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## Overexpression of Smurf2 Stimulates Endochondral Ossification Through Upregulation of $\beta$ -Catenin

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### Abstract

Ectopic expression of Smurf2 in chondrocytes and perichondrial cells accelerated endochondral ossification by stimulating chondrocyte maturation and osteoblast development through upregulation of  $\beta$ -catenin in *Col2a1-Smurf2* embryos. The mechanism underlying Smurf2-mediated morphological changes during embryonic development may provide new mechanistic insights and potential targets for prevention and treatment of human osteoarthritis.

**Introduction:** Our recent finding that adult *Col2a1-Smurf2* mice have an osteoarthritis-like phenotype in knee joints prompted us to examine the role of Smurf2 in the regulation of chondrocyte maturation and osteoblast differentiation during embryonic endochondral ossification.

**Materials and Methods:** We analyzed gene expression and morphological changes in developing limbs by immunofluorescence, immunohistochemistry, Western blot, skeletal preparation, and histology. A series of markers for chondrocyte maturation and osteoblast differentiation in developing limbs were examined by in situ hybridization.

**Results:** Ectopic overexpression of Smurf2 driven by the *Col2a1* promoter was detected in chondrocytes and in the perichondrium/periosteum of 16.5 dpc transgenic limbs. Ectopic Smurf2 expression in cells of the chondrogenic lineage inhibited chondrocyte differentiation and stimulated maturation; ectopic Smurf2 in cells of the osteoblastic lineage stimulated osteoblast differentiation. Mechanistically, this could be caused by a dramatic increase in the expression of  $\beta$ -catenin protein levels in the chondrocytes and perichondrial/periosteal cells of the *Col2a1-Smurf2* limbs.

**Conclusions:** Ectopic expression of Smurf2 driven by the *Col2a1* promoter accelerated the process of endochondral ossification including chondrocyte maturation and osteoblast differentiation through upregulation of  $\beta$ -catenin, suggesting a possible mechanism for development of osteoarthritis seen in these mice.

### Keywords

Smurf2;  $\beta$ -catenin; chondrocyte; osteoblast; endochondral ossification

## INTRODUCTION

The process of endochondral ossification is a complex one that consists of multiple stages. First, the mesenchymal cells aggregate to form condensations, which subsequently differentiate into two types of cells: chondrocytes that form cartilage elements and osteoblast

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lineage cells that form perichondrium surrounding the cartilage rudiment. At 13.5 days postcoitum (dpc) of mouse development, the condensed mesenchymal cells in the center of the condensation undergo differentiation into chondrocytes, which produce cartilage-specific matrix proteins including type 2 collagen (*Col2a1*) and aggrecan. Chondrocytes undergo proliferation and become hypertrophic chondrocytes around 14.5 dpc, and the primary ossification center is formed by 14.5–16.5 dpc. Furthermore, this process of chondrocyte maturation is coupled to osteoblast recruitment and differentiation. In parallel to the process of chondrocyte maturation and matrix ossification, mesenchymal cells in the periphery of the cartilage differentiate and mature into the osteoblast lineage to form perichondrium or periosteum flanking chondrocytes and the primary spongiosa. Overall, this process of endochondral ossification happens in the majority of the skeletal elements, except the flat bones of the skull.

The process of endochondral ossification is carefully regulated by an array of growth factors and transcription factors. *Sox9*, a high-mobility-group (HMG-box) transcription factor, is required for chondrogenic entry of mesenchyme and chondrocyte differentiation and maturation during embryonic development.(1-5) For example, inactivation of *Sox9* before chondrogenic mesenchymal condensation results in an early chondrocyte differentiation arrest; removal of *Sox9* after chondrogenetic condensation leads to lack of proliferating chondrocytes and a severe reduction of cartilage matrix production in the growth plate.(1,2) In addition, *Sox9* also plays a role in negatively regulating the transition of chondrocytes into hypertrophic chondrocytes in the growth plate, leading to an enlarged hypertrophic zone and accelerated immature ossification in the heterozygous *Sox9* mutant mice.(2,6) Consistent with the *in vivo* studies that *Sox9* is essential for chondrogenesis, *in vitro* studies have shown that *Sox9* binds to and activates chondrocyte-specific enhancer elements in *Col2a1*, *aggrecan*, and *CD-AP* genes.(7-10) Indian Hedgehog (*Ihh*) is indispensable for endochondral ossification. *Ihh* expressed by prehypertrophic chondrocytes coordinates with PTH-like peptide (*PthrP*; *Pthlh*) expressed by periarticular chondrocytes to form a negative feedback loop regulating the growth and differentiation of chondrocytes.(11) In addition, *Ihh* directly signals to both chondrocytes and osteoblast lineage cells in perichondrium and regulates chondrocyte proliferation and osteoblast development.(12,13) *Runx2*, a transcription factor with Runt domain, is required for osteoblast cell fate determination and sufficient to induce osteoblast-specific marker genes including *collagen type 1 (Col1a1)*, *bone sialoprotein (BSP)*, and *osteocalcin (OC)*.(14-16) *Osterix (Osx1)*, an osteoblast-specific transcription factor, acts downstream of *Runx2* to ensure full differentiation of *Runx2*-expressing osteoprogenitor cells along the osteoblast lineage.(17)

Canonical Wnt signaling has been implicated in multiple steps of the endochondral bone formation cascade. The canonical Wnt signaling pathway signals through  $\beta$ -catenin. At the chondrogenic mesenchymal condensation stage (E11-E13),  $\beta$ -catenin is normally expressed in perichondrium surrounding the cartilage rudiments, with relatively lower levels detected in the condensed chondrogenic mesenchymal cells, suggesting a potential role of  $\beta$ -catenin in differentiation of mesenchymal precursors toward the osteoblast lineage.(4,5) More direct evidence for the involvement of  $\beta$ -catenin in osteoblastogenesis is that removal of  $\beta$ -catenin from either mesenchymal progenitors for both chondrocytes and osteoblast ( $\beta$ -catenin<sup>fllox/fllox</sup>; *Dermo-Cre*) or chondrogenic progenitors ( $\beta$ -catenin<sup>fllox/fllox</sup>; *Col2a1-Cre*) results in a complete loss of mature osteoblasts and bone formation.(5,13) Interestingly, in both types of mutant embryos, along with other two similar studies from  $\beta$ -catenin<sup>fllox/fllox</sup>; *Prx1-Cre* and  $\beta$ -catenin<sup>fllox/fllox</sup>; *Osx1-Cre* mouse mutants,(4,18) ectopic chondrocyte differentiation instead of osteoblast commitment occurred in the area where bone is normally formed, suggesting a fundamental role for  $\beta$ -catenin in repression of chondrogenic potential in osteochondroprogenitors located within the periosteum. Similar to loss-of-function studies, stabilization of  $\beta$ -catenin in either mesenchymal cells ( $\beta$ -catenin  $\Delta$ ex3<sup>Prx+</sup>) or chondrogenic

precursors ( $\beta$ -catenin<sup>+floxp(ex3);Col2a1-Cre</sup>) also results in defects in cartilage and bone formation.(3,4) However, ectopic overexpression of *Wnt14* driven by the *Col2a1* promoter promotes osteoblast differentiation and chondrocyte maturation, leading to accelerated endochondral ossification in the *Col2a1-Wnt14* mutant long bones.(5) Thus,  $\beta$ -catenin level in the mesenchymal precursors or osteochondroprogenitors is critical for controlling cell fate determination, cell differentiation, and maturation through regulation of Sox9 and Runx2 expression.(3-5) In general,  $\beta$ -catenin levels are upregulated by Wnt ligands.  $\beta$ -catenin levels are normally kept low through continuous proteasome-mediated degradation of phosphorylated  $\beta$ -catenin, which is catalyzed by the enzymes glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) in a “destruction complex.”(19,20) On Wnt ligand binding to its receptors, the activity of GSK-3 $\beta$  is suppressed, and nonphosphorylated  $\beta$ -catenin accumulates in the cytoplasm and translocates into the nucleus as a co-activator to regulate expression of Wnt target genes.(21) Thus, any mechanism by which normal  $\beta$ -catenin regulation is uncoupled from Wnt signaling control could result in dysregulation of osteoblast/chondrocyte function in the human, leading to abnormal bone mass or degenerative joints.(22-24) However, our understanding of the abnormal regulation of  $\beta$ -catenin in these diseases and others remains incomplete.

Smurf2, an E3 ubiquitin ligase, induces multiple targets for ubiquitination and degradation and modulates various signaling pathways in vitro. Smurf2 directly interacts with Smad1 and Smad2 and indirectly interacts with the type I receptor of TGF $\beta$  and  $\beta$ -catenin through an adaptor Smad7 for ubiquitination and proteasome-dependent degradation.(25-28) Our previous study that overexpression of Smurf2 in articular chondrocytes activated cell maturation indicated an important role of Smurf2 in development of osteoarthritis (OA).(29) Because activation of articular chondrocyte maturation and cartilage degeneration during OA recapitulates many of the events that occur during embryonic development, Smurf2 may also regulate the process of embryonic development. In this study, we showed that overexpression of *Smurf2* under the control of *Col2a1* promoter accelerates endochondral ossification through up-regulation of  $\beta$ -catenin during embryonic development.

## MATERIALS AND METHODS

### Skeletal preparation

Embryos at 13.5, 14.5, 16.5, and 18.5 dpc and newborns were harvested in cold PBS, fixed in 95% ethanol for 2–5 days, and stained with 0.3% Alcian blue 8GS and 0.1% alizarin red S for 3–6 days. The embryos were cleared in 1% KOH in a series concentration of glycerol solution.

### Histology and in situ hybridization

Embryonic limbs at 16.5 and 18.5 dpc, as well as adult knee joints, were dissected in cold PBS, fixed in 4% paraformaldehyde at 4°C for 1–3 days, and embedded in paraffin for histology and in situ hybridization. Immunofluorescence staining was performed according to a modified protocol.(30) Briefly, antigen was unmasked by incubating the sections in sodium citrate buffer at 95°C for 15 min followed by cooling to room temperature. The primary monoclonal anti-flag M2 antibody (Sigma) was diluted at 1:600, and polyclonal anti- $\beta$ -catenin antibody (Cell Signaling) was diluted at 1:120 in PBS/Triton. The secondary antibodies used were FITC-conjugated donkey anti-mouse or -rabbit (H&L; Rockland) diluted at 1:2000. The coverslip slides and Vectashield mounting medium were from Vector Laboratories.

Immunohistochemistry was performed as described previously.(13) Briefly, sections were incubated with a monoclonal anti-GSK-3 $\beta$  (Cell Signaling) diluted at 1:100 after antigen retrieval. Signal was detected using horseradish peroxidase (HRP) streptavidin and romulin AEC chromagen (Biocare Medical). Alizarin red, safranin O, and hematoxylin staining were performed using standard histological methods. We assessed chondrocyte number per unit area

by counting cell number from three randomly chosen regions of each section. We used six sections at a similar level from six independent samples. For in situ hybridization, after fixation, the samples were decalcified with diethylpyrocarbonate (DEPC)-treated 10% EDTA and embedded in paraffin, and 6- $\mu$ m sections were cut. A riboprobe was labeled with [<sup>35</sup>S]UTP (Amersham), and in situ hybridization was performed using a technique that we have described previously.(31)

### Western blot

Protein was extracted from limbs as described previously.(32) Briefly, the 16.5 dpc limbs were pulverized in liquid nitrogen using a mini-pestle (Clontech) before protein extraction with RIPA buffer. Protein concentration of the disrupted tissues was determined using the Bradford method. Fifty-microgram aliquots of protein extract were separated by SDS-PAGE and transferred to a PVDF membrane (Schleider and Schuell). The blots were probed overnight at 4°C with anti- $\beta$ -catenin pAb and anti-GSK-3 $\beta$  mAb (Cell Signaling) at a 1:1000 dilution. Blots were further incubated for 1 h at room temperature in the presence of HRP-conjugated secondary antibodies against rabbit or mouse (BioRad) at a dilution of 1:2000. The immune complexes were detected using ECL-Femto (Pierce) and visualized through exposure of X-OMAT AR film (Kodak).

## RESULTS

### Acceleration of endochondral ossification in *Col2a1-Smurf2* embryos

The human flag-*Smurf2* cDNA was cloned downstream of *Col2a1* promoter, and *Col2a1-Smurf2* transgenic mouse lines were generated. The adult transgenic mice showed OA phenotype. Hematoxylin and Alcian blue staining indicated that normal articular cartilage surface is smooth in 7.5-mo-old wildtype mice (Fig. 1A), whereas cartilage degeneration and osteophytes were detected in the transgenic littermates (Fig. 1A', arrows and arrowheads, respectively). The *Smurf2* transgene was mainly expressed by the articular chondrocytes and much less by the growth plate chondrocytes in the adult mice (data not shown); however, it was highly expressed in all chondrocytes in the 16.5 dpc transgenic limbs compared with that in the wildtype littermates (Figs. 1B and 1B'). In addition, consistent with previous findings about the *Col2a1* promoter function,(5,33) the expression of *Smurf2* was highly detected in the osteoprecursors in the perichondrium/periosteum where osteoblasts arise (Fig. 1B', arrows). This suggests that *Col2a1* promoter not only works in all chondrocytes but also in some osteoprogenitors, which have the potential to differentiate into the chondrogenic lineage.(4,5, 18) Therefore, we took the advantage of the *Col2a1* expression profile and studied the impact of *Smurf2* on chondrocytes and osteoprogenitors during embryonic development. During embryogenesis, no apparent difference was observed between wildtype and transgenic embryos at 13.5 dpc by whole mount preparation stained with Alcian blue, which specifically stains cartilage matrix blue (data not shown). This suggests that mesenchymal condensation and differentiation into the skeletal anlagen is not affected. However, from 14.5 dpc onward, we found that the ossification in all skeletal elements derived by endochondral bone formation was accelerated in the transgenic embryos, and the size of transgenic embryos was shortened over the wildtype littermates, which is shown by skeletal preparation stained with Alcian blue and Alizarin red, which specifically stains mineralized matrix in a red color (Figs. 1C–1F'). In general, at 14.5 dpc, chondrocyte hypertrophic differentiation occurred in the middle of most long bones (Figs. 1D–1F, black arrows), or ossification had just started in some proximal long bones such as the humerus (Fig. 1D, red arrow). In contrast, in the 14.5 dpc transgenic mouse embryos, the ossification domain was apparent and expanded in proximal long bones such as scapula, humerus of the forelimb, and femurs of the hind-limb, as well as ribs (Figs. 1D'–1F', red arrows). The ossification in the transgenic distal long bones such as radius and ulna of the forelimb, and tibia and fibula of the hind-limb was less accelerated than that in the transgenic

proximal long bones (Fig. 1, compare E' with D'), which is caused by the dynamic mesenchyme migration from the lateral plate mesoderm, leading to delayed development of distal versus the proximal elements (Fig. 1, compare E with D).(4,19) By 16.5 dpc, the difference in overall appearance between the wildtype and transgenic embryos was less than that at 14.5 dpc (Figs. 1G and 1G'), but the continuous acceleration of endochondral ossification in the transgenic long bones still could be observed. Specifically, the length of the ossification domain in the 16.5 dpc transgenic forelimbs and hindlimbs is increased 11% and 16%, respectively, compared with wildtype littermates (Fig. 1, compare H' with H and I' with I), although the full length of the transgenic limbs was shorter (Figs. 1H', 1H, 1I', and 1I; data not shown). Despite this, no gross phenotypic difference was observed between the wildtype and transgenic littermates after birth (data not shown). These data suggest that ectopic overexpression of *Smurf2* under the control of *Col2a1* promoter enhances the process of endochondral ossification during the early stage of skeletal development.

### Smurf2 promotes osteoblast development

Accelerated endochondral ossification could have been caused by advanced osteoblast differentiation or/and maturation in the transgenic mice. We tested this hypothesis by examining a panel of markers activated at various stages of osteoblast development. *Colla1*, an early marker for the osteoblast lineage,(34) was expressed at a lower level in early osteoblast progenitors in the perichondrium toward the epiphysis (Fig. 2A, red arrow) and was subsequently upregulated in more mature osteoblast lineage cells in the perichondrium surrounding the prehypertrophic and hypertrophic chondrocytes (Fig. 2A, red arrowhead) in the bone collar flanking the marrow cavity (Fig. 2A, white arrowhead), as well as in the primary spongiosa (Fig. 2A, asterisk), consistent with the previous studies.(13,32) In the 16.5 dpc transgenic littermates, however, the expression levels of *Colla1* were dramatically increased in the perichondrium, which is from the immature area toward the epiphysis (Fig. 2A', red arrow) to the mature area flanking the hypertrophic chondrocytes (Fig. 2A', red arrowhead), in the bone collar (Fig. 2A', white arrowhead), and the primary spongiosa (Fig. 2A', asterisk). Like *Colla1*, *Runx2*, another marker of early stage of osteoblast lineage, was normally expressed in a steady low level in the perichondrium toward the epiphysis and flanking the prehypertrophic and hypertrophic chondrocytes (Fig. 2B, red arrow and arrowhead, respectively), in the bone collar, and the primary spongiosa at 16.5 dpc (Fig. 2B, white arrowhead and asterisk, respectively). Similar to the expression pattern of *Colla1* in the 16.5 dpc wildtype and its transgenic siblings, the *Runx2* levels were also significantly increased in the bone collar and the primary spongiosa in the transgenic limbs (Fig. 2B', white arrowhead and asterisk, respectively) compared with that in the wildtype counterparts. *Osx*, a specific marker of osteoblast lineage and functioning downstream of *Runx2*,(17) was normally expressed in the perichondrium where *Runx2* was expressed (Fig. 2C). By comparing the expression pattern of *Osx* in the 16.5 dpc wildtype with that in the transgenic siblings, we found that the expression domain in the transgenic limbs was enlarged (Fig. 2, orange line, compare C' with C). *BSP*, a marker gene for late stage of the osteoblast lineage, was expressed in the perichondrium surrounding the prehypertrophic and hypertrophic chondrocytes, in the bone collar, and primary spongiosa (Fig. 2D, red-, white-arrowhead, and asterisk, respectively). In addition, *BSP* was also expressed in hypertrophic chondrocytes (Fig. 2D, triangle). Consistent with the overexpression pattern of *Colla1*, and *Runx2* in the 16.5 dpc transgenic limbs, *BSP* expression levels were significantly increased in the primary spongiosa and slightly increased in the perichondrium in the transgenic limbs (Fig. 2D', asterisk and red arrowhead, respectively). In addition, *BSP* expression was also increased in the transgenic hypertrophic chondrocytes over the wildtype counterparts (Fig. 2, triangle, D' and D), which was further confirmed by the histological evidence that more hypertrophic cells were in the hypertrophic zone of the transgenic limbs (Figs. 2C and 2D). *Oc*, an osteoblast terminal differentiation marker, was detected in the perichondrium flanking hypertrophic chondrocytes and in the bone

collar and primary spongiosa in the 16.5 dpc wildtype limbs (Fig. 2E). Although the expression levels of *Oc* in the transgenic limbs were low, the expression domain was enlarged over the wildtype littermates (Fig. 2, orange line, E' and E). Notably, we found the similar expression pattern of these marker genes in the 18.5 dpc wildtype and their transgenic littermates (data not shown). All these data suggest that ectopic overexpression of *Smurf2* under the control of *Col2a1* promoter accelerated osteoblast differentiation and stimulated immature osteoblast maturation. Consistent with this, Alizarin red staining showed that the mineralization domain is significantly expanded in the 16.5 dpc transgenic limbs versus the wildtype littermates (Fig. 2, black line, F', and F), further confirming that *Smurf2* accelerated endochondral ossification by promoting osteoblast development.

### Smurf2 accelerates chondrocyte maturation

The process of endochondral ossification consists of development and maturation of both chondrocytes and osteoblasts; therefore, an increase in chondrocyte maturation rate could be another reason for the acceleration of endochondral ossification. To test this hypothesis, we examined a panel of stage-specific chondrocyte maturation markers by in situ hybridization and performed histological analysis. In the 16.5 dpc transgenic limbs, we found that the hypertrophic zone is longer than that of the wildtype littermates (Figs. 4D, 4D', 4C, and 4C'). Furthermore, we observed that the number of prehypertrophic and hypertrophic chondrocytes in a defined area was increased in the transgenic growth plates versus that in the wildtype counterparts (Figs. 4D', 4C', and 4E). These morphological changes suggest that chondrocytes are undergoing immature hypertrophic differentiation, similar to the phenotype in the heterozygous *Sox9* mutant mice.<sup>(6)</sup> In situ hybridization showed that the domain of *type 10 collagen (Col10a1)*, a marker gene for hypertrophic chondrocytes, was stronger and wider in the 16.5 dpc transgenic limbs than that in the wildtype counterparts (Figs. 3A' and 3A), consistent with the histological evidence that the transgenic embryo possessed a longer hypertrophic zone with more hypertrophic chondrocytes in the hypertrophic zone of the growth plates (Figs. 4C and 4D). *Ihh*, a marker for prehypertrophic chondrocytes, was expressed at normal levels in the 16.5 dpc transgenic limbs, compared with that in the wildtype littermates (Figs. 3B' and 3B). However, the two domains of either *Col10a1* or *Ihh* were further from each other in the transgenic limbs than that in the wildtype littermates, although the entire length of the transgenic limb was shorter (Fig. 3, orange line, compare A' with A and B' with B). Furthermore, the distance from the domain of either *Col10a1* or *Ihh* to the end of the long bones was reduced in size in the transgenic limbs over the wildtype counterparts (Fig. 3, yellow line, compare A' with A and B' with B). Like *Col10a1* and *Ihh*, the region between the two domains of *VEGF* and *MMP13*, markers for the terminally differentiated hypertrophic chondrocytes, was expanded in the 16.5 dpc transgenic limbs versus that in the wildtype littermates, and the distance between the *VEGF/MMP13* domain and the articular surface was shortened in the 18.5 dpc transgenic limbs versus that in the wildtype littermates (Fig. 3, orange line, compare C' with C and D' with D; data not shown). All these results indicated that overexpression of *Smurf2* in chondrogenic lineage stimulated chondrocyte maturation during endochondral ossification.

### Chondrocyte differentiation is inhibited in *Col2a1-Smurf2* embryos

To understand further whether chondrocyte differentiation and proliferation was affected by overexpression of *Smurf2* under the control of *Col2a1* promoter, we analyzed chondrocyte differentiation markers and performed BrdU in vivo pulse and proliferating cell nuclear antigen staining. *Sox9*, a marker gene for early chondrocyte differentiation and essential for activation of cartilage matrix expression, was highly detected in all chondrocytes including the resting chondrocytes in the articular region and growth plate chondrocytes from the proliferative zone and the prehypertrophic zone. However, *Sox9* was absent from the hypertrophic chondrocytes at 16.5 dpc (Fig. 4A). In the 16.5 dpc transgenic littermates, the *Sox9* mRNA expression levels

were significantly decreased in these chondrocytes (Figs. 4A' and 4A). *Col2a1* was normally expressed coincidentally with the expression of *Sox9*. Similar to the expression pattern of *Sox9* mRNA in both wildtype and transgenic littermates at 16.5 dpc, *Col2a1* mRNA was highly expressed in all chondrocytes except hypertrophic chondrocytes in the wildtype limbs, and a significant decrease was detected in all chondrocytes in the transgenic littermates (Figs. 4B' and 4B). Safranin O staining (stains aggrecan protein in a red color) showed that the protein levels of aggrecan, a specific marker for chondrocytes, was decreased in the 16.5 dpc transgenic limbs compared with that in the wildtype littermates (Figs. 4D, 4D', 4C, and 4C'). The similar expression pattern of these genes was also found in the 18.5 dpc transgenic embryos and wildtype littermates (data not shown). These results suggest that chondrocyte differentiation is inhibited in the *Col2a1-Smurf2* transgenic limbs. We also examined whether chondrocyte proliferation was affected in the transgenic embryos. BrdU and proliferating cell nuclear antigen staining showed that chondrocyte proliferation was not significantly affected in the transgenic limbs compared with that in the wildtype littermates (data not shown).

### **β-catenin protein levels are upregulated in *Col2a1-Smurf2* limbs**

To study the molecular mechanism underlying *Smurf2*-mediated acceleration of endochondral ossification, we analyzed several signaling pathways. *Ihh*, expressed by prehypertrophic chondrocytes, regulates the growth and differentiation of chondrocytes directly or through the control of other factors such as *Pthrp*; *Pthlh*. In addition, *Ihh* also acts directly on perichondrium to initiate an osteogenic program in osteoblast progenitors. *Ihh* signaling is initiated by binding to *Patched* (*ptc*), which is a receptor and transcriptional target, and normally expressed in the proliferating chondrocytes, the primary spongiosa within the marrow cavity, and the perichondrium surrounding the *Ihh* domain (Fig. 3E).(11,13) We examined the mRNA levels of *Ihh*, *Ptc*, *Pthrp*; *Pthlh*, and *Pthrp receptor (PP-R)* by in situ hybridization; however, no significant difference could be found between the transgenic limbs and the wildtype littermates at 16.5 dpc (Figs. 3B', B, E, and E'; data not shown), suggesting that *Ihh* and *Ihh-Pthrp* signaling pathways were not responsible for the phenotypic changes in osteoblasts and chondrocytes in the *Col2a1-Smurf2* transgenic embryos.

The phenotypic similarities between *Col2a1-Wnt14* and *Col2a1-Smurf2* mouse embryos prompted us to examine β-catenin in the *Col2a1-Smurf2* embryos. Immunofluorescence staining showed that β-catenin protein was highly expressed in the perichondrium/periosteum flanking the prehypertrophic and hypertrophic chondrocytes, as well as the primary spongiosa (Fig. 5A, arrows, data not shown), whereas its expression was relatively low in the chondrocytes in the 16.5 dpc wildtype limbs (Fig. 5A, asterisk). In the 16.5 dpc transgenic littermates, however, the protein levels of β-catenin in the perichondrium/periosteum adjacent the prehypertrophic and hypertrophic chondrocytes was dramatically increased (Fig. 5B, arrows). Notably, β-catenin was also expressed with relatively higher levels in the perichondrium flanking the immature chondrocytes toward the epiphysis in the transgenic limbs (Fig. 5B, arrowheads) than that in the wildtype counterparts. Upregulation of β-catenin was detected not only in the perichondrium/periosteum but also in the chondrocytes including resting, proliferating and prehypertrophic chondrocytes of the growth plate in the *Col2a1-Smurf2* limbs (Figs. 5B, 5B', 5A, and 5A'). In addition, high magnification of the immunofluorescence staining showed that β-catenin nuclear translocation was increased in the perichondrial cells and the chondrocytes of the growth plate in the transgenic limbs (Figs. 5B' and 5A'; data not shown). To confirm the observed upregulation of β-catenin in the transgenic limbs shown by immunofluorescence, we extracted protein from limbs containing cartilage and perichondrium/periosteum and performed Western blot analysis. As expected, the transgenic limbs showed a 3-fold increase in the protein levels of β-catenin compared with the wildtype littermates (Figs. 5C and 5C').

## Upregulation of $\beta$ -catenin by Smurf2 occurs at protein levels

To understand whether the increase in  $\beta$ -catenin protein levels in the perichondrium/periosteum and chondrocytes was caused by an increase in transcription of the gene, we examined the mRNA levels of  *$\beta$ -catenin* in the limbs by in situ hybridization. Consistent with the expression pattern of protein in the 16.5 dpc wildtype limbs, the mRNA levels of  *$\beta$ -catenin* were very high in the perichondrium/periosteum surrounding the hypertrophic chondrocytes, and the marrow cavity (Fig. 5D, arrow; data not shown); the mRNA levels were relatively low in the perichondrium flanking the prehypertrophic chondrocytes (Fig. 5D, arrowhead) and very low in the chondrocytes (Fig. 5D, asterisk) in the 16.5 dpc wildtype limbs. In contrast to the dramatic increase in the protein levels in the transgenic limbs, no significant increase in the mRNA levels of  *$\beta$ -catenin* could be detected in the transgenic limbs including the perichondrium/periosteum, the primary spongiosa, and the chondrocytes (Figs. 5D' and 5D), indicating that the increase in  $\beta$ -catenin protein in the transgenic limbs is not caused by an increase in its transcription.

Because  $\beta$ -catenin protein levels are regulated through continuous phosphorylation and degradation of  $\beta$ -catenin by GSK-3 $\beta$ , (19) we examined the protein levels of GSK-3 $\beta$  in 16.5 dpc limbs. Immunohistochemistry showed that GSK-3 $\beta$  is detected in the perichondrium and the proliferating chondrocytes (Figs. 5E, arrow, and 5E'; data not shown) and is strongly detected in the maturing chondrocytes (Fig. 5E, arrowhead) in the 16.5 dpc wildtype limbs. However, the protein levels of GSK-3 $\beta$  were significantly decreased in either perichondrium or chondrocytes (Figs. 5F, 5F', 5E, and 5E'; data not shown) in the transgenic littermates. Western blot analysis showed that the protein levels of GSK-3 $\beta$  in the 16.5 dpc transgenic limbs were 2.2-fold lower than that in the wildtype littermates (Figs. 5H and 5H'), consistent with the result by immunohistochemistry. No significant difference in the protein levels of GSK-3 $\alpha$  could be detected between the transgenic limbs and the wildtype littermates (Fig. 5G). These data suggested that upregulation of  $\beta$ -catenin in *Col2a1-Smurf2* transgenic limbs is caused by a decrease in GSK-3 $\beta$  by Smurf2.

## DISCUSSION

### Stimulation of osteoblast differentiation and chondrocyte maturation in *Col2a1-Smurf2* limbs

Perichondrial cells that surround the developing growth plate differentiate into osteoblasts during endochondral ossification. Recently, several lines of evidence have shown that *Col2a1*, a marker gene for chondrocyte differentiation, is also expressed in the perichondrium where osteoblasts arise. (5,13) The *Col2a1*-expressing osteochondroprogenitors in the perichondrium normally differentiate into osteoblasts, but will indeed differentiate into chondrocytes after removal of  $\beta$ -catenin from the cells. (4,5,13,18) Therefore,  $\beta$ -catenin signaling is needed to repress the chondrogenic potential of osteochondroprogenitors and to ensure full differentiation along osteoblast lineage by upregulation of *Runx2* and *Osx* in the perichondrium. Indeed, we found that the transgene *Smurf2* driven by the *Col2a1* promoter was expressed not only in chondrocytes but also in the perichondrium surrounding the prehypertrophic and the hypertrophic chondrocytes at 16.5 dpc (Fig. 1). Thus, the *Col2a1-Smurf2* mouse model has allowed us to study the impact of Smurf2 on both chondrocytes and osteochondroprogenitors in a developing long bone. A panel of osteoblast lineage markers is expressed by the perichondrial cells while these cells are undergoing osteoblast differentiation. The expression levels of *Colla1*, *Runx2*, and *BSP*, markers for early and late stages of osteoblast differentiation, were dramatically increased in the transgenic perichondrium/periosteum and the primary spongiosa (Fig. 2), suggesting that overexpression of *Smurf2* in the perichondrial cells stimulates osteoblast differentiation. The expression levels of *Oc*, a marker gene for the terminally differentiated osteoblasts located in the bone collar and the primary spongiosa, was not increased in the transgenic bone collar or the primary spongiosa at 16.5 dpc, but its expression domain was extended from the perichondrium/periosteum



adjacent to the marrow cavity and hypertrophic chondrocytes to that around the proliferating chondrocytes (Figs. 2E and 2E'). Therefore, ectopic expression of *Smurf2* in the perichondrium/periosteum appears to stimulate immature osteoblast maturation through upregulation of Runx2/Osx signaling (Fig. 2).<sup>(5)</sup> In addition, progression of chondrocyte maturation was also accelerated in the *Col2a1-Smurf2* long bones. Specifically, the expression domains of *Col10* and *Ihh*, markers for the hypertrophic and prehypertrophic chondrocytes, respectively, were separated faster so that the distance between the *Col10* or *Ihh* domain and the long bone end was shorter than that in the wildtype littermates. *VEGF* and *MMP13*, markers for terminally differentiated chondrocytes, show a similar expression pattern in the transgenic and wildtype littermates to that of *Col10* and *Ihh*, suggesting that chondrocyte maturation was accelerated (Fig. 3). Consistent with accelerated osteoblast development and chondrocyte maturation in the *Col2a1-Smurf2* embryos, endochondral ossification was advanced accordingly (Figs. 1 and 2F').

### Inhibition of chondrocyte differentiation through downregulation of Sox9 in *Col2a1-Smurf2* chondrocytes

*Sox9*, a transcription factor essential for chondrocyte differentiation and cartilage formation, marks the earliest stage of the chondrocyte lineage.<sup>(1)</sup> *Col2a1*, a specific marker for the chondrocyte lineage, is normally coexpressed with *Sox9*. The transcription levels of both *Sox9* and *Col2a1* were significantly decreased in the transgenic limbs (Figs. 4A' and 4B'), suggesting an inhibition of chondrocyte differentiation in the transgenic embryos. Like *Col2a1*, aggrecan, another major component of the cartilage matrix, is also decreased in the transgenic limbs (Fig. 4D). It is likely that the decrease in the expression levels of *Col2a1* and aggrecan is caused by the decrease in *Sox9* signaling in the transgenic chondrocytes, because *Sox9* is responsible for activation of the promoter and enhancer of these cartilage matrix genes.<sup>(7,8,10)</sup> Consistent with the phenotypic changes of an enlarged hypertrophic zone and immature endochondral ossification observed in the *Sox9* heterozygous mutant mice,<sup>(6)</sup> we found similar histological evidence in the *Col2a1-Smurf2* transgenic embryos, namely, a longer hypertrophic zone with more hypertrophic chondrocytes and advanced endochondral ossification (Figs. 4D, 4C, 4E, and 1C-1I'). Thus, it seems that overexpression of *Smurf2* under the *Col2a1* promoter inhibits chondrocyte differentiation and stimulates chondrocyte maturation through downregulation of *Sox9* in the chondrocytes.

### Upregulation of $\beta$ -catenin at the protein level in *Col2a1-Smurf2* embryos

A recent study by Day et al.<sup>(5)</sup> showed that ectopic over-expression of *Wnt14* under the *Col2a1* promoter stimulated osteoblast differentiation, chondrocyte maturation, and endochondral ossification through  $\beta$ -catenin signaling in a developing long bone, which is largely similar to the phenotype we observed in the developing long bones of *Col2a1-Smurf2* embryos (Figs. 1-3). The phenotypic similarities between *Col2a1-Wnt14* and *Col2a1-Smurf2* prompted us to examine  $\beta$ -catenin mRNA and protein levels in the limbs. Immunofluorescence staining showed that the protein levels of  $\beta$ -catenin were dramatically increased in all stages of the osteoblast lineage in the perichondrium/periosteum in the *Col2a1-Smurf2* transgenic limbs, which may be responsible for the observed immature osteoblast maturation (Fig. 2) and consistent with the previous study by Day et al.<sup>(5)</sup> Most importantly, upregulation of  $\beta$ -catenin occurred in all chondrocytes of the transgenic growth plates (Fig. 5B). However, its mRNA level was not increased accordingly in the transgenic limbs shown by in situ hybridization (Fig. 5D'), suggesting a mechanism underlying upregulation of  $\beta$ -catenin protein in the transgenic limbs occurred at the protein levels. Supporting this hypothesis, the protein levels of GSK-3 $\beta$  in the transgenic limbs were significantly decreased versus that in the wildtype counterparts (Figs. 5E, 5F, 5H, and 5H'). Given *Smurf2* is an E3 ubiquitin ligase, *Smurf2* may decrease GSK-3 $\beta$  through induction of GSK-3 $\beta$  ubiquitination and proteasomal degradation, leading to an increase in  $\beta$ -catenin.

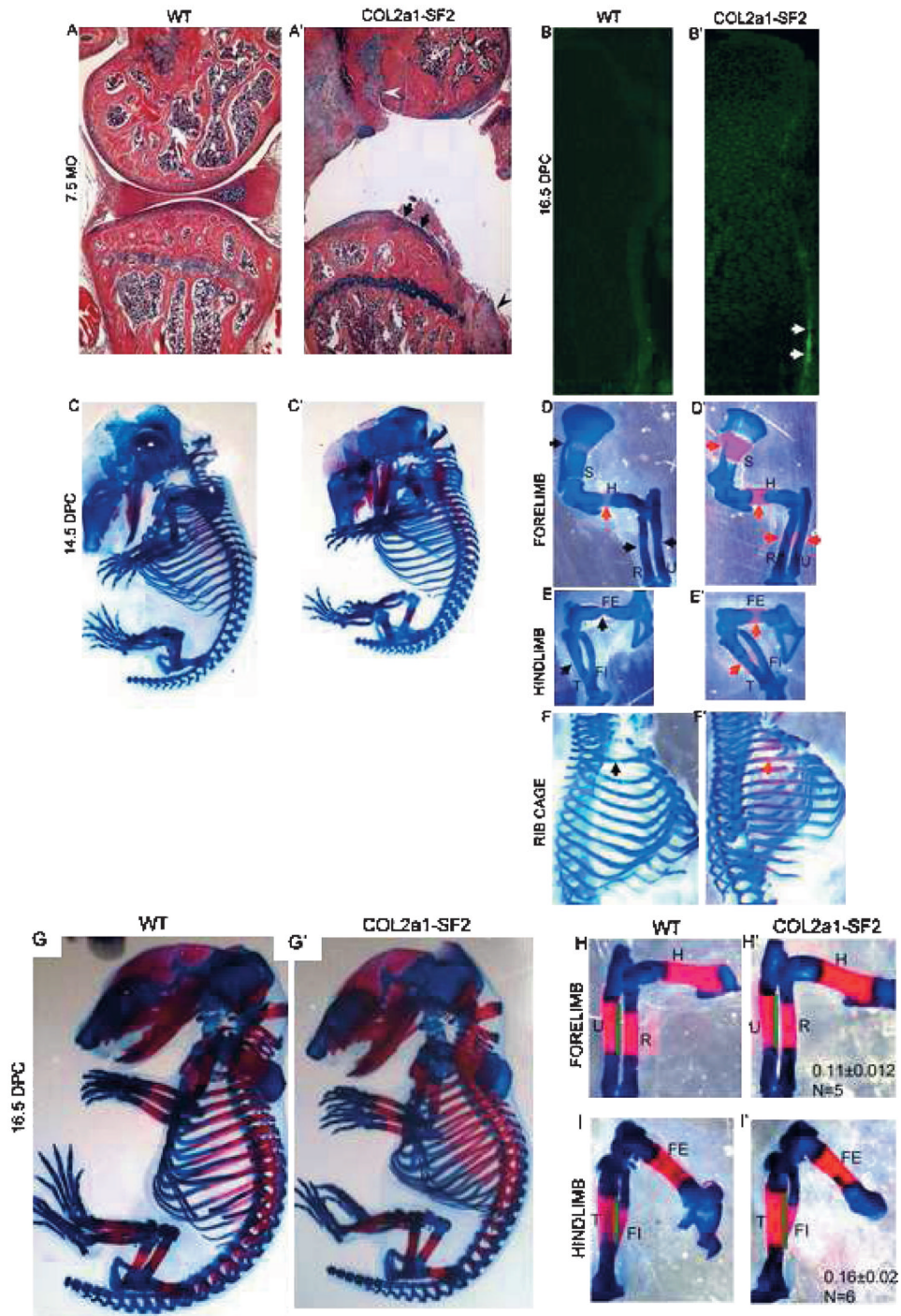
## ACKNOWLEDGMENTS

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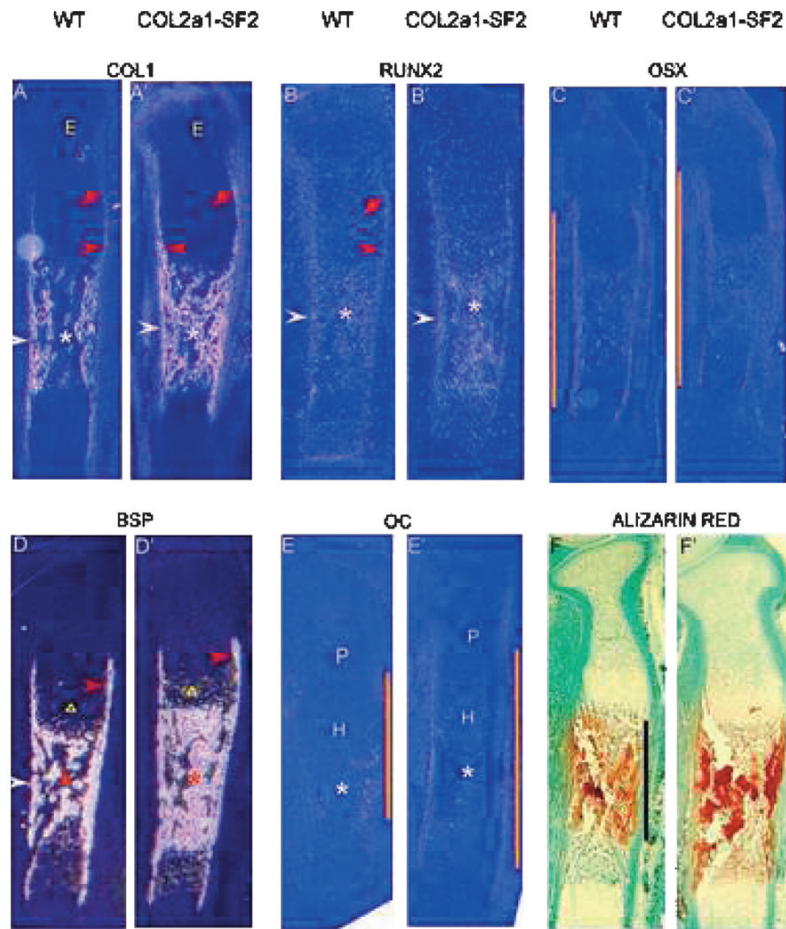
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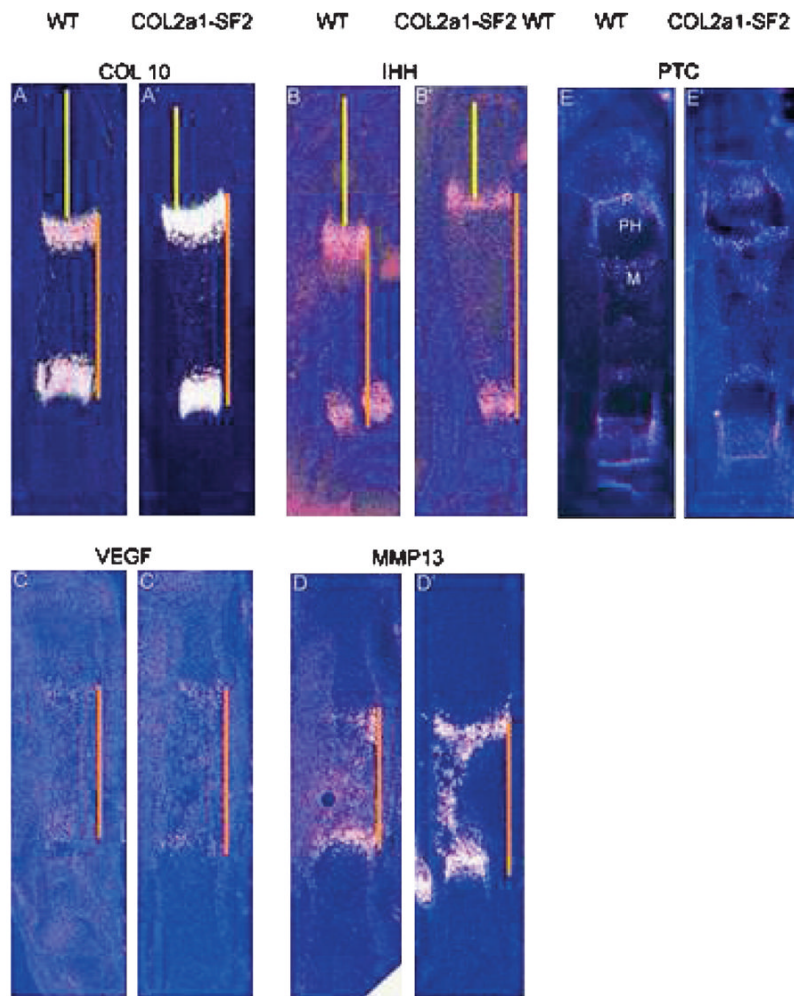
**FIG. 1.**

Ectopic overexpression of *Smurf2* under *Col2a1* promoter promotes endochondral ossification. (A and A') Phenotype of knee joints from 7.5-mo-old wildtype (A) and *Col2a1-Smurf2* (A') mice. Arrows and arrowheads indicate cartilage degeneration and osteophytes, respectively. (B and B') Immunofluorescence of wildtype (B) and *Col2a1-Smurf2* (B') tibias at 16.5 dpc. Arrows indicate overexpression of *Smurf2* in the perichondrial cells. (C–I') Skeletal preparation of mouse embryos. Wildtype (C and G) and *Col2a1-Smurf2* (C' and G') embryos were stained with Alcian blue and alizarin red at 14.5 (C and C') and 16.5 dpc (G and G').

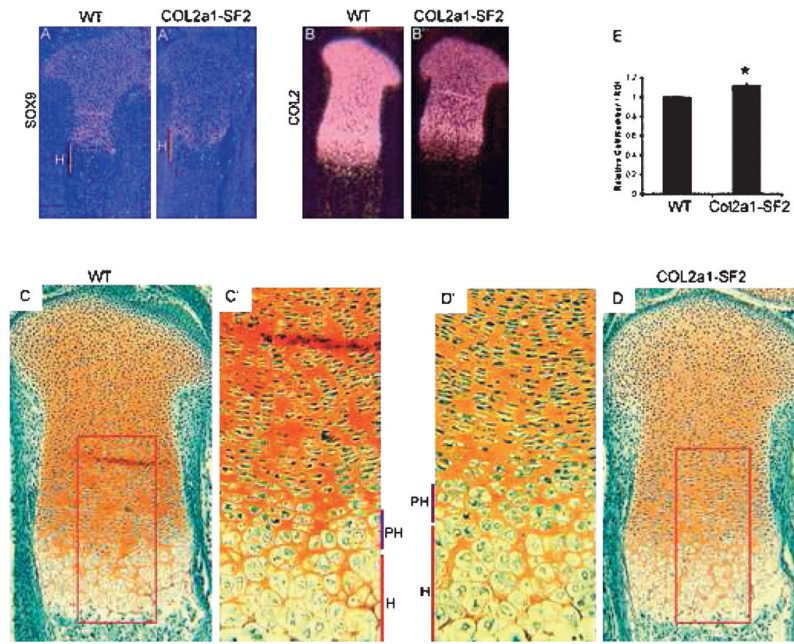
Forelimbs (D, D', H, and H'), hindlimbs (E, E', I, and I'), and rib cages (F and F') were separated from C, C', G, and G' with the aid of a dissecting microscope. Black arrows indicate the hypertrophic region; red arrows indicate the ossification region. S, scapula; H, humerus; R, radius; U, ulna; FE, femur; T, tibia; FI, fibula. Green line indicates the length of ossification domain.

**FIG. 2.**

Accelerated osteoblast differentiation and bone matrix mineralization in *Col2a1-Smurf2* embryos. (A–E') Analyses of osteoblast differentiation by in situ hybridization. In situ hybridization was performed on longitudinal sections through the tibia from wildtype (A–E) and *Col2a1-Smurf2* (A'–E') embryos. Red arrow and red arrowhead indicates the perichondrium toward the epiphysis and that surrounding the prehypertrophic and hypertrophic chondrocytes, respectively. Triangles indicate the prehypertrophic zone. White arrowheads and asterisks indicate the perichondrium/periosteum surrounding the primary spongiosa and the primary spongiosa, respectively. E, epiphysis; P, proliferation zone; H, hypertrophic zone. The orange line indicates the length of the domain of gene expression. (F and F') Alizarin red staining was performed on the sections from wildtype (F) and *Col2a1-Smurf2* (F') embryos. Black line indicates ossification domain.

**FIG. 3.**

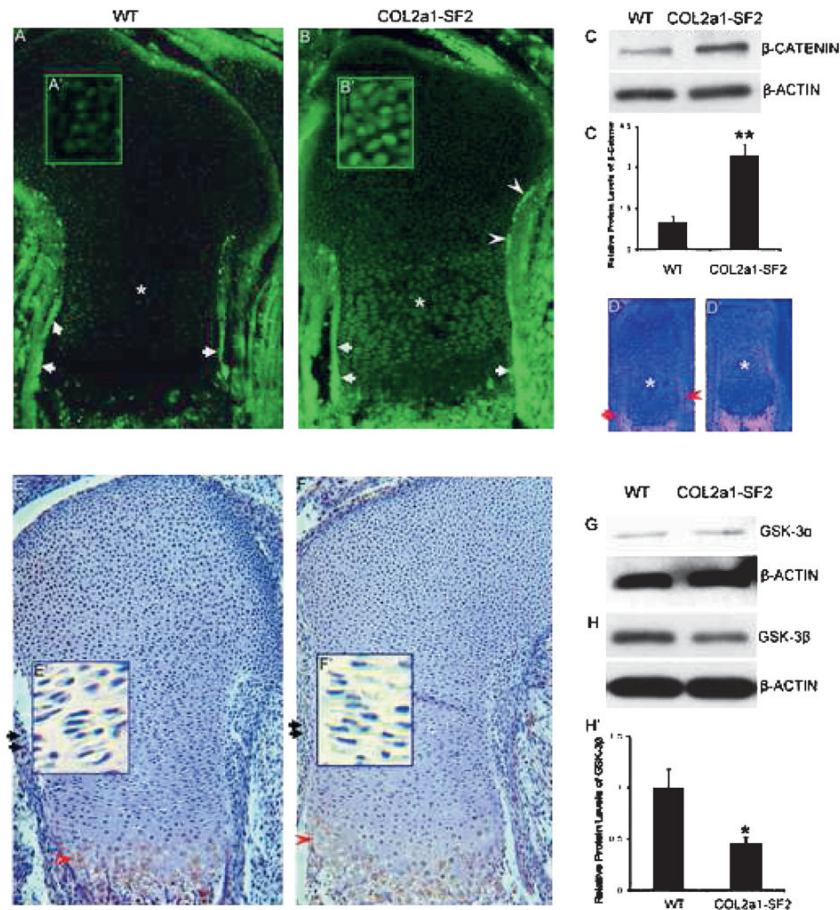
Advanced chondrocyte maturation in *Col2a1-Smurf2* embryos. (A–D') Chondrocyte maturation markers were analyzed on longitudinal sections through the tibia from wildtype (A–D) and *Col2a1-Smurf2* (A'–D') embryos by in situ hybridization. The orange line indicates the distance between the separated domains of *Col10* (A and A'), *Ihh* (B and B'), *VEGF* (C and C'), and *MMP13* (D and D'), and the yellow line indicates that from the domain to the articular surface. (E and E') *Ptc* expression was analyzed on sections from wildtype (E) and *Col2a1-Smurf2* (E') by in situ hybridization. P, proliferation zone; PH, prehypertrophic zone; M, marrow cavity.



**FIG. 4.**

Inhibition of chondrocyte differentiation in *Col2a1-Smurf2* limbs. (A–B') Expression of chondrocyte differentiation markers *Sox9* (A and A') and *Col2* (B and B') was analyzed on sections through the tibia from wildtype (A and B) and *Col2a1-Smurf2* (A' and B') embryos by in situ hybridization. H, hypertrophic zone (orange line). (C–D') Safranin O staining of tibias from wildtype (C) and *Col2a1-Smurf2* (D) embryos. C' and D' are higher-magnification images of boxed areas in C and D. PH, prehypertrophic zone (purple line); H, hypertrophic zone (red line). (E) Relative cell number per unit area of hypertrophic zone. Data are expressed as mean  $\pm$  SD ( $n$  [H11505] 6). Statistical significance is assessed by an unpaired Student's *t*-test ( $p < 0.01$ ).



**FIG. 5.**

Upregulation of  $\beta$ -catenin protein levels in *Col2a1-Smurf2* limbs. (A–C')  $\beta$ -catenin protein levels were analyzed by immunofluorescence (A–B') and Western blot (C and C') in wildtype and *Col2a1-Smurf2* limbs as indicated. A' and B' are higher magnification of resting chondrocytes. (D and D') Analysis of  $\beta$ -catenin expression by in situ hybridization in wildtype (D) and *Col2a1-Smurf2* (D') limbs. Arrows and arrowheads indicate the perichondrium surrounding the hypertrophic and the prehypertrophic chondrocytes, respectively. Asterisks indicate the proliferating chondrocytes. (E–H') Analysis of GSK-3 $\beta$  (E–F' and H and H') and GSK-3 $\alpha$  (G) by immunohistochemistry (E–F') and Western blot (G and H) in wildtype and *Col2a1-Smurf2* limbs as indicated. Arrows and arrowheads indicate perichondrium and maturing chondrocytes, respectively. E' and F' are higher magnification of proliferating chondrocytes. The protein levels of  $\beta$ -catenin and GSK-3 were quantitated by scanning densitometry (normalized  $\beta$ -actin). Data are expressed as mean  $\pm$  SD of three independent experiments. Statistical significance is assessed by an unpaired Student's *t*-test (\*\* $p < 0.01$ ; \* $p < 0.05$ ).