DEVELOPMENTAL REGULATION OF WNT/β-CATENIN SIGNALS IS REQUIRED FOR GROWTH PLATE ASSEMBLY, CARTILAGE INTEGRITY, AND ENDOCHONDRAL OSSIFICATION

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Running title: Wnt/β-catenin signals and limb cartilage development

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Studies have suggested that continuous Wnt/β-catenin signaling in nascent cartilaginous skeletal elements blocks chondrocyte hypertrophy and endochondral ossification, while signaling starting at later stages stimulates hypertrophy and ossification, indicating that Wnt/β-catenin roles are developmentally regulated. To test this conclusion further, we created transgenic mice expressing a fusion mutant protein of β-catenin and LEF (CA-LEF) in nascent chondrocytes. Transgenic mice had severe skeletal defects, particularly in limbs. Growth plates were totally disorganized, lacked maturing chondrocytes expressing Indian hedgehog and collagen X, and failed to undergo endochondral ossification. Interestingly, the transgenic cartilaginous elements were ill defined, intermingled with surrounding connective and vascular tissues, and even displayed abnormal joints. However, when activated β-catenin mutant (Δ−β-catenin) was expressed in chondrocytes already engaged in maturation such as those present in chick limbs, chondrocyte maturation and bone formation were greatly enhanced. Differential responses to Wnt/β-catenin signaling were confirmed in cultured chondrocytes. Activation in immature cells blocked maturation and actually de-stabilized their phenotype, as revealed by reduced expression of chondrocyte markers, abnormal cytoarchitecture and loss of proteoglycan matrix. Activation in mature cells instead stimulated hypertrophy, matrix mineralization and expression of terminal markers such as MMP-13 and VEGF. Since proteoglycans are crucial for cartilage function, we tested possible mechanisms for matrix loss. Δ−β-catenin expression markedly increased expression of MMP-2, MMP-3, MMP-7, MMP-9, MT3-MMP and ADAMTS5. In conclusion, Wnt/β-catenin signaling regulates chondrocyte phenotype, maturation and function in a developmentally-regulated manner, and regulated action by this pathway is critical for growth plate organization, cartilage boundary definition, and endochondral ossification.

Skeletogenesis continues to attract much
research interest owing to its fundamental roles in embryonic development and growth and its susceptibility to pathologies. The process initiates with formation of mesenchymal or ectomesenchymal cell condensations at specific sites and times in the early embryo. As exemplified by the limb, the mesenchymal condensations then undergo chondrogenesis and give rise to cartilaginous long bone anlagen (1,2). The chondrocytes become organized in growth plates, proliferate, and mature into pre-hypertrophic and hypertrophic chondrocytes. Pre-hypertrophic chondrocytes express genes such as Indian hedgehog, and hypertrophic chondrocytes express a number of characteristic genes, including collagen X, alkaline phosphatase, osteopontin and metalloprotease-13 (MMP-13).

Eventually, the hypertrophic chondrocytes mineralize their matrix by deposition of apatitic crystals and are replaced by marrow, vascular and bone cells via an endochondral ossification process. While these step-wise endochondral events proceed, diarthrodial synovial joints form at each epiphyseal end. Joint formation begins with appearance of a mesenchymal interzone that comes to separate adjacent cartilaginous anlagen (3). Though the above principles and general mechanisms are clear and well established, much remains to be understood about skeletogenesis, particularly at the molecular signaling level.

Wnt proteins are powerful secreted signaling factors that regulate a number of developmental processes (4,5). The vertebrate Wnt family currently comprises 20 members. Wnt proteins act by binding to Frizzled and LRP cell surface receptors (4,5). Upon Wnt binding, Frizzled receptors transduce signals via β–catenin-LEF/TCF pathway (5,6), Ca^{2+}-calmodulin-PKC pathway (7) or JNK-dependent pathway (8). The β-catenin-LEF/TCF pathway is the best characterized to date and is conserved from Drosophila to humans (5,6). In absence of Wnt binding to Frizzled receptors, β-catenin is found to form complexes with cytoplasmic proteins such as glycogen synthase kinase 3β, Axin and adenomatous polyposis coli, and remains phosphorylated (9,10). Phosphorylated cytoplasmic β-catenin molecules become ubiquitynated and are directed for proteasome-assisted degradation (11). On the other hand, when Wnt proteins bind to the receptors, degradation is inactivated and a “free” form of β-catenin accumulates in the cytoplasm (9,10,12). Accumulated β-catenin molecules translocate to the nucleus and serve as a co-activator of resident LEF/TCF transcriptional factors, resulting in complexes that bind to response sequences and modulate expression of target genes (6,12).

Wnt proteins are involved in skeletogenesis. Seminal observations by Dealy and coworkers first showed that Wnt-7a is expressed by early limb bud ectoderm and influences skeletal patterning along the dorsal-ventral axis (13). Wnt-3a was found to share similar expression patterns and possibly similar roles (14). Subsequent studies indicated that Wnt-1, -4 and -7a exert inhibitory influences on differentiation of mesenchymal cells into chondrocytes, while Wnt-5a and -5b stimulate it (15,16). Wnt-5a and Wnt-5b were also shown to have strong influences on skeletogenesis in chick and mouse embryos (17-19). Of particular relevance to the present study are two recent reports on Wnt/β-catenin roles in skeletogenesis using transgenic approaches (20,21). Akiyama et al. (20) found that continuous expression of an activated β-catenin form (Δ–β-catenin) in nascent chondrocytes using collagen II promoter sequences blocks further chondrocyte development and also ossification, as indicated by low expression of chondrocyte master regulator Sox9 and bone regulator Runx2. A detailed analysis of the chondrocyte phenotype and maturation program was, however, not performed. Guo et al. (21) used a similar approach...
and found severe defects in long bone joint formation also. On the other hand, previous studies from our groups indicated that when Wnt/β-catenin signaling is experimentally activated at later stages and specifically in chondrocytes already undergoing and advancing toward maturation, activation resulted in a marked stimulation of hypertrophy (22,23). Together, the above studies lead to the intriguing and important notion that Wnt/β-catenin signals may not have a single role in skeletogenesis, but their interventions in the regulation of this complex multi-step process may be multiple and developmentally prescribed. The present study was carried out to test these hypotheses more directly using transgenic approaches in mice and gain-of-function studies in chick embryo limbs and chick chondrocytes in culture. The results provide support for our hypotheses and reveal also that the Wnt/β-catenin signaling pathway has critical roles in maintenance of cartilage tissue integrity and formation of proper tissue boundaries.

Materials and Methods

**Generation of transgenic mice** - DNA fragments encoding a constitutive active form of mouse LEF-1 (CA-LEF) that includes amino acids 695-781 of β-catenin fused to the C-terminus of murine LEF-DNA (24), were cloned into the NolI site of a Col2α1-based expression vector (25). Synthetic Nar I site was introduced into a Hind III site of the above vector. The resulting vector contains CA-LEF expression unit including 5'-Nar I site-Col2α1 promoter (nucleotide 1940-2971 of M65161), β-globin intron cassette, CA-LEF, SV40 poly A, Col2α1 enhancer (4930-5571 of M65161), Nar I site-3' in pNASSβ backbone (Clontech, California, USA). CA-LEF was tagged with HA antigen at the C-terminal site to monitor transgene expression. The expression unit of CA-LEF was excised by Nar I digestion, purified and injected into pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6 X C3H). Transgenic embryos were identified by PCR using two pairs of primers: 5'TGC AGC TTT ATC CAG GCT GGT CAG-3' and 5' CAC CCA TCT CAT GCT CCA TCA TCA TAG G-3' that amplify 345 bp fragment of CA-LEF; 5'TGG TCA TCA TCC TGC CTT TCT C-3' and 5' ATT AGG TCA CTG TCC GTG TGG G-3' that amplify 256 bp fragment of part of β-globin intron and LEF1 DNA binding domain. Genomic DNA was prepared from liver tissue. Expression of the transgene at protein level was analyzed by immunostaining for HA antigens. Seventy two embryos out of 377 embryos carried CA-LEF transgene and 11 embryos expressed the transgene products.

**Skeletal, histological, and immunohistochemical examinations** - For anatomical examination, whole skeletons of E18.5 embryos were stained with alizarin red S and Alcian blue as described (25). For light microscopy, tissues from E18.5 embryos were fixed in 4% paraformaldehyde/0.1M phosphate buffer, and resulting paraffin sections (6 µm in thickness) were stained with hematoxylin and eosin (H-E). For immunohistochemistry, sections were de-masked by treatment with 0.2% pepsin in 0.02N HCl for 15 min at 37°C, and were incubated with rabbit anti-mouse tenascin-C antibody (26) or anti-MMP-2 antibody (Chemicon International Inc., Temecula, CA) for 16 h at 4°C. After rinsing, sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and then with Cy3-conjugated Streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA); each incubation was for 30 min at room temperature.

**In situ hybridization** - Tissue section in situ hybridization was carried out using digoxigenin-conjugated or 35S-labeled riboprobes (25,27). cDNA fragments of mouse type IX (317-805 of NM_007740) and X (1302-1816 of NM_009925) collagens, aggrecan (880-1733 of...
NM_007424), Sox9 (569-1520 of AF421878), Ihh (897-1954 of NM_010544), Gas1 (1559-2452 of NM_008086), GDF5 (1585-2172 of U08337), ChM-1(156-1195 of U43509), and ERG (1007-1029 of AB07380) were amplified by RT-PCR from E15 mouse limb bud cDNA, subcloned into pGEM-T vector (Promega Corporation, Madison, WI) and used as templates to generate RNA probes. Chick CD44 (438-915 of NM_204980), and Gli3 (688-1131 of AF0022818) were amplified from E8 chick wing bud cDNA by RT-PCR and subcloned into pGEM-T vector.

**Cell cultures and viral infection** - Chondrocytes isolated from the caudal (for immature cells) and cephalic (for mature cells) portions of Day 17 chick embryo sternae (SPAFAS, c/o) were cultured in high-glucose DMEM containing 10% FBS (28). The cells were infected with concentrated RCAS virus encoding active form of Δ-β-catenin that lacks the amino-terminal domain (β-catenin) (14) or chick Wnt-14 (29) (kindly provided by Dr. C. J. Tabin, Harvard University) and subcultured at the density of about 1.0-1.5 x 10^4/cm² by trypsinization after one week, and maintained in complete medium containing 10 µg/ml of ascorbic acid. By passage one, more than 85% of the cells were routinely infected as revealed by immunocytochemistry of viral antigens (30); expression of introduced genes was confirmed by western blot. For matrix mineralization, cultures were provided with 1 mM β-glycerophosphate (31). Proteoglycan accumulation and matrix mineralization in cell layer were visualized by histochemistry (30).

Freshly isolated immature and mature chondrocytes were cultured until subconfluent and were then transfected with Ax2-Luc promoter reporter vector that contains Axin-2 promoter, exon1 and intron1 (32) in the absence or presence of pUSE expression vector encoding Δ-β-catenin or dominant negative form of LEF (that lacks amino acids 17-264 of murine LEF-1) (24). Transfections were carried out with TransIT-LT1 transfection reagent (Mirus Bio Corp., Madison, WI) according to manufacturer's instructions. Luciferase activity was measured 2 days after transfection by standard methods.

**RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)** - Total RNA was isolated by the guanidine isothiocyanate method (31). First strand cDNA was synthesized from 1 µg of total RNA with 1 µM of random 9 mer primer (Perkin Elmer) using SuperScript II TM reverse transcriptase ( Gibco BRL, Gaithersburg, MD) at 42 °C for 45 min. Subsequent amplification was performed with Premix Taq (TaKaRa EX Taq version, Takara Mirus Bio Inc. Madison, WI) for 25-35 cycles under the following conditions: 94 °C for 30 sec, 58-60 °C for 1 min and 68 °C for 1 min. Primer sequences for RT-PCR amplification are follows: 5'-TTCTGCTCTAGTTGATCCACTG-3' and 5'-CGATCTCCTATCGTGACGTTGTA-3' for 13-324 of VEGF (AB011078), 5'-GCAAACAGAAACGAGGAGCAAAGCAT-3' and 5'-CAGGGAGAAGAATGTTCCACCG TC-3' for 438-915 of CD44 (NM_204980), 5'-AGCGGATGCTCAAGGGTTTCTATG-3' and 5'-GGTGCAATGTCGCACTTGTATGAG-3' for 1814-2409 of chordin (NM_204980), 5'-TGTCTATCTGCCGCTGGTCTGATG-3' and 5'-CGACCTTCGGTCTTGACTTTGGTGCG-3' for 688-1131 of Gli3 (AF0022818), 5'-TCCTTTGCTCTGATCCAGGCGT-3' and 5'-CTCTGGTTCTTCTGTTGGGTGACGCTT-3' for 523-960 of MMP3 (XM_417175), 5'-TGCAGGCAGATGGAAATG-3' and 5'-GCACCTACCCGACATCCAATGGA-3' for 295-805 of MMP7 (Ensembl database ID: ENSGALT0000027770), 5'-GCCCTATGAAGACATCGCCGAGA-3' and 5'-TGGAGGTTTCCCGTGTACCCACTC-3' for 84-407 of MT2-MMP (ENSGALT0000001489), 5'-TCACCTCCCACCTTCTACCCGAGAT-3' and 5'-ACCACATCGCTGTGGCCACCATTG-3' for
452-856 of MMP9 (AF222690), 5'-GCCTTGCTCCTTCTTTGTTTCT-3' and 5'-AAAGTCCACCGTGAGTTGTTGCGT-3' for 1837-2365 of cMT3-MMP (NM_205197), 5'-GGAGGCGTTCACTTTGCTTCT-3' and 5'-TCCACCACACACCCCATTCATA-3' for 832-1257 of ADAMTS5 (ENSGALT00000025470). The resulting products were sequenced to confirm specific amplification of the target transcripts. All other PCR primers were described previously (22,33).

RESULTS

Skeletal dis-morphogenesis in transgenic mice

First, we generated transgenic mice in which a constitutive active form of β-catenin (CA-LEF) was expressed in nascent chondrocytes in developing mouse embryo skeletal elements, using cartilage-characteristic collagen II promoter/enhancer sequences to drive the transgene (25). CA-LEF is a fusion protein consisting of the DNA binding site of mouse LEF-1 and the transactivation domain of β-catenin, and has activity in chondrocytes similar to Δ−β-catenin used in previous studies (24).

Transgenic mice expressing CA-LEF died at birth of respiratory failure and displayed severe dwarfism with shortened limbs and a flattened skull (Fig. 1B) compared to wild type littermates (Fig. 1A). Skeletal staining with alizarin red and Alcian blue revealed that mineralization was markedly reduced in trunk and limbs and affected ribs, vertebrae, and long bones (Figs. 1A and 1B). Under-mineralization was also evident in cranial base (Fig. 1D, arrow) compared to wild type (Fig. 1C), but calvaria mineralization appeared largely unaffected (Figs. 1A and 1B). In addition to mineralization defects, transgenic skeletons displayed abnormalities in overall patterning, development and growth, particularly in the limbs. Transgenic scapula, humerus, radius and ulna were considerably short (Fig. 1H) and hind limb elements were sharply hypoplastic and totally un-mineralized (Fig. 1J) compared to corresponding wild type elements (Figs. 1G and 1I). Ulna and radius had undergone fusion in some embryos (Fig. 1H, arrow) and digit joints were poorly formed (Figs. 1H and 1J, arrowheads). Interestingly, the extent and overall patterns of Alcian blue staining suggested that transgenic cartilages and their extracellular matrices were actually not uniform and homogenous (Fig. 1L, arrows), while wild type cartilages displayed typical seamless matrix homogeneity (Fig. 1K).

Growth plate defects

Histologically, long bone elements in wild type E18.5 limbs had a typical organization consisting of a well-formed articular cap and joints, well-defined zones of chondrocyte maturation in the growth plates, and active enchondral ossification in the diaphysis (Figs. 2A, 2C, 2E and 2G). In contrast, transgenic elements were completely dis-organized (Figs. 2B, 2D, 2F and 2H). There was no clear histological demarcation of articular cap and joints, and typical growth plates were absent. Most if not all the chondrocytes, regardless of their localization with a given element, displayed a uniform, round and relatively small-sized morphology and shape (Figs. 2D and 2H, arrows), thus differing sharply from wild type chondrocytes displaying their characteristic and marked changes in cytoarchitecture and phenotype in different growth plate zones (Figs. 2C and 2G). Interestingly, transgenic cartilage was not clearly distinguishable from adjacent tissues, and there was common occurrence of an apparent inter-mixing of cartilage tissues with adjacent connective and vascular tissues (Figs. 2D and 2H, arrowheads). The latter probably accounts for the lack of uniform alcian blue staining seen anatomically in similar transgenic specimens above (Fig. 1L).

To verify that the above histological
defects were associated with expression of CA-LEF in cartilage, sections were processed for immunohistochemistry using antibodies to HA tag present in CA-LEF-encoding transgene. Transgenic cartilages were clearly stained by the HA antibodies, while surrounding tissues were not (Fig. 2L). In addition, every tissue in wild type specimens was negative (Fig. 2K), reaffirming specificity of expression and staining.

**Gene expression patterns**

The severe histological defects and chondrocyte cellular uniformity seen in transgenic growth plate cartilage suggested that the maturation program of chondrocytes had been markedly affected by CA-LEF expression. To characterize such possible changes, longitudinal sections of E18.5 limb skeletal elements were processed for histochemical staining with Safranin O to depict matrix proteoglycan deposition and for in situ hybridization to define the molecular cell phenotypes. Wild type specimens stained strongly with Safranin O both in their articular cap and growth plate, and low staining was seen in subadjacent endochondral bone and surrounding connective tissues as expected (Fig. 3A). In contrast, transgenic cartilages stained in a dull and substandard manner, and staining of cartilaginous masses was often not uniform (Fig. 3B, arrows). In situ hybridization showed that wild type articular cap and growth plate chondrocytes strongly expressed genes encoding cartilage-characteristic matrix macromolecules, such as collagen IX and aggrecan, and showed a prescribed reduction in expression of these genes in the hypertrophic zone (Figs. 3C and 3E). The cartilage master regulator Sox 9 displayed an identical and typical pattern (Fig. 3G), and the important signaling factor Indian hedgehog (Ihh) was expressed in the pre-hypertrophic zone (Fig. 3I). Transgenic cartilage displayed very low and scattered expression of collagen IX and aggrecan (Figs. 3D and 3F), poor expression of Sox9 (Fig. 3H), and undetectable expression of Ihh (Figs. 3J). Not surprisingly then, expression of genes associated with chondrocyte hypertrophy and endochondral ossification, that is collagen X, was completely absent in transgenic cartilage (Fig. 3L), while the gene exhibited a strong and restricted pattern in wild type (Fig. 3K).

In addition to the above defects in growth plate gene expression, we observed changes in expression of developing synovial joint genes. For example, GDF-5 transcripts were much more abundant in transgenic tissues and were present throughout the entire cartilaginous elements, including tarsal, metatarsal and phalangeal elements (Fig. 4B, arrows), while GDF-5 transcripts in wild type specimens displayed were characteristically limited to nascent joints and their interzones (Fig. 4A, arrows). Similar abnormal expression patterns were observed for two other joint markers: growth arrest specific gene 1 (GAS1) (34) and ERG (35) (Figs 4C-4F).

**Blood vessel analysis**

To further examine the mechanisms leading to tissue boundary alterations at epiphyseal ends and along the shaft of long bone elements, we processed longitudinal sections for immunostaining with antibodies to tenascin-C, a protein characteristic of developing articular cartilage and present at tissue-tissue interfaces also (25,36). Wild type E18.5 limb sections displayed strong and characteristic tenascin-C staining, with the protein decorating the epiphyseal articular layer and metaphyseal perichondrium (Figs. 5A and 5D). In transgenic sections, however, the protein was much more abundant and widely distributed in cartilage and surrounding tissues (Figs. 5B and 5E), a clear sign of chaotic tissue-tissue interfaces and relationships. Thus, we examined next the distribution of blood vessels, using antibodies to the cell surface marker CD34 (which is expressed by mesenchymal and vascular
endothelial cells) and CD31 (which is restricted to endothelial cells). In wild type sections, CD34- or CD-31-positive cells were present in peri-articular tissue, perichondrium and periosteum (Figs. 5G and 5J, arrows) and were absent from articular cap cartilage itself and most of the growth plate cartilage (Figs. 5G and 5J). There was, however, clear and well defined staining at the chondro-osseus boundary reflecting ongoing vascular invasion and endochondral bone and marrow development (Figs. 5G and 5J, arrowheads). In transgenic specimens, CD34- or CD-31-positive cells were distributed in a broader, ill defined and scattered manner (Figs. 5H-5I and 5K-5L). Some of the positive cells were in very close proximity and essentially within the cartilaginous tissue, appearing to be in the process of actively entering it (Figs. 5H and 5K, arrows). Given the nature of CD-31 and CD-34 markers, the positive cells intermingling with cartilage likely represented a mixture of vascular and mesenchymal cells.

We asked then whether abnormal vascular distribution of transgenic cartilage is associated with, and may be due to, changes in expression of genes regulating angiogenesis. ChM-1 is known as an angiogenic inhibitor and was originally identified in bovine cartilage (37). In wild type sections, ChM-1 transcripts were abundant and present from epiphyseal articular cartilage through much of the underlying growth plate, with an obvious reduction in the hypertrophic zone (Fig. 6A). ChM-1 expression was significantly reduced in transgenic specimens (Fig. 6B). We also examined the distribution of MMP-2, a protease responsible for extracellular matrix degradation during vascular formation (38). MMP-2 staining was clear and prominent in pre-hypertrophic and hypertrophic zones of wild type growth plates (Figs. 6C and 6E), but was much more prominent and actually widespread in transgenic tissue and was not restricted to cartilage only (Figs. 6D and 6F).

**Chick limb studies**

The transgenic data above indicate that continuous expression of CA-LEF in nascent chondrocytes driven by collagen II gene promoter sequences blocks further chondrocyte maturation and hypertrophy in transgenic mice, while our previous studies suggested that CA-LEF expression in cultured growth plate chondrocytes (already engaged in and pursuing maturation) accelerated their hypertrophy. The data raised the possibility that chondrocyte responses to Wnt/β-catenin signaling activation depend on the cell developmental stage/status at the moment of activation. To test this possibility, we mis-expressed a constitutive-active form of β-catenin (Δ−β-catenin) in Day 5.5 chick embryo wing cartilaginous skeletal anlagen; at this stage of embryogenesis, humerus, radius and ulna contain: diaphyseal growth plates with chondrocytes already engaged in maturation; and epiphyseal caps containing resting/pre-articular chondrocytes. We reasoned that simultaneous Δ−β-catenin mis-expression in diaphyseal and epiphyseal chondrocytes may elicit different responses and have different developmental consequences in diaphysis versus epiphysis.

Retroviral RCAS particles encoding Δ−β-catenin were microinjected in the vicinity of cartilaginous anlagen in Day 5.5 chick embryo limbs in ovo and embryos were monitored and examined over time. Longitudinal sections of control Day 11 embryos revealed that ulna had a typical elongated shape with an epiphyseal articular cap (Fig 7C), a prominent growth plate extending from metaphysis to diaphysis (Fig. 7A), and a small amount of invading bone/marrow cell population in the diaphyseal center (Figs. 7A and 7D, arrow). In Δ−β-catenin-expressing ulna, however, there was extensive ossification and marrow deposition in the diaphysis (Figs. 7B and 7F, arrow). However, the epiphyseal region had not undergone ossification and marrow deposition.
and was instead composed of seemingly immature cartilage with ill-defined boundaries and weakly staining with Safranin O (Fig. 7E, arrowheads). Joint markers genes CD44 (39) and Gli3 (40) were widely expressed (Figs. 7I and 7J), whereas they were confined to developing articular layers in control (Figs. 7G and 7H). Immunostaining with β-catenin antibodies (that recognize Δ−β-catenin as well) showed that the protein was widely distributed in epiphyseal and diaphyseal cartilage and adjacent tissues in virally-infected specimens (Figs. 7M and 7N), while it was much less abundant and restricted in distribution in control tissues as expected (Figs. 7K and 7L).

**Differential responses in immature and mature chondrocytes in vitro**

To test even more directly whether chondrocyte responses to Wnt/β-catenin signal activation are developmentally regulated, populations rich in immature or mature chondrocytes isolated from caudal and cephalic sternal regions (31) were infected with Δ−β-catenin-encoding RCAS viruses chondrocytes and were monitored over time in culture. Companion control cultures infected with insert-less viruses behaved as expected over time; the caudal cells maintained an immature and differentiated phenotype (Fig. 8A), whereas the cephalic cells displayed a hypertrophic phenotype (Fig. 8D). The responses of the two cell populations to Δ−β-catenin over-expression differed markedly. The immature cells became fibroblastic and flat-shaped (Fig. 8B), but the mature cells remained differentiated (Fig. 8E). Similar responses were seen when joint-associated Wnt-14 (29) was over-expressed in place of Δ−β-catenin (Figs. 8C and 8F). Histochemical staining showed that Δ−β-catenin over-expression reduced proteoglycan content and accumulation in both immature and mature cultures (Fig. 8G, AB), but stimulated matrix calcification in mature cultures only (Fig. 8G, AR). Again, Wnt-14 over-expression elicited similar responses (Fig. 8G).

To further characterize the cell phenotype, we determined expression of various chondrocyte markers by RT-PCR. Over-expression of Δ−β-catenin or Wnt-14 inhibited aggrecan and type IX collagen gene expression in both immature and mature chondrocytes (Figs. 9A and 9B, respectively). However, it stimulated markers of terminal chondrocyte maturation (MMP13 and VEGF) in mature cultures (Fig. 9D) consistent with data on matrix calcification (Fig. 8G, AR), but had no major effect in immature cultures (Fig. 9C). Type X collagen expression which normally decreases in the mineralizing zone of growth plate was decreased by Δ−β-catenin or Wnt14 (Fig. 9D).

To make sure that Δ−β-catenin and Wnt14 are able to activate the Wnt/β-catenin pathway in immature and mature chondrocytes equally well, control cultures and cultures over-expressing Δ−β-catenin or Wnt-14 were transfected with a promoter reporter construct for Axin-2, a direct target of that pathway (32). Basic activity of Axin-2 reporter was slightly higher in control mature than immature chondrocytes (Fig. 8H), a likely reflection of endogenous differences in ongoing signaling. Reporter activity was enhanced about 5-fold by Δ−β-catenin and about 3-fold by Wnt14 in immature and mature cultures, indicating that the two populations did possess similar Wnt/β-catenin signal activation capacities. This was confirmed by similar responses to over-expression of Wnt-3a or Wnt 8 (Fig. 8H), both of which are well known activators of Wnt/β-catenin pathway. Increases in reporter activity were all counter-acted by co-expression of dominant-negative LEF (Fig. 8H), re-affirming specificity of responses.

**MMP gene expression**

The extracellular matrix is crucial for cartilage structure and function, and thus our data...
indicating a major loss of proteoglycan content and accumulation in cultures over-expressing Δ−β-catenin or Wnt14 was of particular interest. Thus, we asked whether this response is accompanied by increases in gene expression for metalloproteases, enzymes intimately linked to matrix turn-over and remodeling (41-43). Indeed, Δ−β-catenin or Wnt14 over-expression led to sharp increases in expression of MMP-2, -7 and -9, MT3-MMP and ADAMTS-5 in both immature and mature chondrocyte cultures (Figs. 9E and 9F). MMP-3 expression was stimulated in mature chondrocytes only (Fig. 9F), while expression of MT-2-MMP was increased in mature cultures but decreased in immature cultures (Figs. 9E and 9F).

DISCUSSION

We show here that mis-expression of CA-LEF driven by type II collagen promoter/enhancer sequences causes serious defects in organization, structure, histology and molecular phenotype in skeletal elements and inhibits endochondral ossification in transgenic mice. The chondrocytes remain immature, fail to become organized in growth plates, do not undergo an orderly process of maturation, and even fail to assemble typical epiphyseal articular caps. In addition, the cartilage tissue does not establish clear and defined tissue boundaries, resulting in infiltration and invasion by surrounding tissues and abnormal distribution of blood vessels and perichondrial tissues. In contrast, when Δ−β-catenin is over-expressed in the developing chick embryo limb long bone anlagen, endochondral ossification and bone deposition are enhanced in diaphyseal regions, while immature but disorganized cartilage persists at the epiphyseal regions. The data strongly indicate that the responses of chondrocytes to Wnt/β-catenin signaling and the phenotypic and functional consequences are strictly dependent on stage/status of the cells at the moment of signal activation. This is reaffirmed by the very distinct responses to signal activation seen in cultures of chick immature and mature chondrocytes. The data correlate quite well with our previous data showing that while much of endogenous β-catenin is cytoplasmic in proliferative and pre-hypertrophic zones of growth plate, it shifts to a nuclear localization in hypertrophic chondrocytes (22). It is clear then that Wnt/β-catenin signals regulate chondrocyte behavior and function in a developmentally-regulated and maturation-dependent manner and that tight control of this signaling pathway is required for normal chondrocyte behavior and maturation and normal skeletogenesis.

Chondrocyte de-stabilization and tissue boundary loss

A prominent and obvious feature of transgenic CA-LEF-expressing cartilaginous elements is that their chondrocytes are relatively uniform in size and shape, do not display the changes in cytoarchitecture and volume characteristic of normal growth plates, and are tightly packed together, probably a result of suboptimal deposition of extracellular matrix. The tissue itself is not homogenous and not well separated from adjacent tissues. These histological and cellular alterations resemble those seen in thanatophoric dysplasia or achondroplasia (44). In terms of gene expression, the transgenic chondrocytes display poor expression of such typical genes as aggrecan, collagen IX and Sox 9, and virtually undetectable expression of IHH and collagen X. These data indicate that the transgenic chondrocytes were not only unable to mature and become hypertrophic, but were also likely to be undergoing phenotypic de-stabilization. The latter is suggested by the poor expression of Sox9, a transcription factor that acts as a master regulator of chondrocyte phenotype (45), a change also documented by Akiyama et al. (20).
In addition to poor maturation, chondrocytes in CA-LF transgenic mice express joint marker genes widely and ectopically, including GDF5, GAS1 and ERG. These genes are thought to coordinate multiple events needed for joint formation, including formation of interzone, organization of joint structures and maintenance of joint function (27,34,35,46). Our findings are consistent and agree well with recent reports in which Wnt-14 and β-catenin signaling were found to induce ectopic joint marker expression (21,29). These authors interpreted the findings to indicate that Wnt-14 and β-catenin are not only involved in joint formation, but may actually be sufficient to induce it. This conclusion, however, may be premature. In addition to inducing ectopic joint markers, over-expressed Wnt-14 and active β-catenin actually disturbed joint, particularly in the digits where the skeletal elements were fused and the chondrocytes were uniform and packed together. We observed a similar disturbance of joint formation in CA-LF-expressing transgenic cartilaginous limb elements, but did not detect de novo joint formation at ectopic sites. Thus, while Wnt/β-catenin signaling pathway is certainly important for joint formation, it may be insufficient to induce it and may depend on upstream mechanisms dictating site and timing of joint formation initiation.

For the most part cartilage is an avascular tissue, and invasion of cartilage by blood vessels is normally restricted to hypertrophic cartilage during endochondral bone deposition. Thus, lack of clear tissue boundaries and apparent intermingling of cartilage with surrounding perichondrial/connective tissues in CA-LF transgenic mice represent a major departure from normal cartilage physiology. Given the immunostaining patterns we observe, it is likely that transgenic cartilage had been invaded by CD34-positive mesenchymal cells and CD-34/CD-31 vascular endothelial cells. This abnormal process may reflect several factors, including: substandard matrix deposition which may not constitute an effective barrier; abnormally high and widespread expression of MMP-2 likely to further degrade and weaken the matrix; and deficient expression of anti-angiogenic factors such as ChM-1 calcified cartilage. Interestingly, however, the transgenic tissue does not express Indian hedgehog in appreciable amounts (Fig. 3J) and did not express vascular endothelial growth factor either (not shown). Indian hedgehog has several roles in the growth plate and one is likely to be angiogenic (47). VEGF is normally produced by hypertrophic chondrocytes and is thought to be a primary inducer of vascular invasion (48,49). Thus, it seems likely that vascularization and invasion of transgenic cartilage are not directly related to the orderly invasion normally occurring at the chondro-osseous border during endochondral ossification, since they appear to be independent of factors such as IHH, VEGF and MMP-13.

Transgenic cartilage invasion might mimic pathological situations such as those seen in joint degenerative disorders, which exhibit severe loss of cartilage matrix and tissue by itself and invasion by the surrounding tissues. In the present study we demonstrated that Δ−β-catenin over-expression strongly stimulates expression of matrix degradation enzymes such as MMPs and MT-MMPs in chondrocytes. We found also that Wnt/β-catenin signal stimulates expression of MMPs and aggrecanases in mouse chondrogenic cells (manuscript in preparation). In osteoarthritis and rheumatoid arthritis, up-regulation of variety of matrix degradation enzymes including MMPs and aggrecanases is crucial and considered responsible for cartilage matrix loss (42,50). Interestingly, we found that osteoarthritic cartilage displays signs of Wnt/β-catenin activation (submitted). Other investigators have reported that Wnt-1, an activator of β-catenin signal, and other Wnts are up-regulated in synovial cells in rheumatoid arthritis (51), and that functional variants within
sFRP3, which is an endogenous antagonist of Wnt/β-catenin signal, are associated with hip osteoarthritis in females (52). Thus Wnt/β-catenin signals may activate cartilage matrix catabolism and may have roles in cartilage destruction under pathological conditions.

Control of Wnt/β-catenin signaling and action

The data described here and previous reports by others make it quite clear that restriction, activation and modulation of Wnt/β-catenin signaling are crucial for chondrogenesis and skeletogenesis (20-23). The pathway must be kept relatively inactive for condensed mesenchymal cells to differentiate into chondrocytes and for chondrocytes to be able to assemble into well defined skeletal anlagen with characteristic growth plates. The pathway, however, must be activated to allow maturing chondrocytes to complete their development into hypertrophic mineralizing cells which in turn orchestrate their replacement with endochondral bone and marrow. What could then be possible mechanisms activating and modulating Wnt/β-catenin signaling in developing chondrocytes? It should be pointed out that a number of Wnt proteins, including Wnt 2b, Wnt 3a, Wnt 4, Wnt7b and Wnt 8, are expressed by developing chondrocytes or neighboring cells in vivo (14,18,22,53,54) and that these proteins all have the ability to activate Wnt/β-catenin signaling (53-57). In addition, there are well known extracellular components that can dampen degree of signaling by affecting Wnt binding to their receptors (58). These components include secreted forms of frizzleds (SFRPs), Dickkopf (DKK) and WIF. Interestingly, onset of SFRP3/Frzb-1 expression coincides with onset of chondrogenesis (59,60), but expression is down-regulated in the hypertrophic zone of growth plate, and Frzb-1 neutralizes the inhibition of chondrogenesis by mis-expression of Wnt 8 (22). Thus, it is possible that continuous Frzb-1 expression in developing chondrocytes may contribute to prevent Wnt action and may maintain the cells in a healthy and functional state. The cells would be able to form well-defined cartilaginous anlagen, organize growth plates and establish well-defined tissue boundaries. Frzb-1 down-regulation in the hypertrophic zone, however, would allow strong Wnt action, permitting the cells to terminally differentiate and favoring the normal transition from hypertrophic cartilage to bone.

An additional possible regulator of Wnt/β-catenin signaling pathway actions and roles is Wnt 5a. This Wnt is first expressed in cartilage anlagen and expression shifts to perichondrium with further development (16,19). Deletion of Wnt 5a gene in mice causes a phenotype quite similar to that seen in CA-LEF transgenic mice here (17). Wnt5a-null mouse embryos are dwarfs with shorter and deformed limbs, and display cartilaginous elements with low expression of Sox 9, IHH, PTH/PTHrp receptor and type X collagen and no endochondral ossification (17). Wnt 5a has been shown to inhibit Wnt/β-catenin pathway through calmodulin-dependent protein kinase II pathway or an unknown pathway that inhibits β-catenin degradation (61). These findings suggest that Wnt 5a could serve as a strong inhibitor of Wnt/β-catenin signaling during cartilage development. Indeed the limb buds of Wnt 5a-null mouse embryos display ectopic up-regulation of Wnt/β-catenin as revealed by promoter reporter activity (61). Thus, Wnt-5a may be part of normal mechanisms involving also Frzb-1 by which Wnt/β-catenin signaling is maintained relatively low at initial stages of skeletogenesis and cartilage development, and is then markedly up-regulated in hypertrophic mineralizing cartilage.

Lastly, it is important to discuss Sox 9. The factor is continuously expressed starting with pre-cartilaginous mesenchymal cells up to pre-hypertrophic chondrocytes, and is then down-regulated in hypertrophic cells (62). Sox 9...
inhibits Wnt/β-catenin signals by competing with β-catenin binding to LEF/TCF proteins, and experimental ablation of Sox 9 in cartilage causes a phenotype similar to that following chronic activation of Wnt/β-catenin pathway (20). Because Sox 9 expression precedes differentiation of mesenchymal cells into chondrocytes, this molecule also could antagonize Wnt/β-catenin signal from onset of cartilage formation up until the pre-hypertrophic stage. Terminal hypertrophic chondrocyte maturation would thus entail a down-regulation of Sox9, in coordination with down-regulations in Wnt-5a and Frzb1 and concurrent boost in Wnt/β-catenin signaling.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Skeletal structure analyses. Skeletons of E18.5 CA-LEF transgenic mouse embryos (TG; B, D, F, H, J and L) and wild type littermates (WT; A, C, E, G, I and K) were stained with Alcian blue and alizarin red. A and B, whole body view; C and D, back side view of calvaria; E and F, vertebrae and ribs; G and H, forelimb; I and J, hind limb; K and I, magnified view of hind limb.

Fig. 2. Histological analysis of limb skeletons. Longitudinal sections of fore (A-D) and hind (E-J) limb skeletons from E18.5 CA-LEF transgenic mouse embryos (TG; B, D, F, H and J) and the wild type...
littermates (WT; A, C, E, G and I) were subjected to H&E staining (A-H) or immunostaining with anti HA antibody (I and J). Bars represent 1 mm for A, B, E and F, and 0.2 mm for C, D and G-J.

**Fig. 3.** Histochemical and in situ hybridization characterizations of growth plate phenotype. Longitudinal sections of hind limbs from E18.5 CA-LEF transgenic mouse embryos (TG; B, D, F, H, J and L) or tibia from wild type littermates (WT; A, C, E, G, I and K) were subjected to Safranin O staining (A and B) or in situ hybridization for Type IX collagen (C and E), aggrecan (E and F), Sox 9 (G and H), Indian hedgehog (Ihh) (I and J) and type X collagen (K and L) genes. Bar represents 0.2 mm.

**Fig. 4.** Expression of joint marker genes. Longitudinal sections of hind limbs from E18.5 CA-LEF transgenic mouse embryos (TG; B, D and F) or metatarsals in wild type littermates (A, C and E) were subjected to in situ hybridization for GDF 5 (A and B), GAS 1(C and D) and ERG (E and F). Bar represents 0.5 mm.

**Fig. 5.** Distribution of tenascin-C, CD34 and CD31. Longitudinal sections of hind limbs from E18.5 CA-LEF transgenic mouse embryos (TG; B, C, E, F, H, I, K and L) or tibias from wild type littermate (A, D, G and J) were subjected to H&E staining (A-C) or immunostaining for tenascin-C (TN; D and E), CD34; G and H) and CD31 (J and K). C is a magnified image of boxed area in B. F, I and L are phase contrast images corresponding to E, H and K, respectively. Bar represents 0.2 mm for A, B and D-L.

**Fig. 6.** Expression and distribution of ChM-1 and MMP2. Longitudinal sections of hind limbs from E18.5 CA-LEF transgenic mouse embryos (TG; B, D and F) or tibias from wild type littermates (A, C and E) were subjected to in situ hybridization for ChM-1 (A and B) or immunostaining for MMP2 (C and D). E and F are phase contrast images corresponding to C and D, respectively. Bar represents 0.2 mm.

**Fig. 7.** Histological, histochemical and in situ hybridization analyses of chick developing limbs. Longitudinal sections of ulnas from E11 chick embryos infected with Δ−β-catenin RCAS virus (Δ−β-catenin; B, E, F, I, J, M and N) or inset-less virus (Control; A, C, D, G, H, K and L) were subjected to H&E staining (A, B, D and F), Safranin O staining (C and E), in situ hybridization for CD44 (G and H) or Gli3 (I and J), or immunostaining for β-catenin (K-N). Bars represent 0.4 mm for A and B, and 0.2 mm for C-J.

**Fig. 8.** Differential effects of Δ−β-catenin and Wnt-14 on chondrocyte phenotype and maturation. A-G, Freshly isolated immature and mature chondrocytes were infected with RCAS virus encoding Δ−β-catenin or Wnt-14, or insert-less virus (Control). After three days cells were re-plated at a density of 1.0-1.5 x 10^5 cells/cm^2 and cultured in DMEM containing 10% FBS and 10 µg/ml ascorbic acid additional 4 days. On the last day, cultures received 1mM β-glycerophosphate to induce matrix calcification. Cultures were examined by phase microscopy (A-F) and were then stained with Alcian blue (AB) or alizarin red (AR) (G). H, immature and mature chondrocytes were transfected with indicated DNA plasmids along with Axin-2 reporter DNA 2 days after plating, and luciferase activity was measured 2 days later.

**Fig. 9.** Gene expression in chondrocyte cultures. Freshly isolated immature (A, C and E) and mature
chondrocytes (B, D and F) were infected with RCAS viruses encoding Δ−β-catenin or Wnt-14 or with insert-less virus (Control). Three days later, cells were re-plated at a density of 1.0-1.5 x 10^5 cells/cm^2 and cultured in DMEM containing 10% FBS and 10 µg/ml ascorbic acid for an additional 4 days. Total RNAs were subjected to RT-PCR analysis for chondrocyte phenotypic markers (A and B), markers of maturation (C and D) and matrix degradation enzymes (E and F).
Figure 7

Control vs. Δ-β-catenin
Developmental regulation of Wnt/β-catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification
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