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The rate of endochondral bone growth determines final height in humans and is tightly controlled. Glycogen synthase kinase-3 (GSK-3) is a negative regulator of several signaling pathways that govern bone growth, such as insulin/IGF and Wnt/β-catenin. The two GSK-3 proteins, GSK-3α and GSK-3β, display both overlapping and distinct roles in different tissues. Here we show that pharmacological inhibition of GSK-3 signaling in a mouse tibia organ culture system results in enhanced bone growth, accompanied by increased proliferation of growth plate chondrocytes and faster turnover of hypertrophic cartilage to bone. GSK-3 inhibition rescues some, but not all, effects of phosphatidylinositide 3-kinase inhibition in this system, in agreement with the antagonistic role of these two kinases in response to signals such as IGF. However, cartilage-specific deletion of the *Gsk3b* gene in mice has minimal effects on skeletal growth or development. Molecular analyses demonstrated that compensatory up-regulation of GSK-3α protein levels in cartilage is the likely cause for this lack of effect. To our knowledge, this is the first tissue in which such a compensatory mechanism is described. Thus, our study provides important new insights into both skeletal development and the biology of GSK-3 proteins. **(*Endocrinology* 152: 1755–1766, 2011)**

Most bones develop through endochondral ossification, in which a cartilage scaffold is first produced by chondrocytes and then converted to calcified bone tissue by bone-forming cells, osteoblasts. Chondrocytes of the growth plate are responsible for longitudinal growth of endochondral bones (reviewed in Refs. 1 and 2). The growth plate is divided into three distinct zones that can be identified by histological features, rate of cell cycle progression, or marker gene expression. The resting zone is farthest from the midbone (diaphysis) and consists of small chondrocytes with little cytoplasm and relatively low rates of proliferation. Some of these cells mature into proliferative zone chondrocytes that undergo rapid proliferation, resulting in flattened columnar cells surrounded by cartilage matrix. The proliferating chondrocytes then withdraw from the cell cycle and differentiate further into prehypertrophic and ultimately hypertrophic chondrocytes residing at the interface between growth plate cartilage and ossified bone. These hypertrophic cells represent the terminal differentiation stage and secrete a large amount of matrix and regulatory proteins. Finally, the hypertrophic cells die by apoptosis and leave behind a cartilage matrix that is invaded by blood vessels accompanied by osteoblast (bone-forming) and osteoclast (bone-resorbing) precursors, ultimately resulting in the replacement of cartilage by bone tissue (reviewed in Refs. 3–8).

The rate at which the chondrocytes progress through the zones of the growth plate determines the longitudinal growth of bone. This rate is consequently extremely important for normal skeletal development and final height in humans and is therefore tightly regulated both intrin-

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Abbreviations: cGKII, cGMP-dependent kinase II; DMSO, dimethylsulfoxide; e15.5, embryonic d 15.5; GSK-3, glycogen synthase kinase-3; hrp, horseradish peroxidase; IHC, immunohistochemistry; KO, knockout; LY, LY 294002; P0, postnatal d 0; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositide 3-kinase; SB86, SB415286.

sically and extrinsically by a complicated network of signaling pathways (reviewed in Refs. 2 and 9–11).

Many of these signaling pathways have been linked to the regulatory kinase glycogen synthase kinase-3 (GSK-3). Two pathways with relevance to this study are insulin or IGF/phosphatidylinositide 3-kinase (PI3K)/AKT/GSK-3 (12–16) and Wnt/GSK-3/β-catenin (14, 17, 18). GSK-3 is a serine/threonine kinase capable of phosphorylating a large number of substrates including glycogen synthase, from where its name originated, but its role in physiology has expanded exponentially since its discovery. GSK-3 is an unusual kinase in that it is constitutively active and usually negatively regulates pathways (19–21). Mammals have two GSK-3 proteins, GSK-3α and GSK-3β (encoded by different, highly homologous genes), with masses of 51 and 47 kDa, respectively. Regulation of GSK-α and -β can occur through at least two mechanisms: 1) through direct phosphorylation (Ser21 and Ser9 of GSK-3α and -β, respectively), for example by the PI3K/AKT (12) signaling pathway, or 2) through disruption of the protein complex involved in canonical Wnt signaling (22). Potential cross talk between these two pathways is highly debated as is the relationship between the two GSK-3 proteins, GSK-3α and -β. Many studies have demonstrated that phosphorylation of GSK-3 does not affect β-catenin levels, whereas other studies have suggested it can (23–27). Likewise, there is evidence for both overlapping and distinct roles of GSK-3α and GSK-3β (16, 17, 28–31). Tissue-specific roles and relations could explain some of these seemingly contradictory results (16, 32).

Several groups have examined how genetic manipulation of *Gsk3b* affects the skeleton (33, 34). The germline homozygous deletion of *Gsk3b* shows a variable phenotype depending on the genetic background and can result in embryonic lethality (33) or survival to postnatal d 0 (P0) with cleft palate, bifid sternum, and delayed ossification of the sternum, skull, ear bones, and cranial base (34). Heterozygous deletion of *Gsk3b* also causes a skeletal phenotype with increased ossification, clavicle abnormalities and increased bone resorption (35). It would appear that these opposing skeletal phenotypes are *Gsk3b* dosage-dependent effects; however, all these are phenotypes based on germline loss of *Gsk3b*. Consequently it is unclear whether these skeletal phenotypes are cell or tissue autonomous. Interestingly, GSK-3α global knockout (KO) mice have also been created; these are viable and fertile with similar body mass compared with controls (36) but display abnormalities in glucose metabolism and brain structure (16, 36, 37) without any described skeletal abnormalities. The two global KO models suggest that GSK-3β may play a more important role in skeletal development than GSK-3α. Here we address the role of GSK-3 signaling in chondrocytes using an organ culture system and cartilage-specific inactivation of the *Gsk3b* gene.

Materials and Methods

Materials

The following antibodies were used in this study: actin A5441 (Sigma Chemical Co., St. Louis, MO); cyclin D1 RM-9104-S1 (Neomarkers, Fremont, CA); goat antirabbit horseradish peroxidase (hrp) sc-2004, goat antimouse hrp sc-2005, p57/Kipp2 sc-8298 (Santa Cruz Biotechnology, Santa Cruz, CA); GSK-3β no. 9315, pGSK-3β no. 9336, GSK-3α no. 9338, pGSK-3α and -β no. 9331, and β-catenin no. 9562 (Cell Signaling Technology, Danvers, MA). General chemicals and supplies were purchased from Sigma and VWR (Radnor, PA); organ culture reagents were from Invitrogen (Carlsbad, CA).

Mouse breeding and genotyping

Mice homozygous for floxed *Gsk3b* alleles (*Gsk3b*$^{fl/fl}$) have been described previously (16, 38). *Gsk3b*$^{fl/fl}$ mice were crossed with mice expressing cre recombinase under control of the cartilage-specific mouse *Col2a1* promoter, donated by Drs. R. St-Arnaud and G. Karsenty, that we previously used in our lab (39, 40). Mice heterozygous for the floxed *Gsk3b* allele and expressing *Col2a1* cre were backcrossed with homozygous *Gsk3b* floxed mice. The offspring from these crosses were analyzed. Mice were exposed to a 12-h light, 12- h dark cycle and fed tap water and regular chow *ad libitum*. All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. PCR genotyping was performed from ear notch DNA using primers 5′-GGGGCAACCTTAATTTCATT-3′ (forward) and 5′-TCTGGGCTATAGCTATCTAGTAACG-3′ (reverse) for GSK-3β for 30 cycles of 96 C for 55 sec, 56.5 C for 45 sec, and 68 C for 2 min 45 sec to amplify. The cre transgene was detected using the primers 5′-CACACTGTGTAGTGCTTCGT-3′ (forward) and 5′-CCTCCAAACCATCCAAGAT-3′ (reverse) using 40 cycles of 95 C for 45 sec, 58 C for 30 sec, and 72 C for 1 min.

Organ culture

Tibias were isolated from embryonic d 15.5 (e15.5) mice and cultured for 6 d in serum-free medium containing α-MEM, ascorbic acid, β-glycerophosphate, BSA, glutamine, and penicillin-streptomycin as described, without exogenous growth factors (15, 41). After dissection, tibias were incubated in medium overnight and then treated with dimethylsulfoxide (DMSO) (control) or the GSK-3 inhibitor SB415286 (SB86; 10 μM), PI3K inhibitor LY 294002 (10 μM LY), or a combination of the two inhibitors. Please note that SB86 inhibits both GSK-3 proteins (42). Media and inhibitor were changed every 48 h. Length of tibias was measured before start of treatment and at the end of 6 d. Each independent experiment consisted of five to six tibias per treatment; data represent averages from at least three independent experiments. These bones were then either stained with alcian blue/alizarin red or prepared for paraffin embedding, sectioned, and analyzed by immunohistochemistry (IHC).

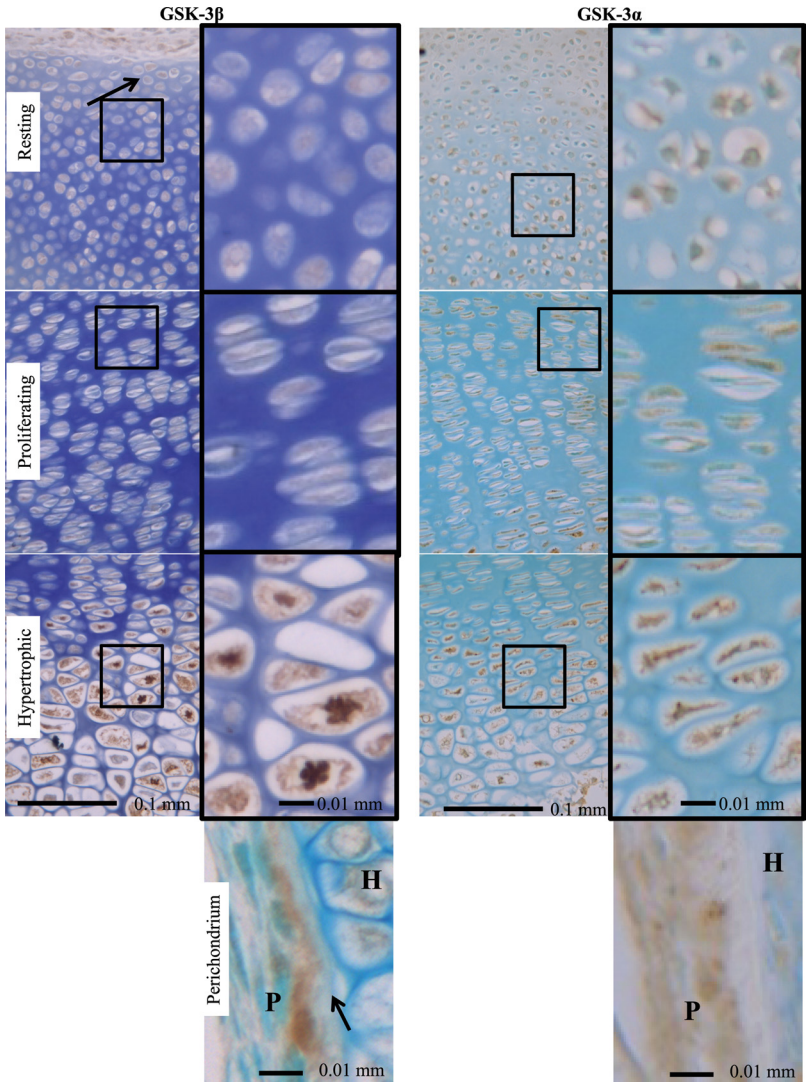

FIG. 1. Expression of GSK-3 proteins in the growth plate. Expression of GSK-3α and -β proteins in the growth plate of wild-type P0 tibia was analyzed by IHC. Although GSK-3β expression was detected in chondrocytes of the articular surface (*arrow*) and in particular in prehypertrophic and hypertrophic chondrocytes, GSK-3α is expressed in all zones of the growth plate. Both proteins are also expressed in the perichondrium (P), although GSK-3β was restricted to the cells closest to hypertrophic chondrocytes (H), and GSK-3α was again uniformly expressed throughout the cells of the perichondrium.

Tibia staining

Tibia staining was performed as described previously (39, 43). Organ culture tibias isolated from e15.5 embryos were dehydrated in 95% ethanol for 24 h, followed by acetone for 24 h. Tibias were stained with 0.015% alcian blue, 0.05% alizarin red, and 5% acetic acid in 70% ethanol. The stained tibias were stored in glycerol/ethanol (1:1). Images were taken with a Nikon SMZ1500 dissecting microscope with a Photometrics (Tucson, AZ) Coolsnap camera using ImageMaster version 5.0 software. At least three independent experiments containing four to six tibias per treatment (n) were used.

Histology and IHC

Organ culture and isolated P0 tibias were fixed in 4% paraformaldehyde overnight and decalcified with 0.1 M EDTA/PBS at room temperature before paraffin embedding and sectioning at the Robarts Research Institute Molecular Pathology Core Facility (London, Ontario, Canada). Five-micrometer sections were dewaxed in xylene followed by a graded series of ethanol washes (100% twice, 95% once, and 70% once). Sections were stained with either hematoxylin and eosin or safranin O/fast green (39, 40, 43, 44). For IHC, sections were incubated in 3% H_2O_2 for 15 min at room temperature, followed by antigen retrieval by incubation in either 10 mM sodium citrate at 95 C for 30 min or 0.1% Triton X-100 for 10 min, followed by blocking with 5% goat serum in PBS. Sections were incubated with primary antibody overnight at 4 C and washed (four times in PBS), and secondary antibody was applied according to manufacturers' recommendations. For detection, diaminobenzidine substrate was used and counterstained with methyl blue. All images were taken with a Leica DME microscope with a Qimaging MircroPublisher 5.0 RTV camera using QCapture Pro version 5.1 software (Surrey, British Columbia, Canada).

Quantification of IHC was conducted depending on the target protein. For quantification of proliferating cell nuclear antigen (PCNA) IHC, the positively stained (brown) and total nuclei were counted to determine the fraction of PCNA-positive cells in the growth plate. The quantification of p57 protein in the growth plate was conducted by measuring the length (proximal to distal) of the prehypertrophic zone where darkly stained (brown) nuclei are present.

Western blot analyses

Fresh calvaria, cartilage from the epiphyseal ends of long bones (humerus, femur, and tibia), and organs were dissected from P0 mice in cold Puck's solution A (39, 45). Samples were flash frozen in RIPA buffer and stored at −20 C overnight and then homogenized, sonicated, and centrifuged. Total protein content was determined, and 25–35 μg total protein (depending on protein yield) was loaded per lane in precast NuPAGE Novex Midi Tris-acetate gels and separated using the XCell Surelock Mini-cell (Invitrogen) system. Gels were blotted using XCell II Blot Module (Invitrogen) as per the manufacturer's instructions. Blots were blocked in 5% BSA Tris-buffered saline/Tween 20 solution for 1 h and then probed with primary antibody overnight at 4 C. After washing (Tris-buffered saline/Tween 20), membranes were incubated with appropriate secondary antibody (hrp conjugated) for 1 h at room temperature, and the resultant signal was detected using the ECL detection system (Amersham, Piscataway, NJ). Representative blots from at least three independent pairs of littermates are shown. Quantitative densitometry analysis was conducted using a ChemiImager 5500 system (Alpha Innotech, Miami, FL), subtracting background and normalizing to β-actin loading control signal. Densitometry results were converted relative to control, allowing comparison between blots and compared statistically by *t* test analysis.

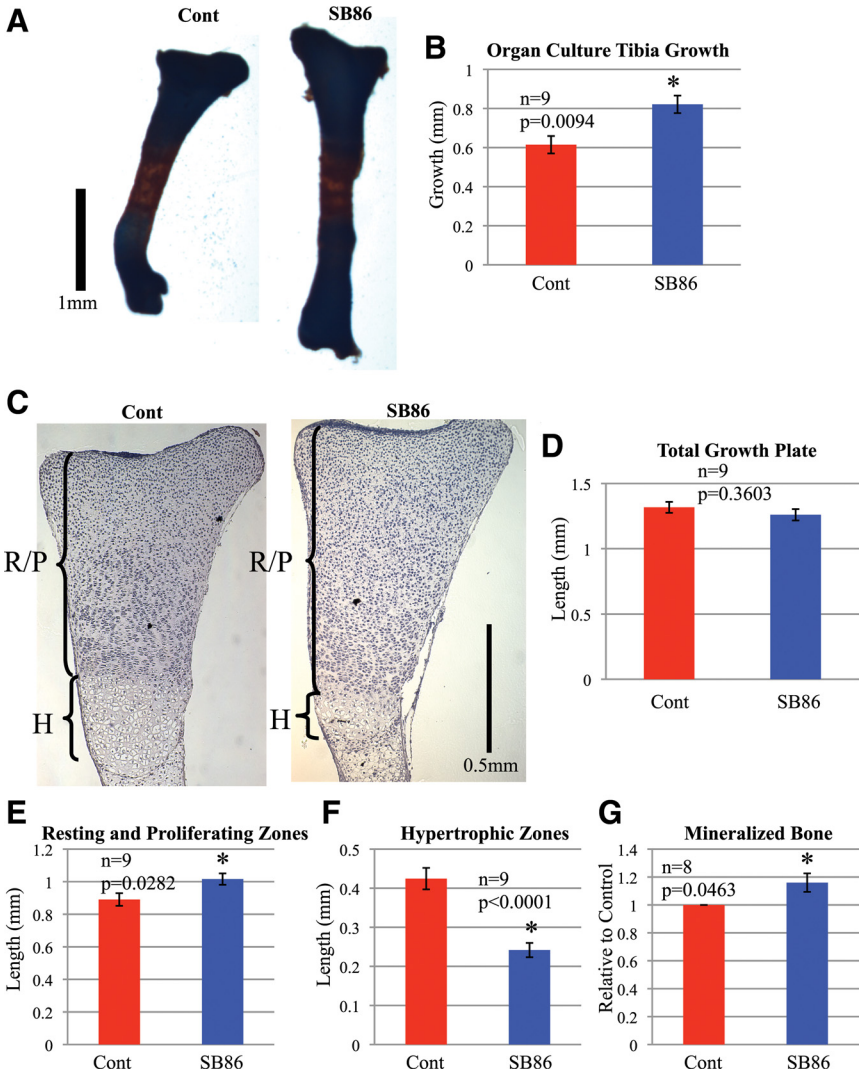

FIG. 2. Inhibition of GSK-3 increases bone growth and changes growth plate organization. The e15.5 mouse tibias were cultured in organ culture for 6 d with a pharmacological inhibitor of GSK-3 (10 μM SB86) or DMSO [control (Cont)]. A, Representative picture of tibias stained with alizarin red (bone) and alcian blue (cartilage); B, quantification of growth (millimeters) of the tibia during the 6 d of treatment; C, Hematoxylin and eosin-stained paraffin sections of organ culture tibias comparing morphology and growth plate zones, *e.g.* resting and proliferating zones (R/P) and hypertrophic zone (H); D, length (millimeters) of the proximal tibia growth plate in control and SB86-treated tibias; E, resting and proliferating zone length (millimeters) in control and GSK-3-inhibited organ culture; F, hypertrophic zone length (millimeters) in control and GSK-3-inhibited organ culture; G, quantification of the proportion of mineralized bone relative to the whole tibia length in control and GSK-3-inhibited organ culture. All data were compared using *t* test analysis, and significant differences are denoted by asterisks (n > 8; *, $P < 0.05$).

Statistical analysis

All data were collected from at least three independent organ culture trial or pairs of littermates. Data are expressed as mean ± SE, and P values < 0.05 were considered significant. For general measurements and comparisons between two groups of data, statistical significance was determined by unpaired *t* test comparing control with treated (SB86) or control with cartilage-specific deletion of GSK-3β (KO) littermates using GraphPad Prism version 3.00 for Windows. Western blot densitometry data were normalized to controls and compared by *t* test. Comparison of multiple treatments was done using one-way ANOVA (normal distribution analyzed) and a Tukey posttest.

Results

GSK-3 expression in the growth plate

To determine the expression patterns of both GSK-3 proteins in the growth plate *in vivo*, we performed IHC on wild-type P0 mouse tibia. GSK-3β was expressed at low levels in chondrocytes at the articular surface and hardly detectable by IHC in resting and proliferating chondrocytes but strongly expressed in prehypertrophic and hypertrophic chondrocytes (Fig. 1). GSK-3β in the hypertrophic chondrocytes appears both cytoplasmic and nuclear with the highest concentration being nuclear. In contrast, GSK-3α showed strong expression throughout the growth plate and appeared to be more cytoplasmic than GSK-3β. Both proteins were also expressed in the perichondrium where GSK-3β appeared confined to the layer of cells closest to hypertrophic chondrocytes and GSK-3α was again more uniformly expressed through the cells of the perichondrium.

GSK-3 inhibition increases bone growth and affects growth plate morphology

To determine the role that GSK-3 plays in endochondral bone growth, e15.5 tibia organ cultures were cultured for 6 d with the pharmacological GSK-3 inhibitor SB86 (10 μM) that inhibits both GSK-3 proteins (42). Longitudinal growth over this time was compared with controls treated with DMSO. Tibia treated with SB86 grew 31% more than controls over the 6 d of organ culture (Fig. 2, A and B). A similar trend, although not statistically significant with a completed n of 3, was also observed using another GSK-3 inhibitor, 10 μM SB216763 (Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). SB86 was used exclusively for the remainder of the experiments. Interestingly, this increase in bone growth did not affect the overall length of the growth plate of the treated tibia (Fig. 2, C and D). However, inhibition of GSK-3 did affect the organization and relative lengths of the zones within the growth plate (Fig. 2, E and F). The division between the

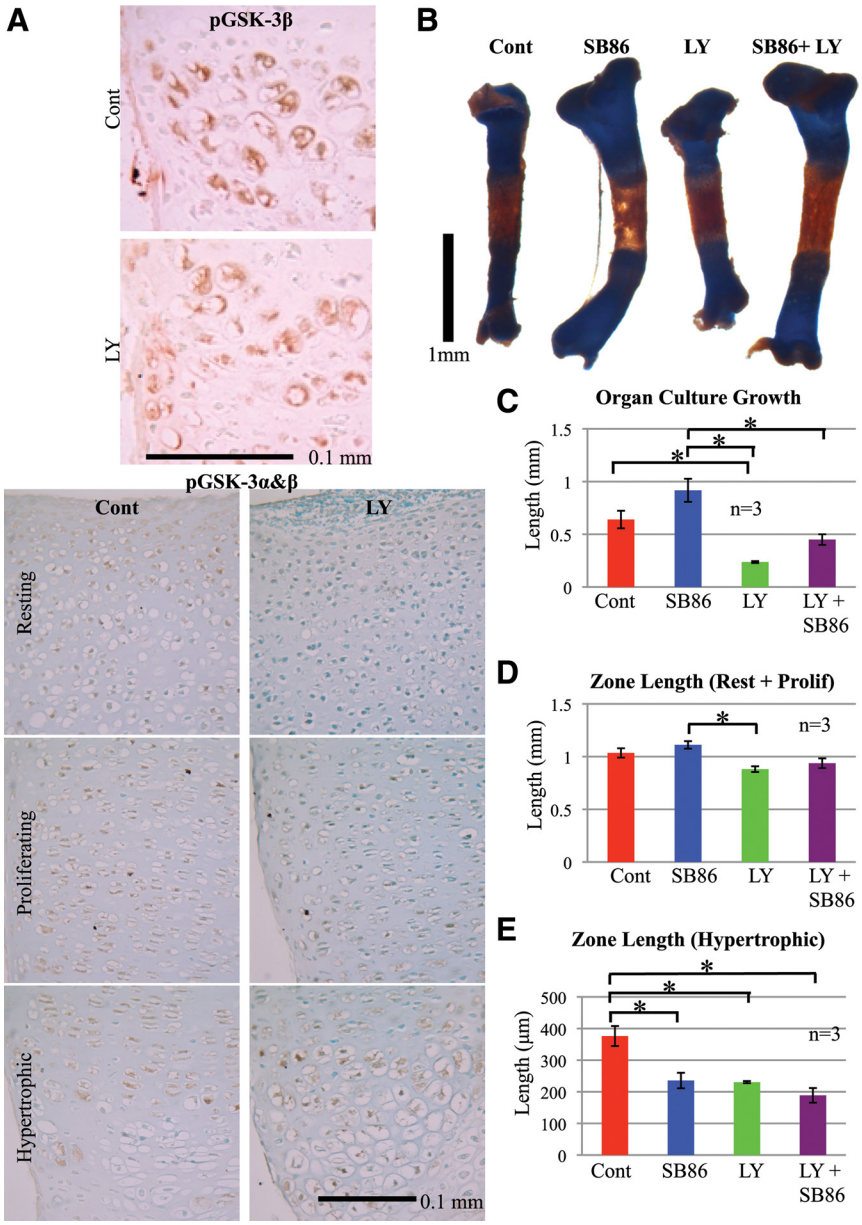

FIG. 3. Interaction of PI3K and GSK-3 signaling in organ culture. Tibia organ cultures (e15.5) were treated with GSK-3 inhibitor (10 μM SB86), PI3K inhibitor (10 μM LY), a combination of both inhibitors (10 μM SB6 plus 10 μM LY), or DMSO [control (Cont)] for 6 d. A, IHC analyses of GSK-3 phosphorylation using phosphospecific antibodies specific for GSK-3β (*top*) or both GSK-3α and β (*bottom*); B, representative picture of tibias after 6 d of culture stained with alizarin red (bone) and alcian blue (cartilage); C, quantification of longitudinal growth of tibias (millimeters) over 6 d of culture; D, resting and proliferating zone length (micrometers) of bones treated with the various inhibitors; E, hypertrophic zone length (micrometers) of bones treated with the various inhibitors. All data were compared using one-way ANOVA with a Tukey posttest analysis; significant differences are denoted by *asterisks* (n = 3; *, $P < 0.05$).

resting and proliferating zones was much less distinct in the SB86-treated bones, and therefore the zone measurements combined both resting and proliferating zones for accuracy of measurements. The resting and proliferating zones of the treated tibia were 13% longer than the controls (Fig. 2E), whereas the length of hypertrophic zone was significantly decreased by 57% (Fig. 2F). Because the inhibition of GSK-3 caused increased tibia growth but the total growth plate length was not increased, we examined the mineralized portion of the tibia (Fig. 2G). The mineralized portion of the tibia was found to be significantly longer as a ratio of total tibia length. These results together suggest that inhibition of GSK-3 promotes long bone longitudinal growth by increasing bone formation.

GSK-3 inhibition rescues most effects of PI3K inhibition in organ culture

GSK-3 activity is regulated through N-terminal phosphorylation by the insulin/PI3K/Akt pathway (12, 46) as well the IGF/PI3K/Akt pathway that is an important regulator of chondrocyte physiology (15, 47). We previously showed that PI3K inhibition decreased bone growth and the length of the proliferating and hypertrophic zones (15). Given that PI3K activates protein kinase B/Akt that in turn phosphorylates and inhibits GSK-3, inhibition of PI3K should activate GSK-3 and therefore have opposite effects as inhibiting GSK-3. Furthermore, if inactivation of GSK-3 is one of the main effectors of PI3K signaling in endochondral bone growth, then GSK-3 inhibition should rescue the effects of PI3K inhibition, at least in part. To explore interactions between PI3K/Akt and GSK-3, the effects of treatments with a PI3K inhibitor (10 μM LY) in combination with the GSK-3 inhibitor (10 μM SB86) on tibia organ cultures were examined (Fig. 3).

To confirm that the inhibition of PI3K would indeed affect the phosphorylation of GSK-3 in our tibia organ culture model, IHC analyses were conducted using antibodies specific to phosphorylated GSK-3β (Fig. 3A, *top*) or to phosphorylated forms of both GSK-3 proteins (Fig. 3A, *bottom*). These data show that phosphorylation for GSK-3β was consistently decreased with LY treatment through the prehypertrophic/hypertrophic zones where GSK-3β is primarily expressed in the growth plate (Fig. 3A, *top*). The IHC detecting both phospho-GSK-3α and -β demonstrated a decrease in the GSK-3 phosphorylation

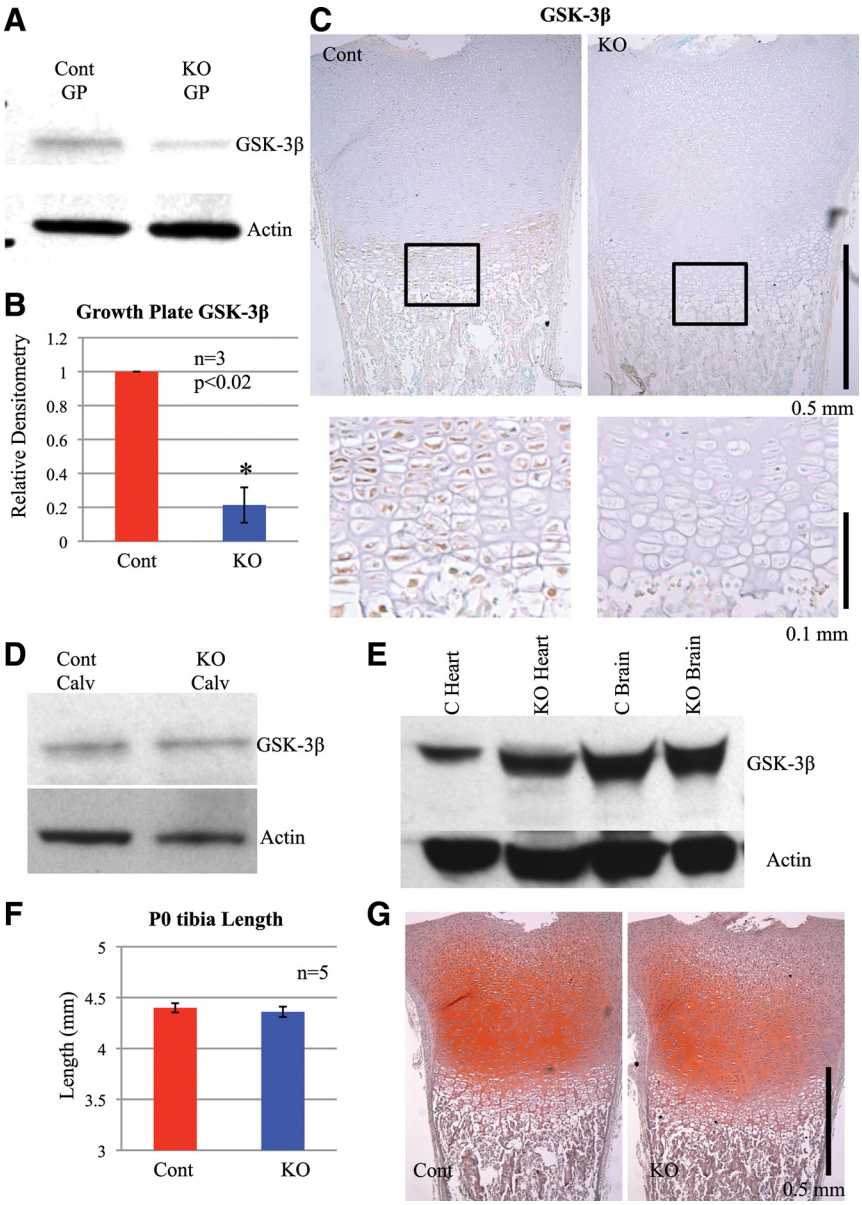

FIG. 4. Cartilage-specific deletion of GSK-3β. Cartilage-specific GSK-3β KO mice carrying two alleles of the floxed *Gsk3b* gene and expressing cre recombinase under the control of the collagen II (*Col2a1*) promoter (KO) were created and compared with the control littermates (Cont). Panel A, Western blot analyses using GSK-3β antibodies conducted on growth plate (GP) protein extracts from newborn littermate mice of $Gsk3b^{fl/fl}$ cre^{+} (KO) and control ($Gsk3b^{fl/fl}$ cre^{-}, or $Gsk3b^{fl/wt}$ cre^{-}) (Cont) genotype; panel B, densitometric quantification of GSK-3β protein in P0 growth plate extracts; panel C, IHC using GSK-3β antibodies on paraffin sections of P0 tibia, with *black box in top image* indicating the location of the lower higher-magnification images; panel D, Western blot analyses of GSK-3β protein in calvarial tissue (Calv); panel E, Western blot analyses of GSK-3β protein in heart, brain, and kidney protein samples of control (C) and KO mice; panel F, tibia length (millimeters) measurements from P0 control and KO mice; panel G, paraffin sections of P0 tibias from control and KO mice stained with safranin O. All Western blot analyses used β-actin as a loading control. Quantification used *t* test analyses where significance was denoted with an *asterisk* ($n > 3$; *, $P < 0.05$).

through the resting and proliferating zones but a less significant change in the prehypertrophic zone (Fig. 3A, *bottom*). This suggests that GSK-3α, which is more widely expressed in the growth plate, is the predominate GSK-3 form targeted by inhibition of the PI3K pathway through the resting and proliferating zones, whereas GSK-3β is the main target in the prehypertrophic zone.

Consistent with our earlier studies, SB86 (Fig. 2) increased and LY (15) decreased tibia growth in culture (Fig. 3, B and C). The effect of the PI3K inhibitor was partially recovered by the combination with the GSK-3 inhibitor (LY plus SB86) (Fig. 3, B and C). None of the treatments had significant effects on the total length of the growth plates (Supplemental Fig. 1B). Similar to the bone growth, the combination of the inhibitors was able to partially recover the effect of PI3K inhibition on the length of the resting/proliferative zone (Fig. 3D), whereas both inhibitors individually and in combination reduced the length of the hypertrophic zone (Fig. 3E).

Chondrocyte-specific ablation of GSK-3β *in vivo*

To determine whether the *ex vivo* tibia organ cultures results would translate into an *in vivo* model and to address the roles of individual GSK-3 proteins, we generated mice with cartilage-specific loss of GSK-3β. Mice homozygote for the floxed alleles of GSK-3β ($Gsk3b^{fl/fl}$) (16, 38) were crossed with mice heterozygote for the floxed allele and expressing cre recombinase gene under control of the mouse collagen II (*Col2a1*) promoter ($Gsk3b^{fl/wt}$ cre^{+}) (39, 48). This breeding scheme produced cartilage-specific GSK-3β deletion in ($Gsk3b^{fl/fl}$ cre^{+}) referred to as knockout mice (KO), heterozygous mice ($Gsk3b^{fl/wt}$ cre^{+}), as well as control (Cont) littermates ($Gsk3b^{fl/fl}$ cre^{-} or $Gsk3b^{fl/wt}$ cre^{-}). Efficiency of GSK-3β deletion was determined through Western blot analysis and immunohistochemistry (Fig. 4). Long bone growth plate extracts from P0 mice demonstrated an 80% reduction in GSK-3β protein in KO cartilage (Fig. 4 A, B). Immunohistochemistry (IHC) of P0 tibia paraffin section demonstrated strong expression of GSK-3β in the prehypertrophic and hypertrophic zones of control mice with virtually a complete loss of signal in KO littermates (Fig. 4 C). GSK-3β

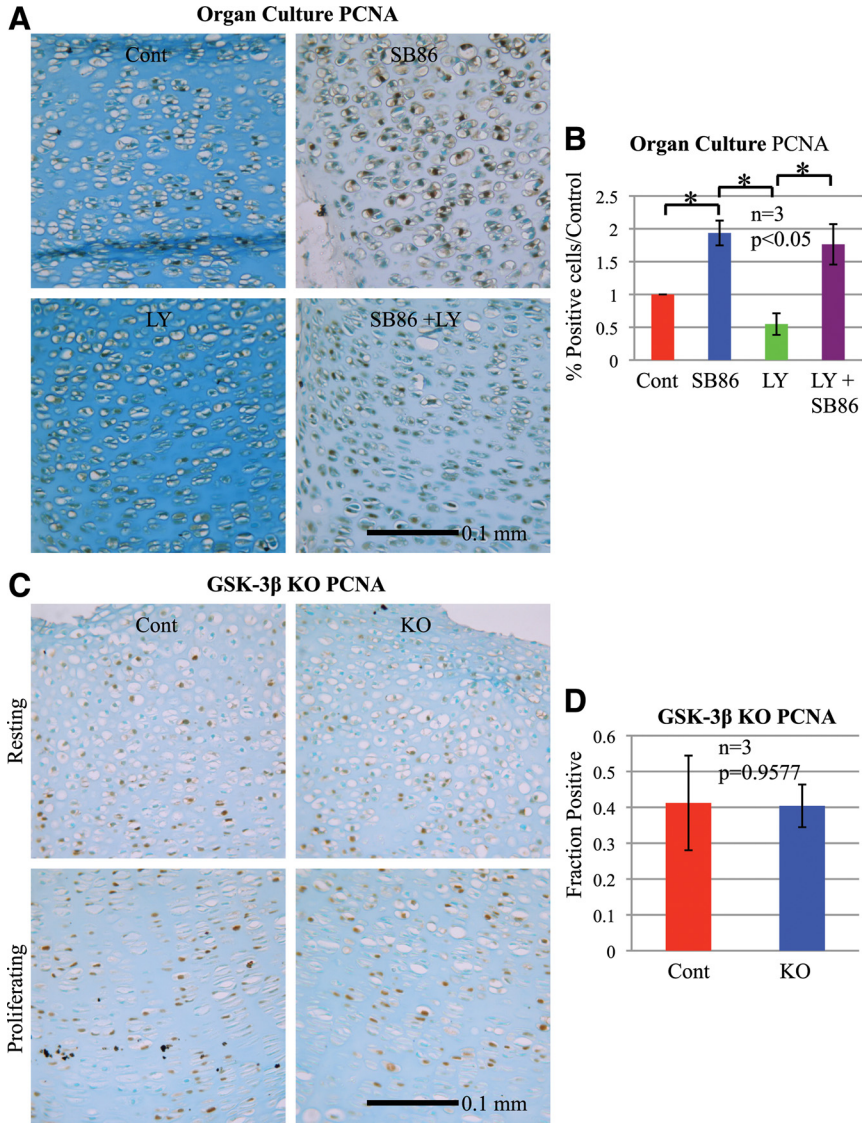

FIG. 5. GSK-3 inhibition increases proliferation in organ culture, whereas GSK-3β deletion in cartilage has no effect on proliferation *in vivo*. PCNA was used to stain proliferating cells in both tibia organ culture experiments and cartilage-specific GSK-3β KO mice. A, IHC analyses of PCNA on paraffin section of tibia organ cultures treated with GSK-3 inhibitor (10 μM SB86), PI3K inhibitor (10 μM LY), a combination of both inhibitors (10 μM SB86 plus 10 μM LY), or DMSO [control (Cont)]. B, Quantification of the PCNA staining from A as percentage of stained cells to total cells. Results were statistically compared using a one-way ANOVA with Tukey posttest analyses, and significance is denoted by *asterisks* (n = 3; *, $P < 0.05$). C, PCNA IHC analyses of P0 tibia from cartilage-specific GSK-3β KO mice and control littermates (Cont). D, PCNA staining quantified as fraction of positive stained cells. Results were compared by *t* test (n = 3; *, $P > 0.05$).

protein levels were explored in many tissues to evaluate the specificity of the deletion (Fig. 4, D and E). Calvaria tissue samples showed that GSK-3β expression was not affected in bone tissue (Fig. 4D). No loss of GSK-3β protein was observed in any other tissues of KO mice (Fig. 4E).

The cartilage-specific GSK-3β KO mice did not display an observable skeletal phenotype (Supplemental Fig. 2, A and B). The size and weight of KO mice were similar to those of control sex-matched littermates throughout their life, from birth to 1 yr (data not shown). The length of the tibias at birth P0 (Fig. 4F) and at P21 (Supplemental Fig. 2C) were not significantly different from control mice. The growth plate zones were also not visually affected upon cartilage-specific GSK-3β deletion (Fig. 4G, Supplemental Fig. 2, D–F).

GSK-3 inhibition increases chondrocyte proliferation

To determine why *ex vivo* inhibition of GSK-3 affects bone growth whereas *in vivo* GSK-3β deletion had no observable effect, the cellular mechanisms controlling growth plate dynamics were explored. Because the rate at which chondrocytes cycle through the growth plate stages determines both zone morphology and bone growth, chondrocyte proliferation was assessed through IHC analyses using PCNA antibodies (Fig. 5). The PCNA IHC was quantified as the fraction of positively stained cells per total number of cells. GSK-3 inhibition (SB86) did increase the amount of actively proliferating cells in the tibia organ culture almost 2-fold (Fig. 5, A and B). Proliferation was not significantly decreased below the control levels upon treatment with the PI3K inhibitor (LY), whereas treatment with both inhibitors increased proliferation significantly over the LY treatment, similar to the effect of GSK-3 inhibition only (Fig. 5B). Similar data were obtained using bromodeoxyuridine labeling and detection (Supplemental Fig. 1G). In contrast, no difference in the fraction of PCNA-positive chondrocytes was observed upon cartilage-specific GSK-3β deletion (Fig. 5, C and D).

Prehypertrophic cell cycle exit increased upon GSK-3 inhibition

Hypertrophic chondrocyte differentiation is initiated by cell cycle exit, giving rise to prehypertrophic chondrocytes (49, 50). The cyclin-dependent kinase inhibitor p57 is both a marker of prehypertrophic chondrocytes and promotes cell cycle exit in the growth plate (49–54). In tibia organ culture, inhibition of GSK-3 or dual treatments of GSK-3 and PI3K inhibitors greatly increased the zone of p57 protein expression in the prehypertrophic zone (Fig. 6A, *black arrows in insets*), whereas the addition of the PI3K inhibitor did reduce this

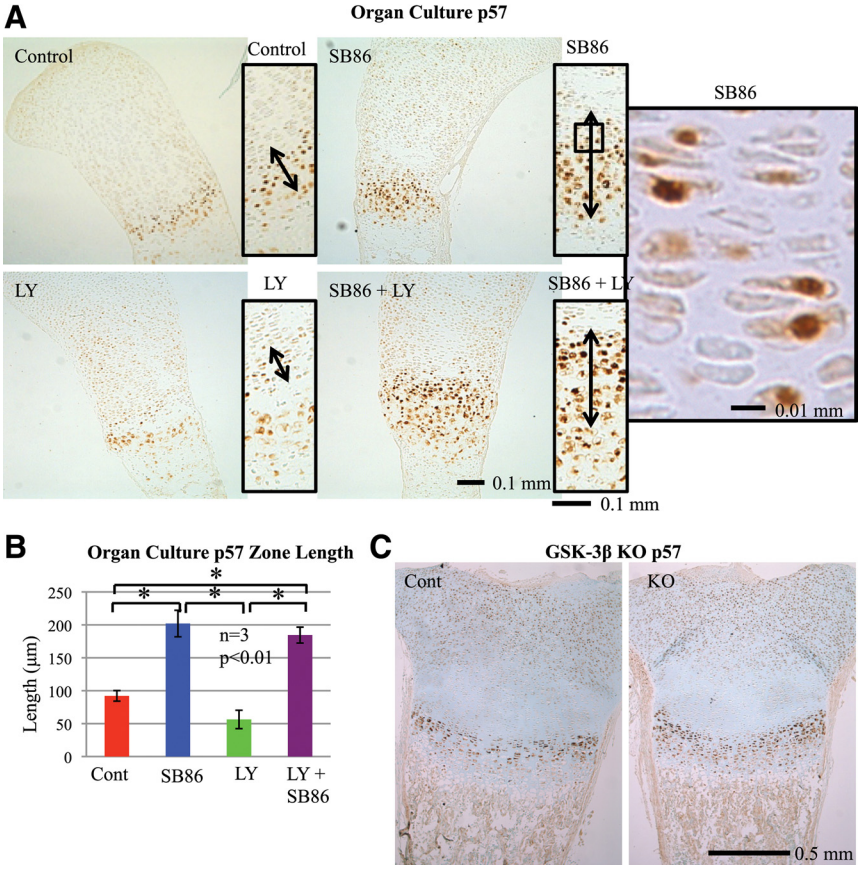

FIG. 6. GSK-3 regulates p57 expression in the prehypertrophic zone *in vitro*, whereas cartilage-specific GSK-3β deletion does not affect p57 expression *in vivo*. A, IHC analyses of p57 expression in paraffin sections of tibia organ culture treated with GSK-3 inhibitor (10 μM SB86), PI3K inhibitor (10 μM LY), a combination of both inhibitors (10 μM SB86 plus 10 μM LY), or DMSO [control (Cont)]. *Black boxed insets* are higher-magnification images depicting the zones of p57 staining (*black arrows*). *Far right image* is a higher-magnification (*boxed area*) of staining to determine cellular localization. B, Quantification of the length (micrometers) of the zone of p57 staining observed in A. Data were analyzed by one-way ANOVA with a Tukey posttest with significance denoted by *asterisks* ($n > 3$; *, $P < 0.01$). C, IHC analysis of p57 expression in paraffin sections of P0 tibias of cartilage-specific GSK-3β KO mice compared with littermate controls (Cont).

zone, although not statistically significantly (Fig. 6, A and B). This p57-positive staining was localized to the cell nucleus in the prehypertrophic zone (Fig. 6A, *far right inset*). In cartilage-specific GSK-3β KO mice, p57 protein expression was similar to that of control littermates (Fig. 6C), and quantification did not show any significant change in the length of the p57-positive zone (data not shown).

β-Catenin expression in prehypertrophic chondrocytes is up-regulated upon inhibition of GSK-3

Arguably the most studied downstream target of GSK-3 is the transcription factor β-catenin, a target of the canonical Wnt pathway that is a central regulator of skeletal development (7, 55–59). An increase in the amount of β-catenin protein was observed specifically throughout the prehypertrophic/hypertrophic zone of tibias in organ culture treated with the GSK-3 inhibitor (Fig. 7A). IHC for β-catenin was also performed on the other two treatments discussed above, LY and SB86 plus LY; however, PI3K inhibition yielded no change, and dual inhibition results were inconclusive (data not shown). Western blot showed no significant change in β-catenin protein levels in the growth plate of cartilage-specific GSK-3β KO mice, although levels consistently appeared increased in mutant cartilage (Fig. 7B). However, IHC suggested that β-catenin levels are increased in specific subsets of chondrocytes in cartilage-specific GSK-3β KO mice, in particular prehypertrophic chondrocytes and the articular surface (Fig. 7C).

Up-regulation of GSK-3α protein in response to GSK-3β deletion *in vivo*

The two forms of GSK-3, α and β, play both overlapping and distinct roles (16, 17, 28, 29, 31). GSK-3α levels and overall phosphorylation of the GSK-3 proteins were examined to understand the apparent lack of phenotype in the cartilage-specific GSK-3β KO mice (Fig. 8). Surprisingly, Western blotting demonstrated an approximately 2-fold increase in GSK-3α protein in KO cartilage (Fig. 8, A and B). IHC further supported these data by showing an increase in GSK-3α protein throughout the prehypertrophic and hypertrophic area as well as the resting zone and articular surface (Fig. 8C). To a lesser extent, an increase in GSK-3α protein was observed in the proliferating zone (Fig. 8C). There was no obvious change in the total phosphorylation of the GSK-3 proteins in all zones (Fig. 8D), providing further evidence that up-regulation of GSK-3α compensates for the loss of GSK-3β in cartilage.

Discussion

This study contributes important and novel data to our understanding of both skeletal development and GSK-3 signaling. Our results demonstrate profound effects of GSK-3 inhibition on bone growth in a tibia organ culture system, which is contrasted by minimal phenotypes observed upon cartilage-specific deletion of GSK-3β. This

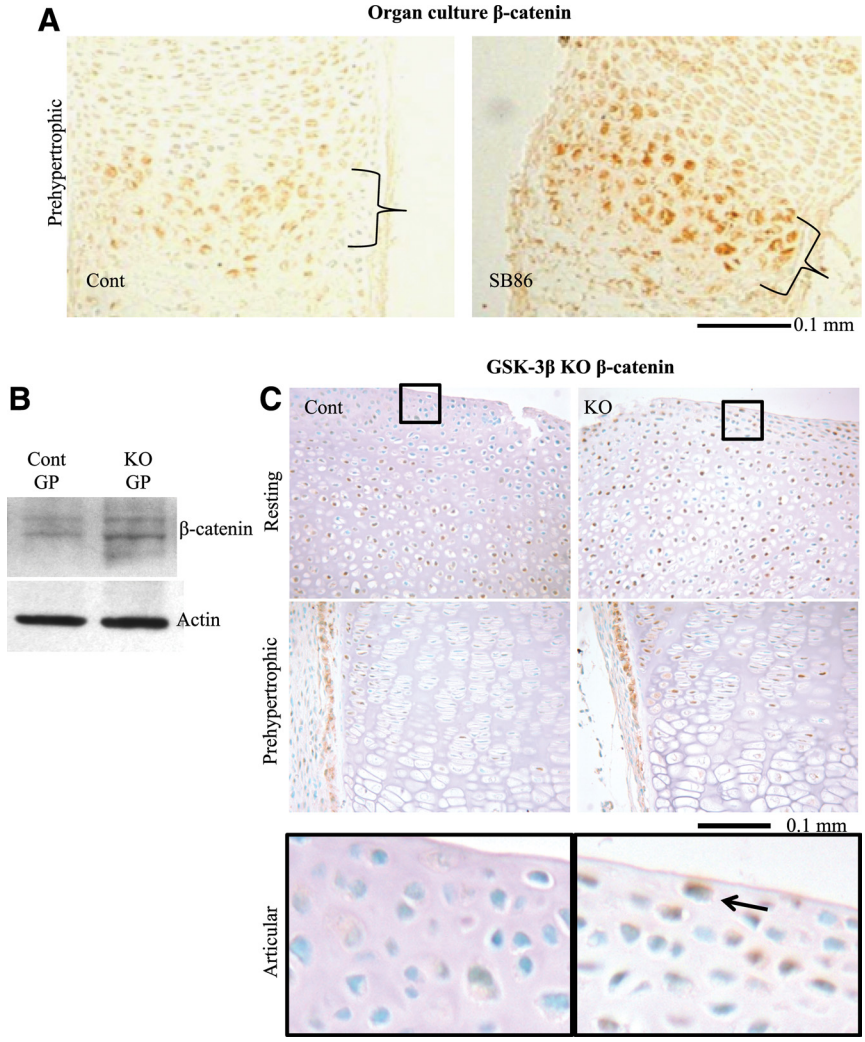

FIG. 7. GSK-3β regulates β-catenin expression in the prehypertrophic zone. A, Sections of tibia organ cultures treated with a GSK-3 inhibitor (10 μM SB86) or DMSO [control (Cont)] were analyzed by IHC using β-catenin antibodies. *Black brackets* indicate the zone of β-catenin-stained cells in the prehypertrophic zone. B, Western blot analyses of P0 growth plate (GP) protein extracts from cartilage-specific GSK-3β KO mice and control (Cont) littermates probed with β-catenin antibodies, with β-actin as loading control. C, Localization of β-catenin protein was conducted by IHC using β-catenin antibodies on paraffin tibia sections from mice described in B.

absence of phenotype is likely due to compensatory up-regulation of GSK-3α expression, a mechanism that has, to our knowledge, not been reported for any other tissue or context.

Our tibia organ culture experiments show that inhibition of GSK-3 results in increased longitudinal growth of endochondral bones. This was likely caused, at least in part, by increased proliferation of chondrocytes in the resting and proliferating zones. The shortened hypertrophic zone resulting from GSK-3 inhibition appears to be counterintuitive to the increase in growth observed by us. However, the larger proportion of PCNA-labeled chondrocytes, the increase in p57 staining, and the relative increase in the length of the mineralized zone all suggest that all processes in the growth plate (proliferation, differentiation, and replacement of cartilage by bone) occur at a faster rate upon GSK-3 inhibition, thus resulting in increased bone growth. The reduced length of the hypertrophic zone would therefore not be due to delayed differentiation but rather to faster turnover of hypertrophic cartilage to bone.

Our observations of increased chondrocyte proliferation and accelerated bone maturation in response to GSK-3 inhibition are opposite to findings from Naski and colleagues (60) who showed that a different pharmacological GSK-3 inhibitor reduced chondrocyte proliferation and differentiation in a metatarsal organ culture system. These differences might be due to the nature and/or concentration of the inhibitors, the duration of treatment, or the identity of the skeletal elements investigated, suggesting that the effects of GSK-3 in cartilage might be context dependent. However, our data showing that GSK-3 inhibition promotes endochondral bone growth, which is unlikely to occur when both proliferation and hypertrophy of chondrocytes are inhibited, are supported by a recent *in vivo* study where loss of one allele of the *Gsk3b* gene rescues the dwarfism of mice deficient for cGMP-dependent kinase II (cGKII), another upstream inhibitor of GSK-3 (24). cGKII is a key mediator of the anabolic effects of C-type natriuretic peptide on endochondral bone growth (61). These data by Kawasaki *et al.* (24) show that inhibition of GSK-3 activity is required for the anabolic effects of cGKII in bone, an effect we mimic by pharmacological inhibition of GSK-3.

Our data also provide insights into the relationship between PI3K and GSK-3 signaling in cartilage. PI3K inhibition resulted in decreased phosphorylation (indicating increased activity) of GSK-3. In agreement with these data, pharmacological inhibition of GSK-3 rescued many of the effects of the PI3K inhibitor, in particular on bone growth. However, this was not true for the length of the hypertrophic zone, which was reduced by either inhibitors individually or in combination. However, these effects on the hypertrophic zone could be due to the different mechanisms, because PI3K inhibition appears to delay chon-

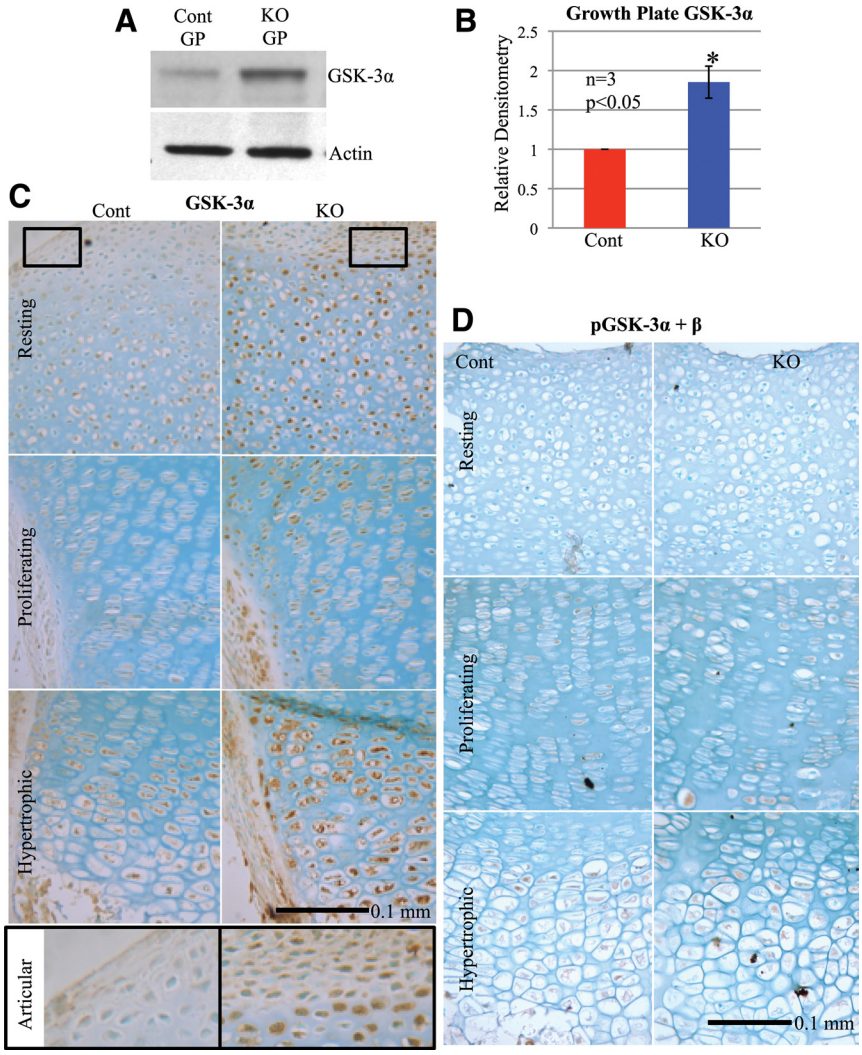

FIG. 8. *In vivo* deletion of GSK-3β causes up-regulation of GSK-3α protein in chondrocytes. A, Western blot analyses of GSK-3α protein in growth plate (GP) protein extracts of cartilage-specific GSK-3β KO and control (Cont) mice, with β-actin as loading control; B, densitometric quantification of GSK-3α Western blot analyses (A); statistical analysis was conducted using *t* test, and significance is indicated by asterisk (*) (n = 3, *: $P < 0.05$); C, IHC analyses using GSK-3α antibodies on paraffin sections of P0 KO and control tibias; D, IHC analysis using antibodies detecting the phosphorylated state of both GSK-3 forms, α and β.

drocyte hypertrophy and the associated bone growth (15) (reviewed in Ref. 62), whereas our data presented here suggest that GSK-3 inhibition accelerates the replacement of hypertrophic cartilage by mineralized tissue. Furthermore, the similar effects of both inhibitors on the hypertrophic zone reflect the fact that PI3K also acts through other downstream effectors in addition to GSK-3 (63), whereas GSK-3 is also involved in the response to signals not mediated by PI3K, such as Wnts.

Given the significant impact of GSK-3 inhibition in organ culture, the absence of a clear phenotype in cartilage-specific *Gsk3b* KO mice was surprising. However, our subsequent molecular analyses revealed the putative explanation. First, the expression of GSK-3β in cartilage is quite restricted, at least in comparison with the more ubiquitously expressed GSK-3α. These different expression patterns were also shown at the mRNA level in our earlier microarray studies on microdissected mouse growth plates (64). More importantly, GSK-3α expression was up-regulated markedly in all zones of the growth plates of *Gsk3b*-mutant mice, even where very low levels of GSK-3β were seen in wild-type mice (such as in the proliferative zone). These data suggest that GSK-3α is able to compensate for most functions of GSK-3β in chondrocytes. However, our results cannot exclude alternative explanations for the lack of a phenotype in our KO mice; analyses of double-KO mice for both *Gsk3* genes will be required to firmly establish redundant functions of these two genes.

The molecular basis of the increase in GSK-3α levels in our mutants is currently unknown. Because GSK-3β regulates the stability of many other proteins (65), it is tempting to speculate that loss of GSK-3β leads to stabilization of GSK-3α protein. However, to our knowledge, no such cross talk between the two GSK-3 proteins has been described. Alternatively, it is plausible that increased GSK-3α expression in our KO mice is due to increased transcription, mRNA stability, and/or translation. Future studies will need to address the mechanisms involved.

The one exception to compensation appeared to be up-regulation of β-catenin protein levels in specific chondrocytes of cartilage-specific *Gsk3b* KO mice, suggesting that GSK-3α is not able to fully substitute for GSK-3β function in all chondrocytes. Because this effect was seen in only a few chondrocytes, it was not reflected in Western blot analyses of extracts from the entire cartilage, but it was very reproducible in immunohistochemical analyses of tissue sections. Notably, increased staining for β-catenin in mutant mice was seen in exactly those cells that express detectable levels of GSK-3β in wild-type mice (*e.g.* articular and pre-hypertrophic/hypertrophic chondrocytes), suggesting that GSK-3α cannot fully compensate for this aspect of GSK-3β function in these cells. The reason for this inability is unclear but might be related to the particular levels of expression of the two GSK-3 proteins in specific chondrocyte populations. Analyses of double mutants for both *Gsk3* genes will provide

further insight into this unexpected finding. Although chondrocyte function is very sensitive to both supra- and superphysiological levels of β-catenin (56, 57), the moderate change in expression observed here appears to be insufficient to induce changes in cartilage development and bone growth, at least over the time frame evaluated here. It will be interesting to examine in the future whether increased β-catenin or other aspects of cartilage-specific loss of GSK-3β alters the susceptibility to cartilage degeneration in osteoarthritis.

In closing, we believe that this study contributes important and novel information to our understanding of the complicated relationship between the two GSK-3 proteins as well as to our understanding of the signaling pathways controlling endochondral bone development. Additional studies, such as the simultaneous inactivation of both *Gsk3* genes in cartilage, will further elucidate the role of these key signaling molecules in the physiology and pathophysiology of cartilage.

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