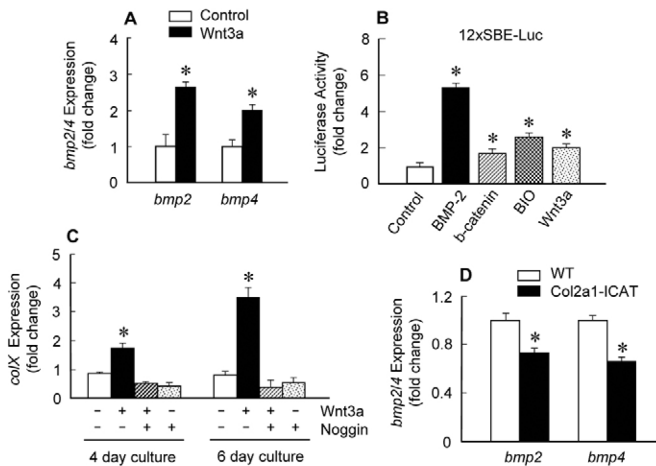

Fig. 6. Wnt3a and β-catenin activate BMP signaling. (A) Primary chondrocytes were treated with or without Wnt3a (100 ng/ml) for 24 hours and the expression of *Bmp2* and *Bmp4* was examined by real-time RT-PCR. Wnt 3a upregulates mRNA expression of *Bmp2* and *Bmp4* in chondrocytes. (B) RCJ3.1C5.18 chondrocytes were transfected with the BMP signaling reporter (12×SBE-Luc) and control vector, and treated with BMP2 (100 ng/ml), BIO (1 μg/ml) or Wnt3a (100 ng/ml). To determine the effect of β-catenin on BMP signaling, RCJ3.1C5.18 cells were also co-transfected with BMP signaling reporter and constitutively active β-catenin (S33Y). Luciferase activity was measured using cell lysates 48 hours after transfection. β-catenin, BIO and Wnt3a stimulated BMP-reporter activity in chondrocytes. BMP2 was used as a positive control. (C) BMP signaling is required for Wnt3a-induced *colX* expression. Primary chondrocytes isolated from WT mice were treated with Wnt3a (100 ng/ml) for 4 and 6 days with or without noggin (300 ng/ml). Type X collagen (*colX*) mRNA levels were measured by real-time RT-PCR and were normalized to β-actin levels. The expression of *colX* was completely inhibited by the BMP antagonist noggin. (D) Expression of *Bmp2* and *Bmp4* mRNA was examined by real-time RT-PCR using primary chondrocytes isolated from *Col2a1-ICAT* transgenic mice and WT littermates after cells were cultured for 2 days. Expression of *Bmp2* and *Bmp4* was significantly decreased in *Col2a1-ICAT* transgenic mice. *$P<0.05$, unpaired *t*-test, $n=4$ (A-D).

age. Compared with the defects in cartilage angiogenesis, only a small difference between WT and *Col2a1-ICAT* transgenic mice was found in the vascularization of liver (Fig. 9C). These results indicate the specific defects in vascularization in the cartilage tissues of *Col2a1-ICAT* transgenic mice.

Discussion

Limb development is a complicated process and is regulated by bone-growth factors, signaling molecules and key transcription factors. β-catenin is a central molecule in canonical Wnt signaling pathway and has a crucial role in the various steps involved in endochondral bone formation. β-catenin activity is regulated by several proteins including ICAT, which inhibits the interaction of β-catenin with TCF/LEF. *Col2a1-ICAT* transgenic mice with chondrocyte-specific transgene expression were generated in order to determine the role of β-catenin signaling in chondrocyte development. A marked effect was observed on postnatal chondrocyte maturation in *Col2a1-ICAT* transgenic mice, establishing for the first time that Wnt/β-catenin signaling is required for postnatal chondrocyte development. In the present study, we generated seven *Col2a1-ICAT* founder mice. We could not establish transgenic lines with two founder mice because their offspring is very small and mice usually died within 1 month after birth. We have established and characterized three lines of the *Col2a1-ICAT* transgenic mice that survive into adulthood and show significant defects in postnatal cartilage development. Other transgenic mice show only a moderate phenotype. These observations suggest a dosage effect for the transgene expression and severity of the phenotype.

The crucial role of β-catenin in endochondral bone formation has been demonstrated in animal models using tissue-specific deletion or conditional activation of the β-catenin gene in chondrocytes (Akiyama et al., 2004). However, the prenatal or perinatal lethality due to the severe developmental defects restricts the usage of these models to further analyze the role of β-catenin signaling in postnatal chondrocyte development. Since ICAT only inhibits canonical Wnt signaling without interruption of cell adhesion and because ICAT might be a reversible inhibitor of β-catenin/TCF interaction, use of the *Col2a1-ICAT* transgenic mouse model provides us with an opportunity to investigate the role of β-catenin signaling in postnatal cartilage development. Our findings clearly demonstrate that β-catenin has an essential role in postnatal chondrocyte maturation and endochondral bone formation within long bones.

In *Col2a1-ICAT* transgenic mice, chondrocyte maturation is delayed and the width of the hypertrophic zone is reduced, especially in 1-week-old and 2-week-old *Col2a1-ICAT* transgenic mice. Consistent with a previous report (Akiyama et al., 2004), chondrocyte proliferation was reduced in *Col2a1-ICAT* transgenic mice, demonstrated by the reduction of Ki-67-positive chondrocytes and the decreased width of the proliferating zone in 1-week-old and 2-week-old *Col2a1-ICAT* transgenic mice. Since cell apoptosis is increased in the hypertrophic zone of *Col2a1-ICAT* transgenic mice, the reduced hypertrophic zone observed in *Col2a1-ICAT* transgenic mice might be owing to the combined effect of decreased chondrocytes entering into the hypertrophic zone and increased chondrocyte apoptosis.

In this study, we described that β-catenin signaling is necessary for normal vascularization of cartilage. Evidence in support include findings that: (1) *Vegf* and *Mmp13* expression was reduced in *Col2a1-ICAT* transgenic mice and both genes have an important role in vascularization and endochondral conversion; (2) PECAM-1 is a vascular endothelial protein whose expression in the growth plate is decreased in *Col2a1-ICAT* transgenic mice; (3) vascular invasion is a crucial initial step for the formation of the secondary ossification center, which was significantly delayed in *Col2a1-ICAT* transgenic mice and; (4) lead perfusion and microCT vascularization analysis demonstrated that blood-vessel formation is dramatically impaired in *Col2a1-ICAT* transgenic mice. These results indicate that vascularization is crucial for postnatal cartilage development and β-catenin signaling plays an important role in this process. The mouse *Vegfa* gene has three alternative splicing products, VEGF120, VEGF164 and VEGF188. Mice expressing only VEGF120 die right after birth (Maes et al., 2002; Maes et al., 2004). Mice expressing only VEGF164 show normal bone development (Maes et al., 2002; Maes et al., 2004). However, mice expressing only VEGF188 display a phenotype similar to that observed by us in *Col2a1-ICAT* transgenic mice, including delayed formation of the secondary ossification center, defects in epiphysis angiogenesis and increased cell apoptosis (Maes et al., 2004).

Using the primary sternal chondrocytes and chondrogenic cell lines, we demonstrated that *Vegf* is the direct downstream target gene for β-catenin/TCF signaling in chondrocytes. Activation of BMP2 and Wnt signaling pathways resulted in stimulation of *Vegf* gene expression. We demonstrated the following: (1) Wnt3a and

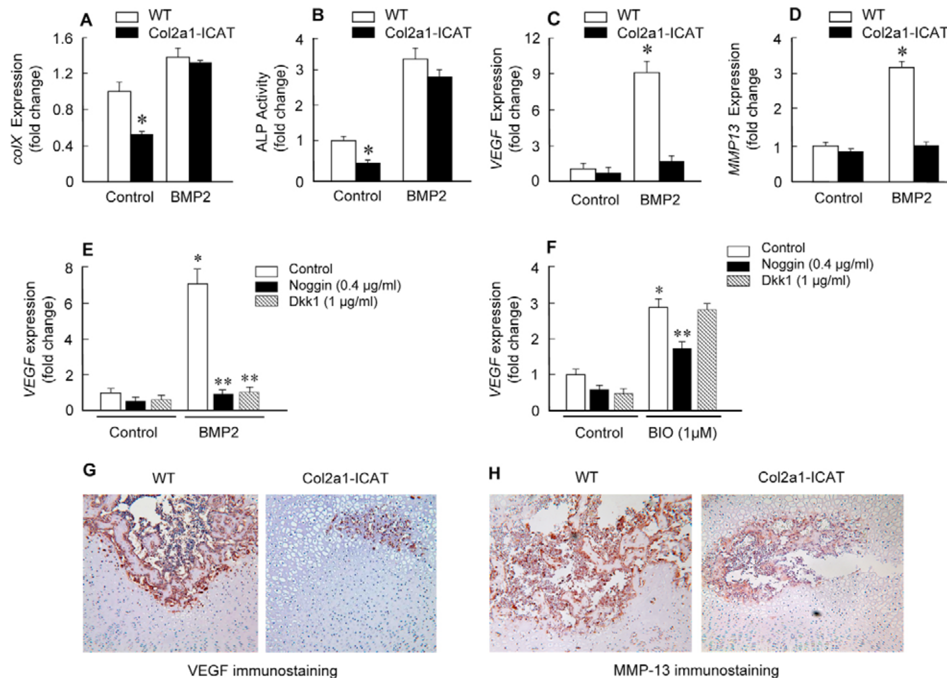

Fig. 7. β-catenin signaling is required for BMP2 to activate *Vegf* and *Mmp13* expression. (A-C) Primary chondrocytes isolated from *Col2a1-ICAT* transgenic mice and WT littermates were treated with BMP2 (100 ng/ml) for 48 hours. The expression of *colX*, *Vegf* and *Mmp13* was determined by real-time RT-PCR and was normalized to β-actin levels. ALP activity was measured using cell lysates from the same cells. BMP2 stimulated the expression of all marker genes in WT chondrocytes and rescued (A) the *colX* expression and (B) ALP activity in chondrocyte isolated from *Col2a1-ICAT* transgenic mice. However, BMP failed to induce the expression of (C) *VEGF* and (D)*Mmp13* in *Col2a1-ICAT* transgenic chondrocytes. *$P<0.05$, unpaired *t*-test, $n=3$. (E) RCJ3.1C5.18 chondrogenic cells were treated with BMP2 (100 ng/ml) for 2 hours with or without the BMP antagonist noggin (300 ng/ml) or Wnt signaling inhibitor Dkk1 (1 μg/ml). Total RNA was extracted and *VEGF* expression determined by real-time RT-PCR normalized to β-actin levels. The induction of *VEGF* expression by BMP2 was completely inhibited by noggin and Dkk1. (F) RCJ3.1C5.18 cells were treated with BIO (1 μg/ml) for 2 hours with or without noggin or Dkk1. The expression of *VEGF* mRNA levels were measured and normalized to β-actin levels. BIO induced *VEGF* expression within 2 hours. Noggin partially inhibited BIO-induced *VEGF* expression. *$P<0.05$, unpaired *t*-test, compared to the untreated group, $n=3$. **$P<0.05$, unpaired *t*-test, compared with BMP2 or BIO treatment groups, $n=3$. (G,H) To further determine changes in VEGF and MMP13 expression in vivo in *Col2a1-ICAT* transgenic mice, we performed immunostaining using the anti-VEGF and anti-MMP13 antibodies. The results showed that the area and intensity of the expression of VEGF and MMP13 proteins were reduced in *Col2a1-ICAT* transgenic mice.

BMP2 stimulate expression of *Vegf* mRNA within 2 hours. (2) In *Col2a1-ICAT* transgenic chondrocytes both basal and BMP2-induced *Vegf* expression is significantly reduced. Although our experiments were performed in isolated primary chondrocytes, β-catenin might have similar effects on *Vegf* expression in mesenchymal cells and neighboring cells in vivo. (3) Wnt3a-induced *Vegf* expression is completely blocked by addition of noggin, a BMP-ligand antagonist. (4) The *Vegf* promoter region responsive to β-catenin is located in the 1-kb region of the *Vegf* proximal promoter. (5) Five potential TCF-binding sites and one potential Smad1/Smad5-binding site have been identified in this region by sequence analysis and – demonstrated by ChIP assay – β-catenin binds to this region. Although further analysis is still required, current findings strongly suggest that BMP2 and Wnt signaling molecules activate *Vegf* gene transcription in a coordinated fashion.

Previously, interactions between Wnt/β-catenin and BMP signaling pathways have been reported; in osteoblasts, BMP2 and β-catenin stimulate osteoblast differentiation synergistically (Mbalaviele et al., 2005; Rawadi et al., 2003). Here, we demonstrated that (1) Wnt3a and β-catenin stimulate *Bmp2* and *Bmp4* mRNA expression; (2) in *Col2a1-ICAT* transgenic mice, *Bmp2* and *Bmp4* expression was reduced; and (3) Wnt3a-induced *colX* expression was completely inhibited by the addition of the BMP antagonist noggin. Whereas these results suggest that BMP signaling is downstream of the Wnt/β-catenin signaling in chondrocytes during the process of chondrocyte maturation, our findings also show that Wnt/β-catenin and BMP signaling molecules act coordinately during the activation of *Vegf* gene transcription. Thus, in chondrocytes, the relationship between these two signaling pathways is complex and, in some cases, gene specific. Our findings establish that these two signaling pathways are highly inter-related and have a crucial role in postnatal chondrocyte development.

Materials and Methods

LacZ staining and immunohistochemistry

β-galactosidase (β-gal) staining was performed in embryos. In brief, specimens were dissected in PBS and prefixed in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, and 0.02% Nonidet P-40 at 4°C for 30-90 minutes. Samples were washed three times in PBS containing 0.02% Nonidet P-40 at room temperature for 30 minutes before they were stained in PBS containing 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 at 30°C for 6-16 hours. The specimens were subsequently fixed in formaldehyde and processed for paraffin or frozen sections.

Generation of *Col2a1-ICAT* transgenic mice

TOP-gal mice were obtained from The Jackson Laboratory (Bar Harbor, ME). To generate the *Col2a1-ICAT* transgenic mice, DNA fragments encoding ICAT were cloned into the *Not*I site of a collagen 2 α1 (Col2a1)-based expression vector PKN185 (Tanaka et al., 2000; Tsuda et al., 2003). The resulting vector contains the Flag-ICAT

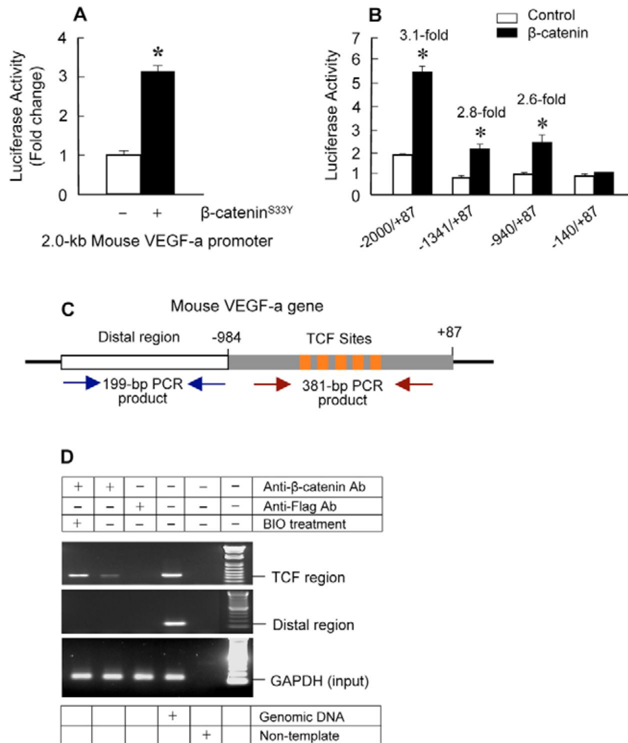

Fig. 8. β-catenin activates the *Vegfa* promoter. 2.0-kb *Vegfa* promoter fragment was cloned into pGL4 vector and deletion mutants were generated. (A) RCJ3.1C5.18 chondrogenic cells were co-transfected with constitutively active β-catenin expression plasmid and the *Vegfa* promoter. Cell lysates were extracted 48 hours after transfection and luciferase was activity measured. β-catenin significantly stimulated VEGF-a promoter activity. (B) RCJ3.1C5.18 chondrocytes were transfected with the deletion constructs of the *Vegfa* promoter and the β-catenin expression plasmid. Luciferase activity was measured 48 hours after transfection. β-catenin activates *Vegfa* promoter activity in 2.0-kb, 1.34-kb and 0.94-kb fragments but not in the 0.14-kb fragment. The results suggest that the β-catenin-responsive region is located in the –940 to –140 region of the *Vegfa* promoter. $^*P<0.05$, unpaired *t*-test, $n=3$ (A,B). (C) The TCF/LEF sites (TREs) in the *Vegfa* promoter are shown. (D) Chromatin IP assay was performed using RCJ3.1C5.18 cells treated with or without BIO (1 μg/ml) for 2 hours. IP was performed using anti-β-catenin antibody, and anti-Flag antibody was used as a negative control. The DNA-protein complex was crosslinked and used as PCR template. The ChIP assay showed that β-catenin binds to the *Vegfa* promoter and that addition of BIO to the cultures enhanced β-catenin binding.

expression unit and includes the 5′ *Nde*I site of the *Col2a1* promoter (nucleotides 1940-2971, GenBank accession number: M65161), the β-globin intron cassette, Flag-ICAT, SV40 poly (A) and the Col2a1 enhancer (nucleotide 4930–5571, GenBank accession number: M65161). The expression unit of Flag-ICAT was excised by *NdeI* and *Hind*III digestion. The *ICAT* transgene was then purified and injected into pronuclei of fertilized eggs (C57BL/6J). Positive transgenic founder mice were identified by PCR and confirmed by Southern blot analysis.

Isolation of primary mouse sternal chondrocytes

Primary mouse sternal chondrocytes were isolated as described (Li et al., 2006b). Briefly, 3-day-old neonatal mice were sacrificed and genotyped using tail tissues obtained at sacrifice. The anterior rib cage and sternum were harvested en-bloc, washed with PBS and then digested with Pronase (Roche Applied Science, Indianapolis, IN) dissolved in PBS (2 mg/ml) in a 37°C water bath with continuous shaking for 60 minutes. This was followed by incubation in a solution of collagenase D (3 mg/ml dissolved in serum-free Dulbecco's modified Eagle's medium (DMEM; Roche Applied Science) for 90 minutes at 37°C. The soft tissue debris was removed and the remaining sterna and costosternal junctions were further digested in fresh collagenase D solution in Petri dishes in a 37°C incubator for 5 hours with intermittent shaking. This step allows remnant fibroblasts to attach to the Petri dish while the chondrocytes remain afloat in the medium. The digestion solution was filtered through Swinex to remove all residual bone fragments. The solution was centrifuged, and the cells were resuspended in complete medium [DMEM with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 100 mM L-glutamine, and 50 μg/ml ascorbic acid, pH 7.1]. Cells were then counted and plated at the appropriate density. To remove any remaining fibroblasts, 24-hour cultures were treated with 0.05% trypsin for 1 minute to lift the fibroblasts from the culture dish while allowing the chondrocytes to remain attached.

Antibodies and reagents

The following antibodies were used in this study: anti-β-catenin monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-cyclin D1 monoclonal antibody (1:1000 dilution, Upstate, Charlottesville, VA), anti-Flag monoclonal antibody (1:200 dilution for immuno-fluorescence assay and 1:1000 dilution for western blotting, Sigma), rabbit anti-Ki67 monoclonal antibody (1:200 dilution for immunostaining, Lab Vision, Fremont, CA), and anti-PECAM-1 antibody (1:100 for immunostaining, Santa Cruz Technology). BIO was purchased from Calbiochem (San Diego, CA), and dissolved in DMSO. BMP2, Wnt3a, noggin and Dkk1 were obtained from R&D Systems (Minneapolis, MN).

Western blot analysis

Cells were lysed on ice for 30 minutes in a buffer containing 50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml asprotinin) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). Proteins were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and detected using the respective antibodies. Bound primary antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies followed by ECL-mediated visualization (Amersham, Piscataway, NJ).

Immunostaining

Mouse tissues were dissected in PBS, fixed in 10% buffered formalin, and paraffin embedded. Tissue sections were subject to immune complex staining using an avidin–biotinylated-enzyme complex according to the manufacturer's protocol (Vector Laboratories, Burlingame, CA). Rabbit monoclonal anti-Ki-67 was used in the analysis. Bound primary antibodies were detected with fluorescein-conjugated secondary antibodies (Amersham, Piscataway, NJ). Immunostaining of cultured cells was performed using indirect fluorescent staining technique. Briefly, cells were fixed with 4% paraformaldehyde for 10 minutes and treated with 0.5% Triton X-100 for 15 minutes and 50 mM glycine for 10 minutes followed by blocking with the PBS-Triton buffer containing 3% BSA for 30 minutes. Samples were then incubated with primary antibody for 1 hour and fluorescent conjugated secondary antibody for 45 minutes and mounted with vectashield (Lab Vision, Fremont, CA). The anti-Flag mouse monoclonal antibody was used as primary antibody.

Alkaline phosphatase (ALP) activity assay

Primary sternal chondrocytes isolated from wild-type (WT) or *Col2a1-ICAT* transgenic mice were plated into 12-well culture plates and grown to 60% confluence. The cells were treated with the growth factors as indicated. 48 hours after incubation, cells were washed twice with PBS, and cell lysates extracted with passive lysis buffer (Promega, Madison, WI). ALP activity in cell lysates was measured using a Sigma ALP assay kit (Sigma, St Louis, MO) and normalized by the protein content.

Total RNA extraction and real-time reverse transcriptase (RT)-PCR analysis

Total cellular and tissue RNA was prepared by Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One microgram total RNA was used to synthesize cDNA using iScripts cDNA Synthesis Kit (Bio-Rad, Hercules, CA). PCR primers were: VEGF Fw: 5-TTACTGCTGTACCTCCACC-3, Rev: 5′-ACAGGA-CGGCTTGAAGATG-3′; MMP13 Fw: 5′-TTTGAGAACACGGGGAAGA-3′, Rev: 5-ACTTTGTTGCCAATTCCAGG-3′; Col-X Fw: 5′-ACCCCAAGGACC-TAAAGGAA-3′, Rev: 5′-CCCCAGGATACCCTGTTTTT-3′; BMP2 Fw: 5′-GCTTTTCTCGTTTGTGGAGC-3′, Rev: 5′-TGGAAGTGGCCCATTTAGAG-3′; BMP-4 Fw: 5′-GAGGAGGAGGAAGAGCAGAG-3′, Rev: 5′-TGGGATGTTCT-CCAGATGTT-3′; ALP Fw: 5′-TGACCTTCTCTCCTCCATCC-3′, Rev: 5′-CTT-CCTGGGAGTCTCATCCT-3′.

Transferase UTP nick-end labeling (TUNEL) staining

A TUNEL staining kit (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI) was used to assess cell death by catalytically incorporating fluorescein-12-dUTP at 3′-OH DNA ends using the terminal deoxynucleotidyl transferase and recombinant enzyme (rTDT). After paraffin removal, the tissue sections were placed in equilibration buffer and then in a solution containing the equilibration buffer, nucleotide mix, and rTDT enzyme and incubated at 37°C for 1 hour. The reaction was stopped with 2× saline sodium citrate (SSC). Hoechst dye 33342 was used to stain the nuclei. Fluorescence microscopy (Zeiss, Axiovert 40 CFL, Chester, VA) was used to identify apoptotic cells.

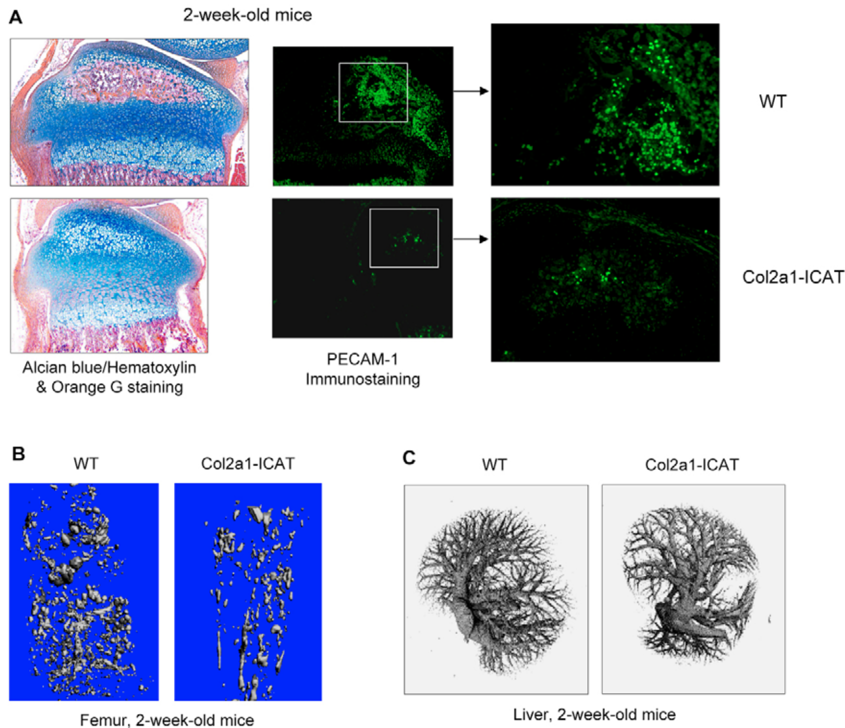

Fig. 9. *Col2a1-ICAT* transgenic mice have reduced angiogenesis. (A) PECAM-1 was detected by immunostaining using an anti-PECAM-1 antibody in tissue sections from 2-week-old mice. PECAM-1 expression was significantly decreased in *Col2a1-ICAT* transgenic mice at the secondary ossification center area of the femoral epiphysis compared with WT mice. (B,C) Microfil perfusion was performed using 2-week-old *Col2a1-ICAT* transgenic mice and their WT littermates. Femurs (B) and liver (C) were imaged using microCT after decalcification of femurs. The images of vessel structure showed a reduced vessel networks in the cartilage tissue (B) but not liver (C) in *Col2a1-ICAT* transgenic mice. The results indicate that inhibition of Wnt signaling by ICAT leads to reduced angiogenesis during endochondral bone formation.

DNA plasmids

We used the 2.7 kb mouse BMP2 promoter and 2.0 kb mouse BMP-4 promoter (Feng et al., 1995; Feng et al., 2003; Garrett et al., 2003), constitutively active β-catenin, β-catenin^{S33Y} (gift from Kenneth Kinzler) (Morin et al., 1997), and the BMP signaling reporter, 12×SBE-luc (Zhao et al., 2003).

Luciferase assay

Cells were transfected by Lipofectamine 2000 (Invitrogen) with various combinations of plasmids. 0.5 μg of reporter plasmids (TOP-flash, 12×SBE-luc) and 0.01 μg of internal control SV-40 *Renilla* plasmid were using in the reporter assays. The luciferase activity was measured using the Promega dual system kit.

Chromatin immunoprecipitation (ChIP) assay

Chromatin IP was performed as described in the manual of the ChIP Assay kit (Upstate, Charlottesville, VA). Briefly, RCJ3.1C518 chondrocytes were cultured in 4×15 cm culture dishes to 70-80% confluence. Chondrocytes were then treated with 1 μM BIO for 4 hours, followed by crosslinking using formaldehyde at 37°C for 10 minutes. The crosslink reaction was stopped using glycine buffer, cells were washed with a protease inhibitor cocktail (Roche Applied Science) and harvested. Chondrocytes were then incubated with lysis buffer on ice for 30 minutes and the cell lysate was sonicated to shear the genomic DNA to 200-bp to 500-bp fragments. After centrifugation, the supernatant was incubated with protein G beads saturated with salmon sperm DNA for 30 minutes to pre-clean the cell lysate. A total of 50 μl anti-β-catenin antibody (Santa Cruz Technology) was added to the cell lysate and incubated for overnight at 4°C. Lysates were then incubated with 100 μl of proteinG beads for 2 hours. Beads were pelleted by centrifugation for 2 minutes at 4000 rpm and washed according to the manufacturer's protocol. The protein-DNA complex was eluted with ChIP elution buffer containing 0.01M $NaHCO_3$ and 0.5% SDS. After centrifugation, 5M NaCl was added to the eluted solution to a final concentration of 200 mM and then incubated at 65°C overnight. After reversing the crosslink, the samples were further digested by proteinase K. The DNA was extracted by phenol-chloroform, precipitated with ethanol and prepared for PCR. Primer sequences were: TRE Fw: 5′-ACTCTA-GTTGTCCCTATCCTCA-3′, TRE Rev: 5′-TCTGCGCTTCTCACCGGTAACA-3′; Cont Fw: 5′-AGAGCTTGCCCGAGGAATGT-3′, Cont Rev: 5′-CTCCGATACCT-GTGGGAAGA-3′; GAPDH Fw: 5′-AACGACCCCTTCATTGAC-3′, GAPDH Rev: 5′-TCCACGACATACTCAGCAC-3′.

Quantification of vascularity using microCT analysis

The bone vascular network was examined on tissue sections of animals following perfusion of a lead-chromate-based contrast agent using microCT analysis. Microfil MV-122 contrast medium, a radiopaque silicone rubber compound containing lead chromate, was perfused through the heart together with 4% paraformaldehyde. After perfusion, the hind limbs were decalcified using 10% EDTA solution. After complete decalcification, the samples were scanned again to image vascularization.

We thank Kenneth Kinzler (The Johns Hopkins University Medical Institutions, Baltimore, Maryland) for providing us the β-catenin^{S33Y} plasmid and Tetsu Akiyama (University of Tokyo, Japan) for the ICAT plasmid. This work was supported by grants AR038945 (to R.J.O.), AR053717 (to R.J.O.) and AR054465 (to D.C.) from the National Institute of Health.

References

Akiyama, H., Lyons, J. P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., Deng, J. M., Taketo, M. M., Nakamura, T., Behringer, R. R. et al. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.* **18**, 1072-1087.

Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D. H., McMahon, A. P., Sommer, L., Boussadia, O. and Kemler, R. (2001). Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* **128**, 1253-1264.

Daniels, D. L. and Weis, W. I. (2002). ICAT inhibits beta-catenin binding to Tcf/Lef-family transcription factors and the general coactivator p300 using independent structural modules. *Mol. Cell* **10**, 573-584.

DasGupta, R. and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557-4568.

Day, T. F., Guo, X., Garrett-Beal, L. and Yang, Y. (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev. Cell* **8**, 739-750.

Easwaran, V., Lee, S. H., Inge, L., Guo, L., Goldbeck, C., Garrett, E., Wiesmann, M., Garcia, P. D., Fuller, J. H., Chan, V. et al. (2003). beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res.* **63**, 3145-3153.

Feng, J. Q., Chen, D., Cooney, A. J., Tsai, M. J., Harris, M. A., Tsai, S. Y., Feng, M., Mundy, G. R. and Harris, S. E. (1995). The mouse bone morphogenetic protein-4 gene. Analysis of promoter utilization in fetal rat calvarial osteoblasts and regulation by COUP-TFI orphan receptor. *J. Biol. Chem.* **270**, 28364-28373.

Feng, J. Q., Xing, L., Zhang, J. H., Zhao, M., Horn, D., Chan, J., Boyce, B. F., Harris, S. E., Mundy, G. R. and Chen, D. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. *J. Biol. Chem.* **278**, 29130-29135.

Garrett, I. R., Chen, D., Gutierrez, G., Zhao, M., Escobedo, A., Rossini, G., Harris, S. E., Gallwitz, W., Kim, K. B., Hu, S. et al. (2003). Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. *J. Clin. Invest.* **111**, 1771-1782.

Graham, T. A., Clements, W. K., Kimelman, D. and Xu, W. (2002). The crystal structure of the beta-catenin/ICAT complex reveals the inhibitory mechanism of ICAT. *Mol. Cell* **10**, 563-571.
Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K. and Kemler, R. (1995). Lack of beta-catenin affects mouse development at gastrulation. *Development* **121**, 3529-3537.
Hill, T. P., Spater, D., Taketo, M. M., Birchmeier, W. and Hartmann, C. (2005). Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev. Cell* **8**, 727-738.
Kolpakova, E. and Olsen, B. R. (2005). Wnt/beta-catenin-a canonical tale of cell-fate choice in the vertebrate skeleton. *Dev. Cell* **8**, 626-627.
Komori, T. (2003). Requisite roles of Runx2 and Cbfb in skeletal development. *J. Bone Miner. Metab.* **21**, 193-197.
Krebsbach, P. H., Nakata, K., Bernier, S. M., Hatano, O., Miyashita, T., Rhodes, C. S. and Yamada, Y. (1996). Identification of a minimum enhancer sequence for the type II collagen gene reveals several core sequence motifs in common with the link protein gene. *J. Biol. Chem.* **271**, 4298-4303.
Kronenberg, H. M. (2003). Developmental regulation of the growth plate. *Nature* **423**, 332-336.
Li, T. F., Chen, D., Wu, Q., Chen, M., Sheu, T. J., Schwarz, E. M., Drissi, H., Zuscik, M. and O'Keefe, R. J. (2006a). Transforming growth factor-beta stimulates cyclin D1 expression through activation of beta-catenin signaling in chondrocytes. *J. Biol. Chem.* **281**, 21296-21304.
Li, T. F., Darowish, M., Zuscik, M. J., Chen, D., Schwarz, E. M., Rosier, R. N., Drissi, H. and O'Keefe, R. J. (2006b). Smad3-deficient chondrocytes have enhanced BMP signaling and accelerated differentiation. *J. Bone Miner. Res.* **21**, 4-16.
Maes, C., Carmeliet, P., Moermans, K., Stockmans, I., Smets, N., Collen, D., Bouillon, R. and Carmeliet, G. (2002). Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech. Dev.* **111**, 61-73.
Maes, C., Stockmans, I., Moermans, K., Van Looveren, R., Smets, N., Carmeliet, P., Bouillon, R. and Carmeliet, G. (2004). Soluble VEGF isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. *J. Clin. Invest.* **113**, 188-199.
Mbalaviele, G., Sheikh, S., Stains, J. P., Salazar, V. S., Cheng, S. L., Chen, D. and Civitelli, R. (2005). Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. *J. Cell. Biochem.* **94**, 403-418.
McCrea, P. D., Turck, C. W. and Gumbiner, B. (1991). A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. *Science* **254**, 1359-1361.
Metsaranta, M., Toman, D., de Crombrugghe, B. and Vuorio, E. (1991). Mouse type II collagen gene. Complete nucleotide sequence, exon structure, and alternative splicing. *J. Biol. Chem.* **266**, 16862-16869.
Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-1790.
Ornitz, D. M. (2005). FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev.* **16**, 205-213.
Peifer, M., McCrea, P. D., Green, K. J., Wieschaus, E. and Gumbiner, B. M. (1992). The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. *J. Cell Biol.* **118**, 681-691.
Rawadi, G., Vayssiere, B., Dunn, F., Baron, R. and Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J. Bone Miner. Res.* **18**, 1842-1853.
Satoh, K., Kasai, M., Ishidao, T., Tago, K., Ohwada, S., Hasegawa, Y., Senda, T., Takada, S., Nada, S., Nakamura, T. et al. (2004). Anteriorization of neural fate by inhibitor of beta-catenin and T cell factor (ICAT), a negative regulator of Wnt signaling. *Proc. Natl. Acad. Sci. USA* **101**, 8017-8021.
Tago, K., Nakamura, T., Nishita, M., Hyodo, J., Nagai, S., Murata, Y., Adachi, S., Ohwada, S., Morishita, Y., Shibuya, H. et al. (2000). Inhibition of Wnt signaling by ICAT, a novel beta-catenin-interacting protein. *Genes Dev.* **14**, 1741-1749.
Tanaka, K., Matsumoto, Y., Nakatani, F., Iwamoto, Y. and Yamada, Y. (2000). A zinc finger transcription factor, alphaA-crystallin binding protein 1, is a negative regulator of the chondrocyte-specific enhancer of the alpha1(II) collagen gene. *Mol. Cell. Biol.* **20**, 4428-4435.
Tsuda, M., Takahashi, S., Takahashi, Y. and Asahara, H. (2003). Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. *J. Biol. Chem.* **278**, 27224-27229.
Xue, B., Wen, C., Shi, Y., Zhao, D. and Li, C. (2005). Human NRAGE disrupts E-cadherin/beta-catenin regulated homotypic cell-cell adhesion. *Biochem. Biophys. Res. Commun.* **336**, 247-251.
Yoon, B. S. and Lyons, K. M. (2004). Multiple functions of BMPs in chondrogenesis. *J. Cell. Biochem.* **93**, 93-103.
Zelzer, E., Mamluk, R., Ferrara, N., Johnson, R. S., Schipani, E. and Olsen, B. R. (2004). VEGFA is necessary for chondrocyte survival during bone development. *Development* **131**, 2161-2171.
Zhang, X., Xie, C., Lin, A. S., Ito, H., Awad, H., Lieberman, J. R., Rubery, P. T., Schwarz, E. M., O'Keefe, R. J. and Guldberg, R. E. (2005). Periosteal progenitor cell fate in segmental cortical bone graft transplantations: implications for functional tissue engineering. *J. Bone Miner. Res.* **20**, 2124-2137.
Zhao, M., Qiao, M., Oyajobi, B. O., Mundy, G. R. and Chen, D. (2003). E3 ubiquitin ligase Smurf1 mediates core-binding factor alpha1/Runx2 degradation and plays a specific role in osteoblast differentiation. *J. Biol. Chem.* **278**, 27939-27944.