The American Journal of Pathology, Vol. 175, No. 5, November 2009

DOI: 10.2353/ajpath.2009.081173

Growth Factors, Cytokines, Cell Cycle Molecules

Transient Activation of Wnt/β-Catenin Signaling Induces Abnormal Growth Plate Closure and Articular Cartilage Thickening in Postnatal Mice

Takahito Yuasa,* Naoki Kondo,* Rika Yasuhara,* Kengo Shimono,* Susan Mackem,† Maurizio Pacifici,* Masahiro Iwamoto,* and Motomi Enomoto-Iwamoto*

From the Department of Orthopaedic Surgery, Thomas Jefferson University, College of Medicine, Philadelphia, Pennsylvania; and the Laboratory of Pathology,† National Cancer Institute, Bethesda, Maryland*

Wnt/β-catenin signaling is required for skeletal development and organization and for function of the growth plate and articular cartilage. To further clarify these roles and their possible pathophysiological importance, we created a new transgenic mouse model in which Wnt/β-catenin signaling can be activated in cartilage for specific periods of time. These transgenic mice expressed a constitutive active form of β-catenin fused to a modified estrogen receptor ligand-binding domain under the control of cartilage-specific collagen 11α2 promoter/enhancer. Transient Wnt/β-catenin signaling activation in young adult mice by tamoxifen injections induced growth retardation and severe deformities in knee joints. Tibial and femoral growth plates displayed an excessive number of apoptotic cells and eventually underwent abnormal regression. Articular cartilage exhibited an initial acute loss of proteoglycan matrix that was followed by increases in thickness, cell density, and cell proliferation. In reciprocal studies, we found that conditional ablation of β-catenin in postnatal mice using a Col2-CreER strategy led to hypocellularity in articular cartilage, growth plate disorganization, and a severe reduction in bone volume. Together, these data provide evidence that Wnt/β-catenin signaling has important and distinct roles in growth plate and articular cartilage and that postnatal dysregulation of this signaling pathway causes diverse structural and functional changes in the two cartilaginous structures. *(Am J Pathol 2009, 175:1993–2003; DOI: 10.2353/ajpath.2009.081173)*

The Wnt/β-catenin signaling pathway plays essential roles in animal development, determination of cell lineages, and progression of cell differentiation.[1–3] Previous studies from this and other groups established that this pathway regulates skeletal development and growth and influences skeletal cell behavior.[4–9] Conditional ablation of β-catenin in mouse embryo cartilage was found to cause alterations in growth plate chondrocyte function in developing skeletal elements; the cells exhibited a delay in hypertrophy and consequently both endochondral bone formation and skeletal growth were significantly disturbed.[4–7] Recent studies showed that reduction of Wnt/β-catenin signaling is deleterious for not only growth plate but also articular cartilage[10,11] and that constitutive activation of Wnt/β-catenin signaling in cartilage leads to skeletal deformities and disruption of joint structures.[4,8,12] These and other studies have provided strong support for the conclusion that Wnt/β-catenin signaling must be kept under strict control during skeletal development and growth and that inhibition or hyperactivity of this pathway is incompatible with normal skeletal function and homeostasis and could lead to pathological conditions.

In vivo and *in vitro* studies have indicated that Wnt/β-catenin signaling affects multiple pathways and mechanisms in the chondrocytes. We found that acute experimental activation of Wnt/β-catenin signaling strongly stimulates matrix catabolism and protease activity[8,13]

Supported by the National Institute of Arthritis & Musculoskeletal & Skin Diseases (grants AR050507 and AR046000) and the National Institute on Aging (grant AG025868).

Accepted for publication July 23, 2009.

A guest editor acted as editor-in-chief for this manuscript. No person at Thomas Jefferson University was involved in the peer review process or final disposition for this article.

Current address of T.Y.: Department of Orthopaedic Surgery, Juntendo University, Tokyo, Japan; current address of N.K.: Department of Orthopaedic Surgery, Niigata University, Niigata, Japan.

Address reprint requests to Motomi Enomoto-Iwamoto, D.D.S., Ph.D., Department of Orthopedic Surgery, Thomas Jefferson University, 1015 Walnut St., 501 Curtis Building, Philadelphia, PA 19107. E-mail: motomi.iwamoto@jefferson.edu.

and promotes expression of phenotypic traits associated with chondrocyte hypertrophy and replacement with endochondral bone.[9] Others showed that Wnt/β-catenin signaling affects chondrocyte survival[10,11] and stimulates proliferation.[14] It has remained unclear, however, whether and how these and other cellular changes caused by overactivation or inhibition of Wnt/β-catenin signaling relate to and bring about malfunction and deformities in growth plate and articular cartilage, particularly those associated with aging or skeletal pathological changes. To approach these important issues and questions, we created a new transgenic mouse model in which Wnt/β-catenin signaling can be activated in cartilage at specific time points and specific lengths of time prenatally or postnatally. We used a construct encoding a constitutive-active form of β-catenin fused to a modified estrogen receptor ligand-binding domain (CA-β-catER) that was previously used to create transgenic mice in which Wnt/β-catenin signaling was activated in keratinocytes after tamoxifen injection.[15] Our data indicate that transient activation of Wnt/β-catenin signaling in cartilage in postnatal mice induces rapid and excessive apoptosis in growth plate chondrocytes and irreversible regression of growth plates. The mice also displayed defects in articular cartilage that included rapid proteoglycan loss followed by increases in tissue thickness and cell density. These data suggest that both growth plate and articular cartilage are affected by acute overactivation of Wnt/β-catenin signaling, but their responses are distinct and may have distinct pathological implications.

Materials and Methods

Generation of Col11-CA-β-catER Transgenic Mice

A DNA construct encoding N-terminally truncated β-catenin linked to a modified estrogen receptor ligand binding domain (ER) (ΔNβ-catenin-ERTM, provided by Dr. F. Watt, UK London Research Institution)[15] was cloned into the NotI site of a collagen 11α2 (*Col11a2*) promoter expression vector (provided by Dr. N. Tsumaki, Osaka University).[16] The resulting construct (*Col11-CA-β-catER*) was injected into the pronucleus of fertilized eggs from F1 hybrid mice (C57BL/6) by the Transgenic Core Facility at our institution. Genotyping of transgenic mice was performed by PCR: the primer sequences were 5′-TGCTCCTCAGTGGAT GTTGCCTTTAC-3′ and 5′-AGGTTGTGAAGTGTTCCCGCAGTG-3′. Expression of the transgene was determined by RT-PCR, *in situ* hybridization, and immunohistochemistry. Three positive lines were obtained, and two stable lines are now maintained.

Tamoxifen Treatment

Tamoxifen injection was performed in 2-week-old transgenic mice for 7 to 10 days at doses ranging from 200 to 300 μg/20 to 30 μl volume/mouse. Tamoxifen stock solution was prepared in 99% ethanol at a concentration of 100 mg/ml at 55°C that was diluted to 10 mg/ml with corn oil before use. Control mice received the same amount of cocktail of ethanol (10%) and corn oil (90%). Three wild-type and three transgenic mice were sacrificed 2 weeks after the last tamoxifen administration. Eight wild-type and 11 transgenic mice were sacrificed 4 weeks after the last tamoxifen administration. Corresponding transgenic control mice were sacrificed 2 and 4 weeks after administration of corn oil. Hind limbs were dissected and subjected to anatomical analyses and soft X-ray analysis, and knee and hip joints were subjected to histopathological analyses.

Conditional Ablation of β-Catenin

Mice conditionally deficient in *β-catenin* were created by mating *β-catenin* floxed mice (*β-catenin*$^{fl/fl}$) possessing loxP sites in introns 1 and 6 in the β-catenin gene (6.129-Ctnnb1tmKem/KnwJ line purchased from The Jackson Laboratory, Bar Harbor, ME) with *Col2a1-CreER* mice.[17] Genotyping of the *β-catenin* allele was performed according to protocols from The Jackson Laboratory. To ablate the β-catenin gene, we injected tamoxifen at a dose of 200 μg/20 μl volume/mouse/day on postnatal day (P) 5, 6 and 7, and mice were then sacrificed at 5 weeks of age. Efficiency of Cre recombinase activity was confirmed by analysis of compound *Col2a1-CreER*/*Rosa R26R* transgenic-reporter mice following a similar regimen of tamoxifen injections.

Anatomical Analyses, Soft X-Ray, and Micro-Computed Tomography

Body length was determined by measuring the distance from nose tip to start point of tail. Limb skeletal elements were stained with Alcian blue and alizarin red. Specimens were photographed with a camera-equipped stereomicroscope, and tibia length was measured with computational tools. Fore and hind limbs were dissected and subjected to soft X-ray analysis under automatic exposure conditions using a piXarray 100 Digital Specimen Radiography System (BIOPTICS, Inc., Tucson, AZ). Trabecular bone volume, connectivity, trabecular thickness, and mineral density were measured with a 40 μCT system (Scanco USA, Inc., Southeastern, PA). Samples were scanned at 45 kV and 177 μA, 12-μm scanning thickness, and medium resolution by using a 20.5-mm holder. Two-dimensional slice images were selected and used to generate three-dimensional reconstructions with filter width sigma of 0.8, support level of 1.0, and threshold of 244. The same values were used to analyze wild-type and *Col11-CA-β-catER* transgenic mouse samples.

Histological, Immunohistochemical, and in Situ Hybridization Analyses

Knee and hip joints were dissected after perfusion fixation with 4% paraformaldehyde, decalcified with EDTA for 3 to 5 days, and embedded in paraffin. Serial sections (6-μm-thick) were subjected to staining with H&E, Safra-

nin O, or Alcian blue. Sections were also processed for immunohistochemistry to analyze the presence of C-terminal aggrecan neopeptides using rabbit NITEGE polyclonal antibodies that recognize aggrecanase cleavage products.[18] These antibodies were kindly provided by Dr. John S. Mort (Shriners Hospital for Children, Montreal, QC, Canada). Companion sections were stained with rabbit polyclonal antibodies against the C terminus of mouse estrogen receptor α (sc-542, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or mouse monoclonal antibodies against the C terminus of β-catenin (BD Biosciences, San Jose, CA). To validate specificity of the various antibodies, sections were incubated with corresponding control IgGs at similar concentrations. To facilitate antigen detection and immunostaining, sections were de-masked by treatment with 400 U/ml of testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 minutes or 0.1% pepsin (Sigma-Aldrich) at 37°C for 10 minutes. Bound antibodies were visualized using horseradish peroxidase-conjugated polymer or fluorescence-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). To detect apoptotic cells, we performed a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay using an Apop Tag Red *in situ* apoptosis detection kit (Chemicon International Inc., Temecula, CA).

Gene expression of *CA-β-catER* was analyzed by *in situ* hybridization using ^{35}S-labeled riboprobes.[19] The probe was designed to recognize the C-terminal end of β-catenin plus the N-terminal part of the ER domain. To evaluate cell proliferation, mice received an intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU) (150 μg/ml in PBS, Invitrogen) 2 hours before being sacrificed. Longitudinal long bone sections were incubated with anti-BrdU antibodies (1:200, Roche Diagnostics, Indianapolis, IN) followed by incubation with Alexa Fluor 488-anti-mouse IgG and propidium iodide (1:1000, Invitrogen).

Mouse Chondrocyte Cultures

Chondrocytes were isolated from the epiphyseal portion of femurs and humeri from newborn mice (C57BL/6J) by digestion with 0.25% trypsin and 2 mmol/L EDTA for 15 minutes followed by digestion with 0.15% collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) for 4 to 5 hours. Primary cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. When confluent, cultures were treated with tunicamycin (5 μg/ml, MP Biomedicals LLC, Santa Ana, CA) with or without recombinant mouse Wnt3a protein (100 ng/ml, Millipore, Billerica, MA) or glycogen synthase kinase 3β inhibitor BIO (0.5 μg/ml, Alexis Corporation, Lausen, Switzerland). After 24 hours of treatment, cultures were lysed in SDS sample buffer for immunoblot analysis or fixed with 10% formaldehyde for the TUNEL assay.

Transfection and Reporter Assays

Freshly isolated chondrocytes were cultured until subconfluent and then were transfected with one of the following plasmids using TransIT-LT1 transfection reagent (Mirus Bio Corp., Madison, WI), an *Axin2-Luc* promoter reporter vector that contains an *Axin2* promoter, exon 1, and intron 1[20], Col11CA-β-cateninER, or a dominant-negative β-catenin (EnRβcat) expression vector encoding β-catenin and a repressor element of engrailed.[21] Luciferase activity was measured 2 days after transfection using a Bright-Glo luciferase assay kit (Promega, Madison, WI).

Immunoblots

Equal volumes of cell lysates were separated by electrophoresis on 10% gels and transferred to polyvinylidene difluoride membranes. After blocking, membranes were incubated with polyclonal antibodies against the C terminus of estrogen receptor α (sc-542, 1:250, Santa Cruz Biotechnology, Inc) or with α-tubulin monoclonal antibodies (1:4000, Sigma-Aldrich). Bound antibodies were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) followed by incubation with chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) and detection by luminescent image analyzer LAS-1000plus (Fuji Photo Film Co., Ltd., Tokyo Japan).

RT-PCR and Real-Time PCR

Total RNA isolated by guanidine isothiocyanate was treated with Turbo DNase I (Ambion, Austin, TX). First-strand cDNA was synthesized from 1 μg of total RNA with 1 μmol/L random 9 mer primer (PerkinElmer Life and Analytical Sciences) using AffinityScript reverse transcriptase (Stratagene, La Jolla, CA) at 42°C for 1 hour. Subsequent amplification was performed with Takara PrimeStar HS premix (Takara Mirus Bio Inc., Madison, WI) for 20 to 30 cycles under the following conditions: 95°C for 10 seconds for denaturation and 68°C for 1 minute for annealing and extension. Quantitative PCR was performed with an Applied Biosystems 7900HT Sequence Detection System running SDS 2.1 software using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and following the manufacturer's instructions. Gene expression levels relative to that of glyceraldehyde-3-phosphate dehydrogenase were calculated by dividing the amount of target gene transcript with that of glyceraldehyde-3-phosphate dehydrogenase. Primer sequences were 5′-TGCTCCTCAGTGGATGTTGCCTTTAC-3′ and 5′-AGGTTGTGAAGTGTTCCCGCAGTG-3′ for the CA-β-catER transgene, 5′-GGAATGAAGGCGTGGCAACATAC-3′ and 5′-AGCCCATCAACTGGATAGTCAGCAC-3′ for mouse β-catenin (NM_007614), and 5′-GGAGAAACCTGCCAAGTATGATGACATC-3′ and 5′-ACCACCCTGTTGCTGTAGCCGTATTC-3′ for 799 to 1026 of mouse glyceraldehyde-3-phosphate dehydrogenase (NM_0001001303).

Statistical Analysis

Data were validated by Student's *t*-tests.

Results

Col11-CA-β-catER Mouse Lines

To create transgenic lines in which Wnt/β-catenin activity could be induced conditionally, we linked a cDNA clone encoding a constitutive-active form of β-catenin fused to a modified estrogen receptor ligand-binding domain (CA-β-catER) to promoter/enhancer sequences from the *Collagen 11a2* gene that were previously shown to drive transgene expression specifically in cartilage.[16] To test specificity and effectiveness of the resulting *Col11-CA-β-catER* construct, it was transfected into primary mouse chondrocytes and nonchondrogenic mesenchymal L cells along with an Axin2 Wnt/β-catenin reporter plasmid (*Axin-Luc*).[20] Cultures were then treated with hydroxytamoxifen or vehicle, and reporter activity was measured 48 hours later. Tamoxifen treatment greatly stimulated reporter activity in chondrocytes (Figure 1A, left panel) but not in L cells (Figure 1A, right panel). When companion chondrocyte cultures were cotransfected with the construct and a dominant-negative β-catenin expression vector,[21] tamoxifen treatment failed to elicit reporter activity (Figure 1A), affirming specificity of responses and dependence on β-catenin action.

Three independent *Col11-CA-β-catER* transgenic mouse lines were created in our centralized facility, and two of them were selected based on transgene copy number. Mice from these two lines exhibited normal appearance and behavior and produced transgenic offspring at the expected Mendelian frequency. To verify transgene expression, longitudinal sections of tibial epiphyses from neonatal (P0) *Col11-CA-β-catER* transgenic mice and wild-type littermates were processed for *in situ* hybridization, using a cDNA probe that specifically recognizes the fusion portion of the β-catenin and estrogen ligand-binding domain. Transgenic cartilage displayed abundant transgene transcripts (Figure 1, C and E) that were absent in wild-type cartilage (Figure 1, B and D). To confirm that the corresponding protein was produced, similar longitudinal sections were processed for immunostaining with antibodies against the estrogen receptor α C terminus portion that also recognizes CA-β-catER fusion protein.[15] Positive immunostaining was easily detectable in transgenic cartilage (Figure 1, G and I) but not in wild-type tissue (Figure 1, F and H). Negative staining in control tissue indicates that endogenous estrogen receptor α[22] was produced at levels below detection by this procedure.

To verify that an intact CA-β-catER fusion protein of about 90 kDa was being produced, chondrocytes were isolated from epiphyseal cartilage of *CA-β-catER* transgenic neonatal mice and expanded in primary culture for a few days; analogous cells were isolated from wild-type littermates. Cell homogenates were prepared and subjected to immunoblot (Figure 1J). A 90-kDa band was present in transgenic samples and was recognized by antibodies against the estrogen receptor moiety, but a similar band was absent in wild-type samples (Figure 1J, arrow). In both wild-type and transgenic samples, however, a band of 66 kDa corresponding to endogenous estrogen receptor α was barely detectable (Figure 1J, arrowhead), confirming that endogenous receptor expression was low and difficult to document (Figure 1F).

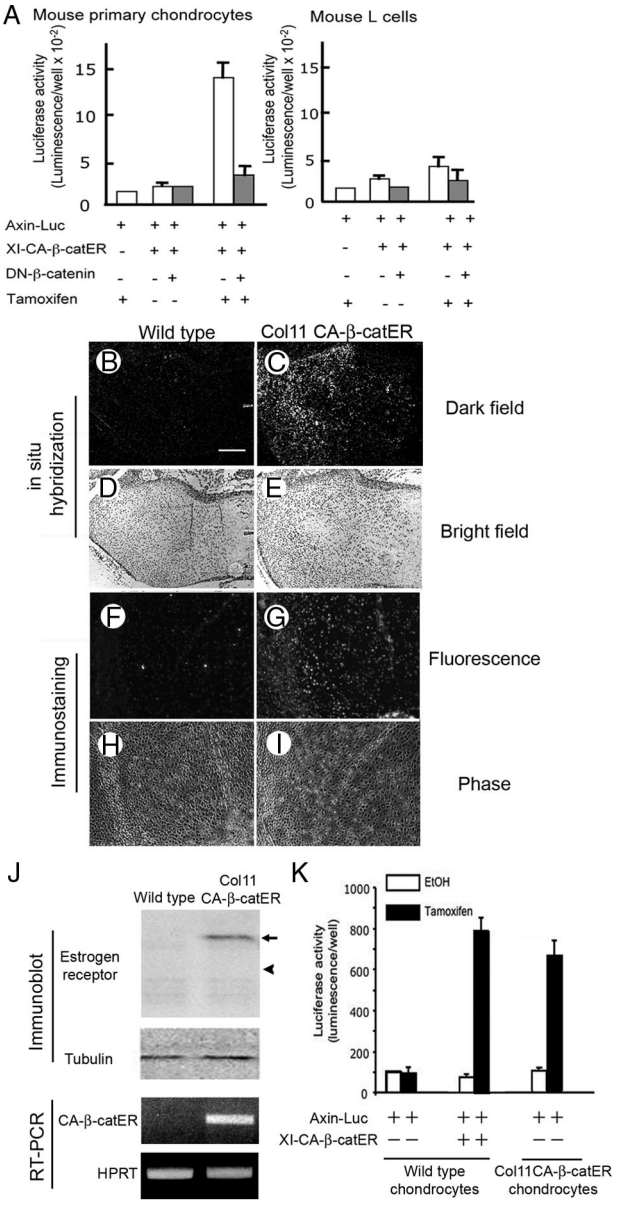

Figure 1. Transgene *Col11-CA-β-catER* expression and activity. **A:** Primary mouse chondrocytes and mouse fibroblastic L cells were transfected with the indicated plasmids and treated with 2 μmol/L 4-hydroxy tamoxifen (Tamoxifen +) or vehicle (Tamoxifen −) 24 hours after transfection. Cultures were incubated for an additional 24 hours and luciferase activity was measured. **B–I:** Longitudinal sections of P0 *Col11-CA-β-cat-ER* transgenic and wild-type tibias were subjected to *in situ* hybridization analysis of transgene expression (**B–E**) or immunofluorescence staining of estrogen receptor α (**F–I**). **B** and **C** are dark-field images corresponding to bright-field images of **D** and **E**, respectively. **F** and **G** are fluorescence images of the phase-contrast images of **H** and **I**, respectively. **J:** Cultures of epiphyseal chondrocytes isolated from P3 to P5 *Col11-CA-β-catER* transgenic mice or their wild-type littermates were subjected to immunoblot analysis for estrogen receptor α (Estrogen receptor) or tubulin expression or RT-PCR analysis of CA-β-catER transgene and *HPRT* expression. The **arrow** and **arrowhead** represent the estimated migrating positions of transgene product and estrogen receptor α, respectively. **K:** Parallel cultures transfected with indicated plasmids were treated with 2 μmol/L 4-hydroxytamoxifen (black bars) or the same volume of ethanol (white bars) 24 hours after transfection. Cultures were incubated for an additional 24 hours, and luciferase activity was measured. Scale bars: 150 μm (**B–E**); 75 μm (**F–I**).

Total RNAs were prepared from companion transgenic and wild-type cultures and subjected to RT-PCR amplification followed by gel electrophoresis. Clearly, only transgenic cultures expressed the *Col11 CA-β-catER* transgene (Figure 1J, RT-PCR). Both cultures, however, expressed the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) that was used as an internal control.

Finally, to be sure that the transgenic chondrocytes responded to tamoxifen, transgenic and wild-type chondrocyte cultures were transfected with the Axin2 Wnt/β-catenin reporter plasmid and treated with hydroxytamoxifen for 24 hours. The treatment did stimulate reporter activity in transgenic but not wild-type cultures (Figure 1K, left and right histograms). When wild-type cultures were cotransfected with both *Col11-CA-β-catER* and Axin2 Wnt/β-catenin reporter plasmids, the cells became responsive to tamoxifen treatment and expressed levels of reporter activity similar to that of transgenic cells (Figure 1K, central histogram). These findings clearly show that *Col11-CA-β-catER* transgenic chondrocytes express a functional CA-β-catER protein and respond to tamoxifen treatment with increases in Wnt/β-catenin signaling.

Growth Retardation

To analyze the possible consequences of transient postnatal activation of Wnt/β-catenin signaling in cartilage, transgenic mice at 2 to 3 weeks of age were subjected to a daily injection of tamoxifen for 7 to 10 days. As controls, transgenic littermates received vehicle alone (corn oil) and age-matched wild-type mice received the same tamoxifen regimen. Starting at about 4 weeks from the last injection, it became apparent by simple visual inspection that the tamoxifen-treated transgenic mice displayed growth retardation compared with either vehicle-treated transgenic mice or tamoxifen-treated wild-type mice (Figure 2A). Body length in male and female tamoxifen-treated transgenic mice was reduced approximately 20 to 35% at 7 weeks of age (Figure 2B, shaded columns) compared with controls (Figure 2B, white columns). Soft X-ray analysis revealed that humerus, ulna, radius, femur, and tibia in tamoxifen-treated *Col11-CA-β-catER* mice were appreciably shorter than those in controls (Figure 2, D–G) confirmed by direct tibia measurement (Figure 2C). In addition, the knee and elbow joints were deformed (Figure 2, F and G), and the growth plates were hardly recognizable (Figure, 2, J and K), whereas they were clearly visible as a translucent structure in wild-type mice (Figure 2, H and I, arrows).

To examine the growth plates in detail, we performed histological and histochemical analyses. Two weeks after the last tamoxifen injection, the height of the proximal tibial growth plate in the 5-week-old transgenic mice was already reduced (Figure 3D, vertical line) compared with that in control mice (vehicle-treated wild-type and transgenic mice or tamoxifen-treated wild-type mice) (Figure 3, A, B, and C, respectively, vertical lines). In the affected growth plates, the columnar alignment of chondrocytes was absent and the hypertrophic zone was particularly

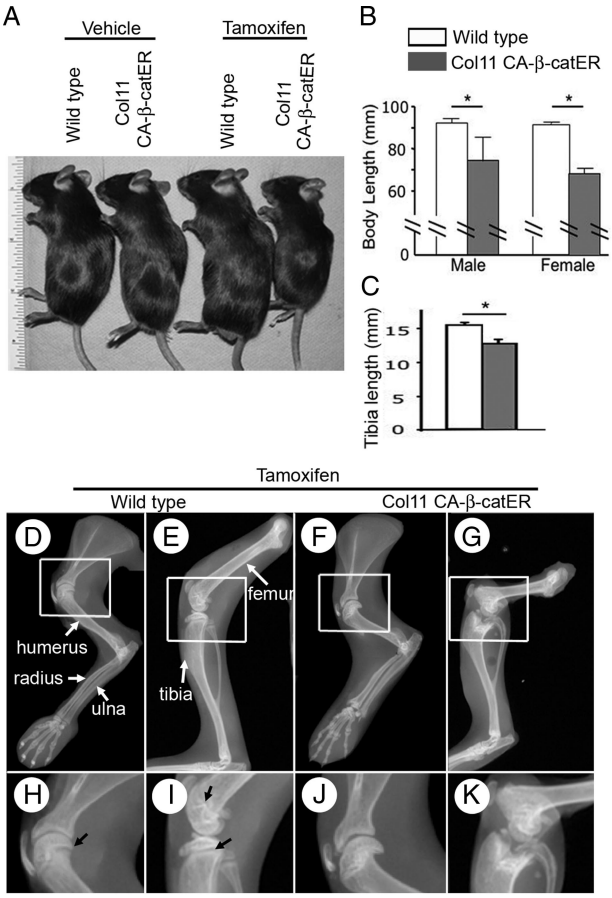

Figure 2. Growth retardation and deformity of long bones in tamoxifen-injected *Col11-CA-β-catER* transgenic mice. Two week-old *Col11-CA-β-catER* transgenic mice and their wild-type littermates received 7 to 10 daily peritoneal injections of tamoxifen (200 μg/20 μl/mouse) (Tamoxifen) or the same volume of corn oil (Vehicle) and were sacrificed at 7 weeks of age. **A:** Gross appearance. **B:** Average body lengths of tamoxifen-treated mice (male: four wild-type mice and four *Col11-CA-β-catER* mice; female: four wild-type mice and seven *Col11-CA-β-catER* mice). $^*P < 0.05$. **C:** Average lengths of three individual tibias of tamoxifen-treated wild-type and *Col11-CA-β-catER* mice. $^*P < 0.05$. **D–K:** Forelimbs (**D** and **F**) and hind limbs (**E** and **G**) were dissected from tamoxifen-injected wild-type (**D** and **E**) and *Col11-CA-β-catER* mice (**F** and **G**) and analyzed by soft X-ray. **H–K** are magnified images of square regions in **D–G**, respectively.

scanty (Figure 3D). By 7 weeks of age (and 4 weeks from the last tamoxifen injection), the growth plates were nearly absent and had thus undergone abnormal closure (Figure 3, E and F). This unexpected finding was consistently observed in seven tamoxifen-treated transgenic mice.

We asked whether the disorganization of growth plates in tamoxifen-treated transgenic mice included excessive cell death. TUNEL staining on sections of growth plates harvested just 1 day after the last tamoxifen injection showed that more numerous apoptotic chondrocytes were present in transgenic tissue and were scattered throughout the hypertrophic zone (Figure 4, B and D, Hyper), whereas apoptotic cells were confined to the chondro-osseous border in controls as expected (Figure 4, A and C, arrows). When companion sections were processed for analysis of chondrocyte proliferation, no major differences were observed (not shown).

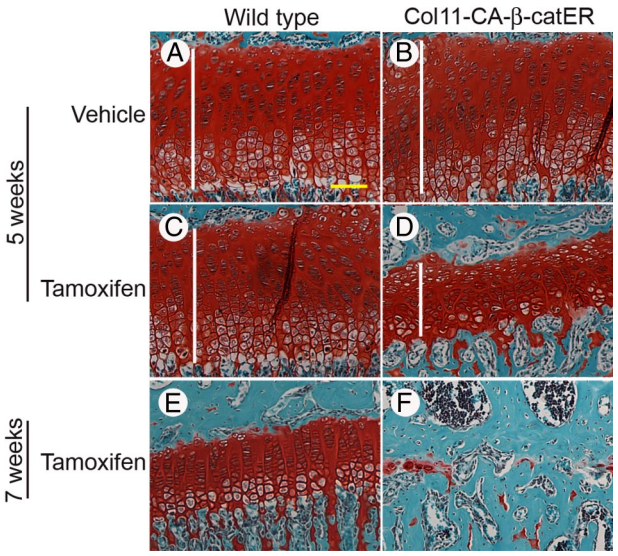

Figure 3. Disarrangement of growth plates in tamoxifen-injected *Col11-CA-β-catER* transgenic mice. Two-week-old *Col11-CA-β-catER* transgenic mice (**B, D,** and **F**) and their wild-type littermates (**A, C,** and **E**) received seven daily peritoneal injections of tamoxifen (200 μg/20 μl/mouse) (**C–F**) or the same volume of corn oil (**A** and **B**) and were sacrificed at 5 weeks (**A–D**) or 7 weeks of age (**E** and **F**). Longitudinal sections of proximal tibias were stained with Safranin O. **Vertical bars** indicate the total width of the growth plate. Scale bar = 100 μm.

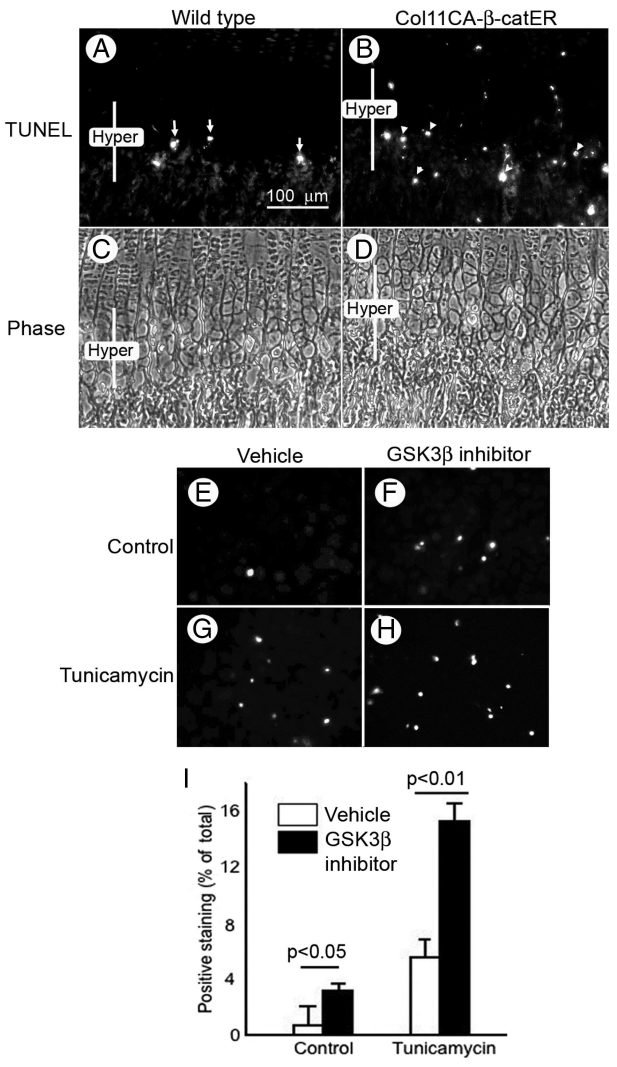

Figure 4. Wnt/β-catenin signaling stimulates cell apoptosis in chondrocytes. **A–D:** Two-week-old *Col11-CA-β-catER* transgenic mice (**B** and **D**) and their wild-type littermates (**A** and **C**) received seven daily peritoneal injections of tamoxifen (200 μg/20 μl/mouse) and were sacrificed 1 day after the last injection. Longitudinal sections of proximal tibia were subjected to TUNEL staining. **C** and **D** are phase-contrast images of **A** and **B**, respectively. **Vertical bars** represent hypertrophic zones. **E–I:** Epiphyseal chondrocytes isolated from P3 neonatal mice were treated with 5 μg/ml of tunicamycin and/or 0.5 μg/ml of the glycogen synthase kinase 3β (GSK3β) inhibitor BIO for 24 hours. Cultures were then subjected to TUNEL assay (**E–H**). Ten random fields were captured and the percentage of TUNEL-positive cells to total cells was calculated (**I**).

To examine whether the increased apoptosis may be a direct response to enhanced Wnt/β-catenin signaling, we treated primary chondrocyte cultures with BIO, a drug that inhibits glycogen synthase kinase 3β and enhances Wnt/β-catenin signaling, and performed TUNEL staining. BIO treatment did significantly increase TUNEL staining compared with that for control cultures (Figure 4, E, F, and I), and this effect was further enhanced by cotreatment with tunicamycin, which is a known apoptosis inducer (Figure 4, G, H, and I).

Joint Abnormalities

Next, we analyzed the limb joints in tamoxifen-treated *Col11-CA-β-catER* and companion control mice. The knee joints were dissected from the wild-type mice or *Col11-CA-β-catER* mice 1 day after the last tamoxifen or vehicle injection. In control wild-type mice treated with tamoxifen or transgenic mice treated with vehicle, knee joints and articular cartilage had normal expected characteristics and organization and strong staining with Safranin O, indicative of abundant proteoglycan content (Figure 5, A and C). However, articular cartilage in companion transgenic mice exhibited a dramatic reduction in Safranin O staining (Figure 5B). To test whether such matrix loss was accompanied by aggrecan degradation, sections were stained with rabbit antibodies against the NITEGE neoepitope created by aggrecanase activity *in vivo*.[18] Indeed, neoepitope levels were far greater in tamoxifen-treated transgenic (Figure 5E) than in control wild-type or vehicle-treated transgenic articular cartilage (Figure 5, D and F). Immunostaining of companion sections with antibodies against anti-estrogen receptor α confirmed that both tamoxifen-treated and untreated transgenic cartilage exhibited strong staining (Figure 5, H–I), whereas wild-type tissue did not (Figure 5G). The specificity of immunostaining procedures was confirmed by use of control rabbit IgGs that elicited only background staining (Figure 5F, inset). Despite the significant drop in proteoglycan content and presence of aggrecan degradation products, the overall appearance of articular cartilage in tamoxifen-treated transgenic mice was still largely normal at this time point (Figure 5B).

When we examined articular cartilage in transgenic mice 2 weeks after the last tamoxifen injection, however, we noted significant and interesting structural changes. The overall thickness of transgenic cartilage was significantly increased compared with control (Figure 6, B ver-

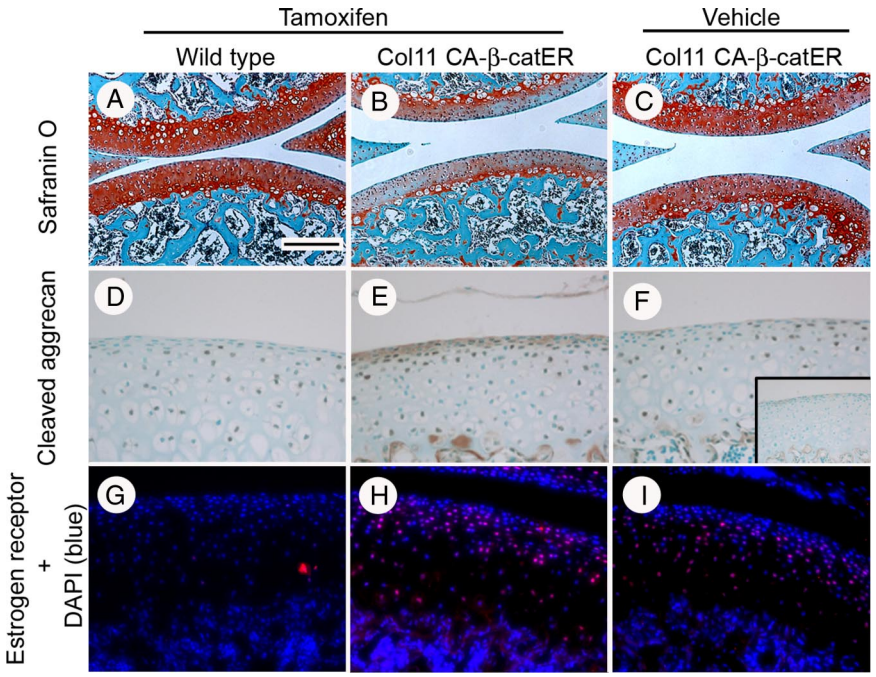

Figure 5. Rapid loss of proteoglycan and increases in aggrecan cleavage neoepitope induced by tamoxifen injection in *Col11-CA-β-catER* transgenic mice. Two-week-old *Col11-CA-β-catER* transgenic mice (**B**, **C**, **E**, **F**, **H** and **I**) and their wild-type littermates (**A**, **D** and **G**) received seven daily peritoneal injections of tamoxifen (200 μg/20 μl/mouse) (Tamoxifen: **A, B, D, E, G,** and **H**) or corn oil (Vehicle: **C, F,** and **I**) and were sacrificed 1 day after the last injection. Longitudinal sections of proximal tibial articular were subjected to Safranin O staining (**A–C**) or immunostaining with antibodies against the neoepitope of cleaved aggrecan (NITEGE) (**D–F**) or estrogen receptor α (**G–I**). The **inset** in **F** shows staining with preimmune rabbit IgG. **D–F** was counterstained with Fast green. **G–I** were double-stained with 4,6-diamidino-2-phenylindole (DAPI, blue). Scale bars: 200 μm (**A–C**); 50 μm (**D–F**); 100 μm (**G–I**).

sus A, vertical green lines). In particular, the superficial zone facing the synovial cavity was composed of three to four layers of flat small-sized cells (Figure 6D, double green arrow), whereas it was composed of a single cell layer in wild-type mice as expected (Figure 6C). BrdU labeling showed that proliferation was increased and widespread in transgenic articular cartilage (Figure 6, I–J), whereas it was restricted to most superficial thin cell layer in controls (Figure 6, G and H, arrows). Similar changes were seen in hip joint articular cartilage of the same animals (not shown). Interestingly, staining with Safranin O indicated that the overall proteoglycan content had been restored to control levels by this time point (Figure 6, E and F).

Postnatal Ablation of β-Catenin

The above results clearly indicate that acute postnatal activation of Wnt/β-catenin signaling is deleterious to articular cartilage and growth plate. Thus, we performed a final set of reciprocal experiments to investigate whether loss of Wnt/β-catenin signaling is deleterious as well. We used transgenic mice expressing tamoxifen-inducible Cre recombinase under control of a collagen 2 α1 promotor/enhancer (*Col2-CreER* mice)[17] and mated them with β-catenin-floxed mice to create conditional cartilage-specific mutants (compound *Col2-CreER/β-catenin$^{fl/fl}$* transgenic mice). To first confirm that the Cre mice responded well to tamoxifen, we mated them with Rosa R26R mice and injected tamoxifen three times staring at P5. Histochemical analysis showed that β-galactosidase activity was strong in articular and growth plate cartilage (Figure 7B) and in some of periosteal cells (Figure 7B, inset, arrows) and was absent in control Rosa mice (Figure 7A). Immunostaining of sections from compound *Col2-CreER/β-catenin$^{fl/fl}$* transgenic mice injected with tamoxifen three times starting at P5 showed that β-catenin levels had been markedly reduced in articular cartilage (Figure 7D) compared with those in control *β-catenin$^{fl/fl}$* mice (Figure 7C). Quantitative analysis indicated that β-catenin expression levels had decreased approximately 80% relative to control levels (Figure 7E).

Limbs were dissected from companion mice at 7 weeks. Clearly, knee joint organization and structure had become markedly deranged in tamoxifen-treated compound transgenic mice. Articular cartilage stained poorly with Alcian blue (Figure 8B) and contained fewer cells (Figure 8D) compared with control cartilage (Figure 8, A and C). In addition, the cartilage surface was rough and uneven (Figure 8D), whereas controls displayed a typical smooth surface (Figure 8C). Notably, the underlying growth plates were affected as well and displayed irregular organization and a severe decrease in hypertrophic chondrocytes (Figure 8F, arrows) and primary spongiosa compared with controls (Figure 8E). The reduction in trabecular bone was confirmed by microcomputed tomography (Figure 8, G and H). We also observed formation of ectopic cartilaginous masses within periosteal tissues surrounding the epiphysis and metaphysis (Figure 8, I and J, arrows), which has also been observed in similar transgenic mice at embryonic stages.[5,6]

Discussion

We report here the creation of *Col11-CA-β-catER* transgenic mouse lines in which Wnt/β-catenin signaling can be activated in cartilage at specific time points. Using this unique experimental tool, we show that postnatal activation of Wnt/β-catenin signaling for 7 to 10 days is sufficient to cause severe growth retardation. The growth

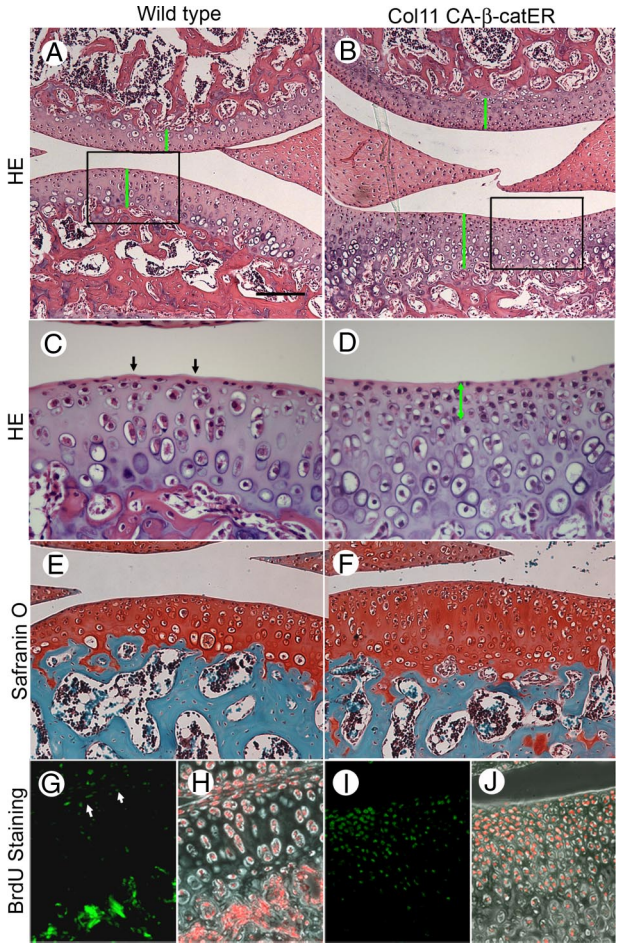

Figure 6. Increases in cell density and proliferating activity in tamoxifen-injected *Col11-CA-β-catER* transgenic mice. Two-week-old *Col11-CA-β-catER* transgenic mice (**B, D, F, I,** and **J**) and their wild-type littermates (**A, C, E, G,** and **H**) received seven daily peritoneal injections of tamoxifen (200 μg/20 μl/mouse) and were sacrificed at 5 weeks. Longitudinal sections of proximal tibial articular cartilage were stained with H&E (**A–D**) or Safranin O (**E** and **F**). **G–J:** Cells were labeled with BrdU as described in *Materials and Methods*. **G** and **I** are visualized BrdU labeling images. **H** and **J** are images merged with phase-contrast and propidium iodide-staining images of **G** and **I**, respectively. Scale bars: 200 μm (**A** and **B**); 60 μm (**C** and **D**); 100 μm (**E** and **F**); 80 μm (**G–J**). **Bar** in **A** and **B** indicate the depth of articular cartilage. **Arrows** and a **double arrowed line** in **C** and **D** indicate the surface layer of articular cartilage. **Arrows** in **G** indicate BrdU-labeled cells.

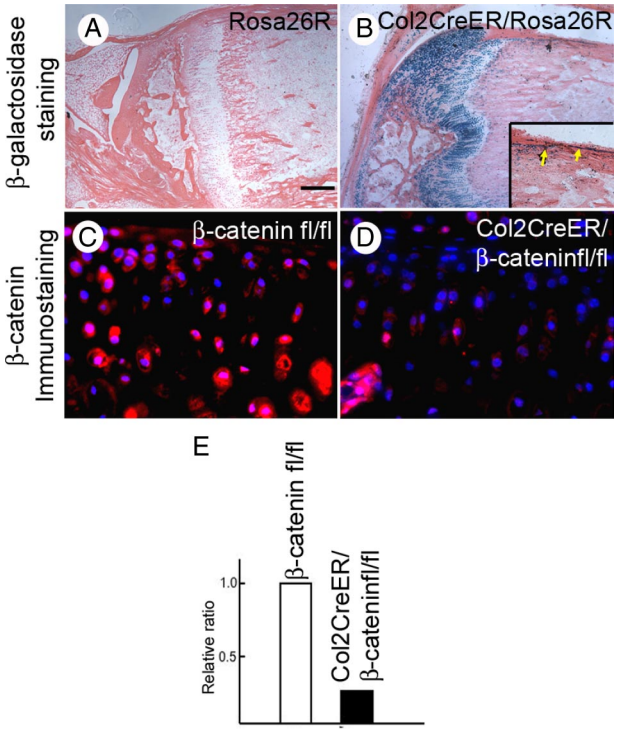

Figure 7. Induction of β-galactosidase activity and suppression of β-catenin expression by tamoxifen-induced Cre recombinase. *Rosa 26R* (**A**), *Col2CreER/Rosa26R* (**B**), *β-catenin*$^{fl/fl}$ (**C**), or *Col2CreER/β-catenin*$^{fl/fl}$ (**D**) mice received three daily peritoneal injections of tamoxifen (200 μg/20 μl/mouse) starting at P5 and were sacrificed at 5 weeks (**A** and **B**) or 7 weeks (**C** and **D**). Longitudinal sections of proximal tibia were subjected to β-galactosidase staining (**A** and **B**) or immunostaining with the monoclonal antibodies against the C terminus of β-catenin (**C** and **D**). Scale bars: 250 μm (**A** and **B**); 16 μm (**C** and **D**). **E:** Epiphyseal chondrocytes were isolated from tamoxifen-injected *β-catenin*$^{fl/fl}$ or *Col2CreER/β-catenin*$^{fl/fl}$ mice at P10 and cultured until confluent. Total RNA was prepared and the mRNA level of β-catenin was semiquantified by real-time PCR as described in *Materials and Methods*. Values represent the ratio to *β-catenin*$^{fl/fl}$ control sample.

plates are deranged structurally and functionally and nearly disappear over time. Articular cartilage is deranged too, but the cells actually exhibit higher cell density and proliferating activity. In complementary studies in which the β-catenin gene was conditionally ablated in postnatal cartilage, we find that joints and growth plates are both affected and this is accompanied by a severe decrease in trabecular bone. Taken together, the data provide clear evidence that normal spatiotemporal patterns and degrees of Wnt/β-catenin signaling are needed to maintain postnatal skeletal growth, joint and articular cartilage organization, and bone homeostasis. The distinct effects of acute Wnt/β-catenin signaling in growth plate and articular cartilage indicate that this pathway does not impose indiscriminate and uniform consequences on every type of chondrocytes, but chondrocyte phenotypic properties, nature, and developmental status influence their responses.

Growth Plate Abnormalities

The severe growth plate changes and the near elimination of the hypertrophic zone occur after either activation of Wnt/β-catenin signaling or conditional ablation of β-catenin, but the underlying pathophysiological responses seem to be distinct. Apoptotic cells are more numerous and broadly dispersed in growth plates after Wnt/β-catenin activation, and the growth plates eventually undergo abnormal closure, but similar events do not occur at early stages after β-catenin ablation. In addition, the consequences on bone volume and density are distinct as well; activation of Wnt/β-catenin signaling does not affect bone volume and density in a significant manner, but β-catenin deficiency strongly impairs bone formation. Given that apoptosis is normally restricted to the posthypertrophic zone of the growth plate before replacement of hypertrophic chondrocytes with bone cells, the widespread increase in apoptotic cells may reflect an acceleration and expansion of this normal process and precocious loss of the hypertrophic zone itself. On the

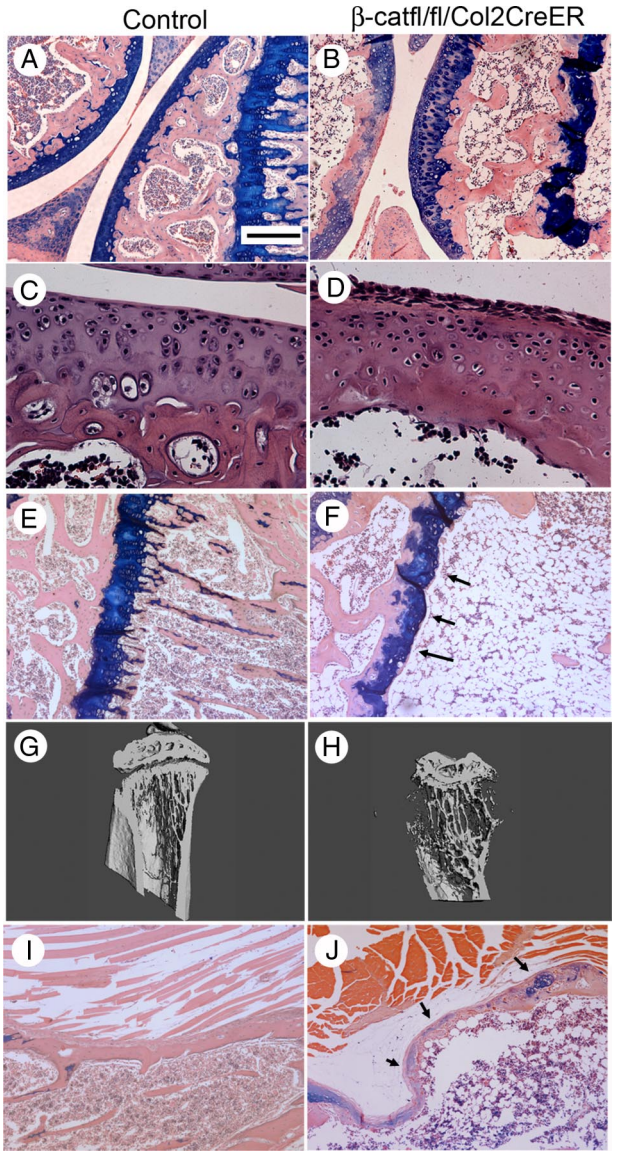

Figure 8. Degeneration of articular cartilage, reduction of bone, and ectopic cartilage formation are induced by postnatal ablation of β-catenin. Five-day-old *β-catenin$^{fl/fl}$* mice (**A, C, E, G,** and **I**), *Col2CreER/β-catenin$^{fl/fl}$* mice (**B, D, F, H,** and **J**) received three daily peritoneal injections of tamoxifen (**A–J**) and were sacrificed at 7 weeks (**A–J**) of age. **A–F** and **J–L**, longitudinal sections of proximal tibia were stained with Alcian blue and Fast red (**A, B, E, F, I,** and **J**), H&E (**C** and **D**). **G** and **H** are three-dimensional cut plane micro-computed tomography images of the proximal part of the tibia. Scale bars: 200 μm (**A, B, E, F, I,** and **J**); 50 μm (**C** and **D**). **Arrows** in **F** indicate loss of hypertrophic cells and **arrows** in **J** indicate ectopic cartilage formation.

other hand, deficient β-catenin expression in our conditionally ablated mice could have prevented formation of hypertrophic chondrocytes and the subsequent matrix calcification and endochondral ossification.

It has been reported recently that chronic inhibition of Wnt/β-catenin signaling by expression of the β-catenin nuclear inhibitor ICAT in cartilage starting during mouse embryogenesis leads to growth retardation and chondrocyte apoptosis.[11] In other systems, Wnt/β-catenin signaling also protects cells from apoptosis by mechanisms that include inhibition of cytochrome *c* release and caspase activation,[23] induction of antiapoptotic factors,[24] and supporting of expression or function of antiapoptotic proteins such as p53, bcl-2, and bcl-xL.[10,25,26] These data are at variance with our observation that activation of β-catenin signaling induces or enhances apoptosis in growth plate chondrocytes *in vivo* and *in vitro*. Intriguingly, this signaling pathway is also able to trigger apoptosis in other cell types. In activated clonal T cells excessive activation of β-catenin signaling induces apoptosis, which leads to suppression of T-cell hyper-reaction.[27] Taking into consideration all of the above studies and our current data, it is likely that Wnt/β-catenin signaling may regulate both cell survival and apoptosis in the growth plate and articular cartilage. Basal β-catenin signaling levels would sustain cell survival and protect the chondrocytes from abnormal apoptotic stimuli in upper growth plate zones, whereas high signaling levels would stimulate apoptosis in the posthypertrophic zone, facilitating the replacement of hypertrophic chondrocytes with bone. Excessive stimulation of Wnt/β-catenin signaling would trigger extensive apoptosis and lead to precocious closure of growth plate. In articular cartilage, basal signaling levels would be required to maintain lifelong function, and excessive levels would initially trigger proliferation and tissue thickening but could become overbearing and dysfunctional over time, leading to pathological changes.

It is well established that endogenous Wnt/β-catenin signaling regulates osteogenic cell differentiation and bone formation.[28] It is also well established that endochondral bone formation strictly depends on stimuli provided by hypertrophic chondrocytes to underlying bone marrow-associated osteoprogenitor cells. Thus, the bone deficiency we observe in β-catenin-deficient mice is likely to reflect lack of functional hypertrophic cells. We cannot exclude, however, the possibility that it may be contributed also by some leakage of Col2-driven Cre activity in osteoprogenitor cells.

Articular Cartilage Changes

Our data show that proteoglycan loss is quite rapid after acute activation of β-catenin signaling in articular cartilage (Figures 5). Such loss is associated with increased levels of aggrecan cleavage products, indicating that acute β-catenin signaling promotes rapid matrix degradation. This is in line with previous findings indicating that β-catenin signaling stimulates activities and/or gene expression of matrix metalloproteinases and aggrecanases in cultured chondrocytes.[8,13] Despite the initial matrix loss, articular cartilage was not completely altered in its histological organization in our transgenic mice and in fact the articular cartilage height was increased. In contrast, a recent report has suggested that constitutive activation of β-catenin signaling triggers loss of articular cartilage.[12] These different observations could be explained by the distinct mode of Wnt/β-catenin signaling activation. Our novel system induces transient activation, whereas the system used in the other study creates constitutive and permanent stimulation. Thus, in our experiments articular chondrocytes started to proliferate and recovered their matrix after stoppage of tamoxifen injec-

tion. In contrast, constitutive Wnt/β-catenin activation and signaling might prevent the cells from initiating such rebound and recovery processes and would cause irreversible degeneration of articular cartilage. It is also possible that age of the mice may have contributed to varying responses seen in different studies, thus suggesting that articular chondrocytes in young mice (such as those used here) have the ability to recover from damage, whereas those in older aged mice cannot.

We and another group have demonstrated that articular cartilage originates and is maintained by a unique cell population,[29,30] indicating that it may possess a self-renewal system. Several studies have suggested that the superficial zone of articular cartilage contains progenitors of articular chondrocytes.[31–33] Our data show that whereas transient Wnt/β-catenin signaling increases cellularity in articular cartilage, conditional β-catenin ablation decreases it. In a previous study, we observed a similar loss of superficial cells after β-catenin loss at embryonic stages.[29] Both findings indicate that Wnt/β-catenin signaling regulates proliferation and maintenance of progenitor cells in articular cartilage, which has been reported in other types of tissues.[34–37] Recent studies have revealed that expression of Wnt16, a stimulator of β-catenin signaling is strongly and exclusively increased in association with articular cartilage injury.[38] Although the pathophysiological significance of this upregulation has not been elucidated, our findings indicate that an acute increase in Wnt/β-catenin signaling would be a beneficial response at the beginning for cartilage repair, but when signaling persists for longer periods, it would trigger irreversible degenerative changes. If so, transient activation of Wnt signaling could represent a future therapeutic method to induce or accelerate a repair process in damaged or aging articular cartilage.

Acknowledgments

We thank Drs. Fiona Watt (UK London Research Institute), Noriyuki Tsumaki (Osaka University), Franklin Costantini (University of Columbia), and Dr. John S. Mort (Shriners Hospital for Children) for providing the *β-catER* plasmid, *collagen 11α2* promoter/enhancer plasmid, *Axin-Luc* reporter plasmid, and the antibodies against aggrecan cleavage product. We also thank Ms. Aishah Hargett, Julie Williams, Rashien Griffin and Diane Pilchak for their technical assistance.

References

1. Clevers H: Wnt/β-catenin signaling in development and disease. Cell 2006, 127:469–480
2. Gordon MD, Nusse R: Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem 2006, 281:22429–22433
3. Huang H, He X: Wnt/β-catenin signaling: new (and old) players and new insights. Curr Opin Cell Biol 2008, 20:119–125
4. Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR, McCrea PD, de Crombrugghe B: Interactions between Sox9 and β-catenin control chondrocyte differentiation. Genes Dev 2004, 18:1072–1087
5. Day TF, Guo X, Garrett-Beal L, Yang Y: Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 2005, 8:739–750
6. Hill TP, Spater D, Taketo MM, Birchmeier W, Hartmann C: Canonical Wnt/β-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev Cell 2005, 8:727–738
7. Hu H, Hilton MJ, Tu X, Yu K, Ornitz DM, Long F: Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development 2005, 132:49–60
8. Tamamura Y, Otani T, Kanatani N, Koyama E, Kitagaki J, Komori T, Yamada Y, Costantini F, Wakisaka S, Pacifici M, Iwamoto M, Enomoto-Iwamoto M: Developmental regulation of Wnt/β-catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification. J Biol Chem 2005, 280:19185–19195
9. Enomoto-Iwamoto M, Kitagaki J, Koyama E, Tamamura Y, Wu C, Kanatani N, Koike T, Okada H, Komori T, Yoneda T, Church V, Francis-West PH, Kurisu K, Nohno T, Pacifici M, Iwamoto M: The Wnt antagonist Frzb-1 regulates chondrocyte maturation and long bone development during limb skeletogenesis. Dev Biol 2002, 251:142–156
10. Zhu M, Chen M, Zuscik M, Wu Q, Wang YJ, Rosier RN, O'Keefe RJ, Chen D: Inhibition of β-catenin signaling in articular chondrocytes results in articular cartilage destruction. Arthritis Rheum 2008, 58:2053–2064
11. Chen M, Zhu M, Awad H, Li TF, Sheu TJ, Boyce BF, Chen D, O'Keefe RJ: Inhibition of β-catenin signaling causes defects in postnatal cartilage development. J Cell Sci 2008, 121:1455–1465
12. Zhu M, Tang D, Wu Q, Hao S, Chen M, Xie C, Rosier R, O'Keefe R, Zuscik M, Chen D: Activation of β-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult β-catenin conditional activation mice. J Bone Miner Res 2009, 24:12–21
13. Yuasa T, Otani T, Koike T, Iwamoto M, Enomoto-Iwamoto M: Wnt/β-catenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: its possible role in joint degeneration. Lab Invest 2008, 88:264–274
14. Li TF, O'Keefe RJ, Chen D: TGF-β signaling in chondrocytes. Front Biosci 2005, 10:681–688
15. Lo Celso C, Prowse DM, Watt FM: Transient activation of β-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours. Development 2004, 131:1787–1799
16. Tsumaki N, Kimura T, Matsui Y, Nakata K, Ochi T: Separable cis-regulatory elements that contribute to tissue- and site-specific α2(XI) collagen gene expression in the embryonic mouse cartilage. J Cell Biol 1996, 134:1573–1582
17. Nakamura E, Nguyen MT, Mackem S: Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER^T to assay temporal activity windows along the proximodistal limb skeleton. Dev Dyn 2006, 235:2603–2612
18. Sztrolovics R, Alini M, Roughley PJ, Mort JS: Aggrecan degradation in human intervertebral disc and articular cartilage, Biochem J 1997, 326(Pt 1):235–241
19. Koyama E, Leatherman JL, Shimazu A, Nah HD, Pacifici M: Syndecan-3, tenascin-C, and the development of cartilaginous skeletal elements and joints in chick limbs. Dev Dyn 1995, 203:152–162
20. Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F: Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 2002, 22:1172–1183
21. Montross WT, Ji H, McCrea PD: A β-catenin/engrailed chimera selectively suppresses Wnt signaling, J Cell Sci 2000, 113(Pt 10):1759–1770
22. Nilsson O, Marino R, De Luca F, Phillip M, Baron J: Endocrine regulation of the growth plate. Horm Res 2005, 64:157–165
23. Chen S, Guttridge DC, You Z, Zhang Z, Fribley A, Mayo MW, Kitajewski J, Wang CY: Wnt-1 signaling inhibits apoptosis by activating β-catenin/T cell factor-mediated transcription. J Cell Biol 2001, 152:87–96
24. Longo KA, Kennell JA, Ochocinska MJ, Ross SE, Wright WS, MacDougald OA: Wnt signaling protects 3T3-L1 preadipocytes from apoptosis through induction of insulin-like growth factors. J Biol Chem 2002, 277:38239–38244
25. Rother K, Johne C, Spiesbach K, Haugwitz U, Tschop K, Wasner M, Klein-Hitpass L, Moroy T, Mossner J, Engeland K: Identification of Tcf-4 as a transcriptional target of p53 signalling. Oncogene 2004, 23:3376–3384
26. Wang H, MacNaughton WK: Overexpressed β-catenin blocks nitric

oxide-induced apoptosis in colonic cancer cells. Cancer Res 2005, 65:8604–8607
27. Huang Z, Wang R, Xie H, Shang W, Manicassamy S, Sun Z: Stabilized beta-catenin potentiates Fas-mediated T cell apoptosis. J Immunol 2008, 180:6586–6592
28. Mak KK, Chen MH, Day TF, Chuang PT, Yang Y: Wnt/β-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation. Development 2006, 133:3695–3707
29. Koyama E, Shibukawa Y, Nagayama M, Sugito H, Young B, Yuasa T, Okabe T, Ochiai T, Kamiya N, Rountree RB, Kingsley DM, Iwamoto M, Enomoto-Iwamoto M, Pacifici M: A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. Dev Biol 2008, 316:62–73
30. Hyde G, Dover S, Aszodi A, Wallis GA, Boot-Handford RP: Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. Dev Biol 2007, 304:825–833
31. Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ, Haughton L, Bayram Z, Boyer S, Thomson B, Wolfe MS, Archer CW: The surface of articular cartilage contains a progenitor cell population. J Cell Sci 2004, 117:889–897
32. Hayes AJ, Dowthwaite GP, Webster SV, Archer CW: The distribution of Notch receptors and their ligands during articular cartilage development. J Anat 2003, 202:495–502
33. Ustunel I, Ozenci AM, Sahin Z, Ozbey O, Acar N, Tanriover G, Celik-Ozenci C, Demir R: The immunohistochemical localization of notch receptors and ligands in human articular cartilage, chondroprogenitor culture and ultrastructural characteristics of these progenitor cells. Acta Histochem 2008, 110:397–407
34. Kalani MY, Cheshier SH, Cord BJ, Bababeygy SR, Vogel H, Weissman IL, Palmer TD, Nusse R: Wnt-mediated self-renewal of neural stem/progenitor cells, Proc Natl Acad Sci USA 2008, 105:16970–16975
35. Schmidt-Ott KM, Barasch J: WNT/β-catenin signaling in nephron progenitors and their epithelial progeny. Kidney Int 2008, 74:1004–1008
36. Denayer T, Locker M, Borday C, Deroo T, Janssens S, Hecht A, van Roy F, Perron M, Vleminckx K: Canonical Wnt signaling controls proliferation of retinal stem/progenitor cells in postembryonic Xenopus eyes. Stem Cells 2008, 26:2063–2074
37. Fleming HE, Janzen V, Lo Celso C, Guo J, Leahy KM, Kronenberg HM, Scadden DT: Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. Cell Stem Cell 2008, 2:274–283
38. Dell'accio F, De Bari C, Eltawil NM, Vanhummelen P, Pitzalis C: Identification of the molecular response of articular cartilage to injury, by microarray screening: wnt-16 expression and signaling after injury and in osteoarthritis. Arthritis Rheum 2008, 58:1410–1421