Wnt/β-Catenin and Retinoic Acid Receptor Signaling Pathways Interact to Regulate Chondrocyte Function and Matrix Turnover* [S]

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Activation of the Wnt/β-catenin and retinoid signaling pathways is known to tilt cartilage matrix homeostasis toward catabolism. Here, we investigated possible interactions between these pathways. We found that all-trans retinoic acid (RA) treatment of mouse epiphysial chondrocytes in culture did increase Wnt/β-catenin signaling in the absence or presence of exogenous Wnt3a, as revealed by lymphoid enhancer factor/T-cell factor/β-catenin reporter activity and β-catenin nuclear accumulation. This stimulation was accompanied by increased gene expression of Wnt proteins and receptors and was inhibited by co-treatment with Dickkopf-related protein-1, an extracellular inhibitor of Wnt/β-catenin signaling, suggesting that RA modulates Wnt signaling at Wnt cell surface receptor level. RA also enhanced matrix loss triggered by Wnt/β-catenin signaling, whereas treatment with a retinoid antagonist reduced it. Interestingly, overexpression of retinoic acid receptor γ (RARγ) strongly inhibited Wnt/β-catenin signaling in retinoid-free cultures, whereas small interfering RNA-mediated silencing of endogenous RARγ expression strongly increased it. Small interfering RNA-mediated silencing of RARα or RARβ had minimal effects. Co-immunoprecipitation and two-hybrid assays indicated that RARγ interacts with β-catenin and induces dissociation of β-catenin from lymphoid enhancer factor in retinoid-free cultures. The N-terminal domain (AF-1) of RARγ but not the C-terminal domain (AF-2) was required for association with β-catenin, whereas both AF-1 and AF-2 were necessary for inhibition of β-catenin transcriptional activity. Taken together, our data indicate that the Wnt and retinoid signaling pathways do interact in chondrocytes, and their cross-talks and cross-regulation play important roles in the regulation of cartilage matrix homeostasis.

Growth plate cartilage and hyaline cartilage have essential roles in skeletal growth and long term function. Growth plate cartilage mediates the formation and elongation of most skeletal elements during prenatal and early postnatal life via endochondral ossification, including vertebral bodies, cranial base, and long bones (1, 2). During this process, growth plate chondrocytes undergo a process of maturation during which the cells first proliferate and then enlarge and become hypertrophic, mineralize their extracellular matrix, and are finally replaced by bone and marrow. As the chondrocytes mature, they change their production of extracellular matrix that is mainly composed of aggrecan and collagen I and XI in upper growth plate zones but becomes enriched with collagen X and other macromolecules in the hypertrophic zone. In comparison with the transient nature of growth plate cartilage, hyaline permanent cartilage persists throughout life at important locations, such as joints and tracheal rings. This tissue also has a very abundant and unique extracellular matrix that is maintained in a stable and functional composition and structure by chondrocyte activity. It is well established that the extracellular matrix in both hyaline and growth plate cartilage is regulated by a fine balance between synthetic and catabolic mechanisms that are important for both maintenance of permanent cartilage function and progression of endochondral bone formation. Indeed, imbalances in such homeostatic pathways can lead to a variety of pathological skeletal conditions that include osteoarthritis and skeletal dysplasias (3–5).

In recent studies, we found that Wnt/β-catenin signaling can strongly affect matrix anabolic and catabolic metabolism in chondrocytes (6). Wnt/β-catenin signaling is a major signal transduction pathway of Wnt proteins (7, 8). Binding of Wnt proteins to cell surface receptor complexes composed of Frizzleds and low density lipoprotein receptor-related protein 5/6 (LRP5/6) activates the downstream intracellular protein Dishevelled. This results in inhibition of glycogen synthase 3β (GSK3β) kinase and β-catenin phosphorylation, thus allowing β-catenin to escape the ubiquitin proteasome degradation pathway and to accumulate in the cytoplasm. Non-phosphorylated β-catenin then translocates to the nucleus, where it interacts with lymphoid enhancer factor/T-cell factor (LEF/TCF) (5).

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© The abbreviations used are: LEF, lymphoid enhancer factor; TCF, T-cell factor; RAR, retinoic acid receptor; RA, all-trans-retinoic acid; rWnt3a, recombinant Wnt3a; ANT, RAR antagonist; HA, hemagglutinin; siRNA, small interfering RNA.
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transcription factors to regulate target gene expression. We found that acute activation of Wnt/β-catenin signaling strongly inhibited gene expression of aggrecan and collagens II and IX and stimulated gene expression and activity of matrix proteases, resulting in matrix loss in vitro and in vivo. Subsequent studies by several groups, including ours, showed that in addition to matrix homeostasis, Wnt/β-catenin signaling normally regulates other chondrocyte functions and influences cartilage development (3, 9–13). What has remained unclear, however, is how Wnt/β-catenin signaling is regulated and modulated during cartilage development, skeletal growth, and skeletal homeostasis and is able to regulate such variety of important processes.

Studies have shown that the Wnt/β-catenin pathway can influence the function of nuclear receptor proteins and affect important biological and pathological processes with broad physiological and clinical relevance (14). Nuclear retinoic acid receptors (RARs) are also positively or negatively affected by Wnt/β-catenin signaling in a variety of cell types, such as colon and breast cancer cells, neuronal cells, and ES cells (15–22). Retinoid signaling has long been known to regulate cartilage development and skeletogenesis (23–28). Genes encoding RARα, RARβ, and RARγ display specific spatiotemporal patterns of expression during cartilage formation in the developing limb (29–31). RARγ and RARα expression is predominant in perichondrially mesenchyme and newly formed cartilage, and RARβ is expressed in perichondrium, and chondrogenic cell differentiation is found to require repressor function of unliganded RARα (29). In good correlation, ablation of genes encoding RARs (32) or enzymes involved in retinoid synthesis or degradation causes a spectrum of skeletal abnormalities (33, 34). In addition, studies in vivo and in vitro have shown that retinoic acid (RA) has strong effects on cartilage matrix homeostasis that includes inhibition of matrix synthesis (35, 36), stimulation of matrix degradation (37, 38), and stimulation of chondrocyte terminal differentiation and calcification (23–25). In sum, the above studies clearly indicate that retinoid signaling is very important for skeletogenesis and progression of endochondral ossification and that dysregulation of this signaling pathway can lead to skeletal aberrations and cartilage degeneration.

Because both retinoid signaling and Wnt/β-catenin signaling strongly affect cartilage development and homeostasis, we tested here whether these pathways actually interact to regulate and modulate chondrocyte function and phenotype. Our results show that this is indeed the case and that RARγ has a key role in these mechanisms.

EXPERIMENTAL PROCEDURES

Chondrocyte and Limb Bud Cell Cultures—Mouse primary epiphyseal chondrocytes were isolated from neonatal C57BL/6 mice as previously described (6). Distal cartilaginous ends of femurs and humeri from neonatal mice were digested by 0.25% trypsin and 2 mM EDTA for 15 min, followed by digestion with 2 mg/ml collagenase type I (Worthington) for 6 h. Cells were plated at 40,000 cells/well in 96-well plates and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (GEMINI, West Sacramento, CA). Cultures were treated with recombinant Wnt3a (rWnt3a) (Chemicon, Temecula, CA), recombinant mouse Dickkopf-related protein 1 (R&D Systems Inc., Minneapolis, MN), RA (Sigma), RAR antagonist (ANT) (synthetic retinoid pan-antagonist AGN/VTP 194310 provided from Allergan Pharmaceuticals and Vitae Pharmaceuticals), or 6BIO (BIO, Enzo Life Sciences International Inc., Plymouth Meeting, PA). For detection of sulfated proteoglycans associated with the cell layer, cultures were fixed with 10% formalin for 10 min and incubated with 70% ethanol for 5 min, followed by incubation with 5% acetic acid (pH 1.0) for 5 min and stained with 1% Alcian blue (Electri Sci Microscopy Science, Hartfield, PA) for 2 h at room temperature. Staining levels were quantified by Image J software. Limb mesenchymal cells were isolated from the fore and hind limbs of embryonic day 10.5 mouse embryos by incubation with 0.25% trypsin and 2 mM EDTA for 15 min. Cells were plated at 20,000 cells/well in 96-well plates and maintained in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 containing 2% fetal bovine serum. Cells were used for a Wnt/β-catenin reporter assay immediately after isolation and did not express chondrogenic characteristics. Freshly isolated limb mesenchymal cells were also inoculated at 2.5 × 10^3 cells/20 μl and cultured in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 containing 2% fetal bovine serum and 100 ng/ml recombinant BMP2 for 7 days to induce chondrogenic differentiation. The cells were then dissociated by digestion with 0.15% collagenase for 15 min, replated at 40,000 cells/well in 96-well plates, and used for the Wnt/β-catenin reporter assay.

Reverse Transfection and Reporter Assays—Reverse transfection of DNA plasmid was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Freshly isolated chondrocytes or limb bud cells were plated at an initial density of 4 × 10^4/well or 2 × 10^4/well, respectively, into 96-well plates that had been coated with a Wnt/β-catenin reporter plasmid (Super 8x TOPFlash, Addgene Inc., Cambridge, MA) in the presence of Lipofectamine 2000. After 24 h, cultures were treated with RA, RAR antagonist rWnt3a, 6BIO, or rDKK-1. To normalize transfection efficiency, we co-transfected the Renilla luciferase-expressing plasmid (pRL-TK-luc) as internal control, and luciferase activities of both Super 8x TOPFlash and pRL-TK-luc reporters were measured using a dual luciferase assay kit (Promega Corp., Madison, WI). Super 8x TOPFlash encodes seven copies of mutated LEF/TCF binding sites linked to firefly luciferase and reflects Wnt/β-catenin signaling activity. Super 8x FOPFlash has seven copies of mutated LEF/TCF binding sites and was used as negative internal control. A mammalian two-hybrid system was used to examine protein-protein interactions using the CheckMate Mammalian two-hybrid system (Promega) according to the manufacturer’s protocol. Gal4/β-catenin expression vectors were described previously (16), and VP16/LEF-1 and dominant negative form expression vector were kindly provided by Dr. A. Hecht (Albert-Ludwigs University). Mouse RARα (American Type Culture Collection (ATCC) (Manassas, VA) catalog number 10324162, GenBank™ ID NM_009024), RARβ (ATCC catalog number 10699549, GenBank™ ID NM_011243), or RARγ (ATCC catalog number MGC-11555, GenBank™ ID
NM_011244) were tagged with hemagglutinin (HA) by subcloning at the EcoRI and KpnI sites of pCMV-HA vector (Clontech/Takara, Mountain View, CA). pCMV-HA empty vector was used as control.

**Construction of RARγ Mutants**—Mouse RARγ1 cDNA was purchased from the ATCC, and deletion mutants were generated by PCR. PCR primer pairs for the deletion mutant of the AF-1 domain (718–1833 of NM_011244) were 5′-CCGAATTCCACAAGCCATGCTTTGTATGCAATGAC-3′ and 5′-GGGGTACCTCAGGGCCCTTGTCAGGT-3′, and those for the deletion mutant of AF-2 domain (457–1701 of NM_011244) were 5′-CCGAATTCACATGGCCAACAATAAAGGAGAGACT-3′ and 5′-GGGTACCTCACTTCTCGGATGACGGGTGG-3′. The resulting PCR products were cloned into pCMV-HA vector at EcoRI and KpnI sites.

**Silencing of RARs**—Mouse RARα-, β-, and γ-siRNA pools and non-targeting siRNA (control siRNA) were purchased from Dharmacon (Chicago, IL). siRNA for each RAR (1 pmol) and control siRNA (1 pmol) (to exclude potential off-target effects caused by siRNA) were co-transfected with Super 8x TOPFlash reporter plasmid and pRL-TK-luc into freshly isolated mouse chondrocytes seeded at an initial density of 4 × 104/well in a 96-well plate by reverse transfection using Lipofectamine 2000.

**RNA Isolation and Gene Expression Assay**—Total RNA was isolated by the guanidine isothiocyanate method as previously described (24). The resulting reverse transcription mixture was used for a reverse transcription-PCR or quantitative PCR assay. Real-time PCR was performed with an Applied Biosystems 7900HT sequence detection system running SDS 2.1 software using SYBR Green (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. The average threshold cycle value (Ct value) was calculated from 4-fold reactions. Averaged Ct values were then normalized to the averaged Ct value of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Standard curves were generated using 10-fold serial dilutions of cDNA of each gene with a correlation coefficient of >0.98. Relative expression levels were calculated based on standard curves and represented ratios of experimental over control values. Primer sequences for real-time PCR amplification were as follows: 5′-CTG AGG ACT TTC CAG GTG TTG ACT CAA G-3′ and 5′-TGG TTC TGC CAT AGC ACA TGC TGA AC-3′ for 1362–1623 of mouse matrix metalloproteinase 3 (Mmp3) 5′-TCA GTT TCT TTA TGG TCC AGG CGA TG-3′ and 5′-TCA GTC TCT TCA CCT TTT GGA GTA CTC-3′ for 799–1160 of mouse Mmp13, 5′-TCT GGA AAT GAC AAC CCC AAG CAC A-3′ and 5′-GCG TAA CCA TGC CCA CCC TGG AAC T-3′ for 5463–5939 of mouse aggrecan, and 5′-AAG CCC ATC ACC ACC TTC CAG GAG-3′ and 5′-ATG AGC CCT TCC ACA ATG CCA AAG-3′ for 258–568 of glyceraldehyde-3-phosphate dehydrogenase, using proper filters to visualize fluorescent markers.

To profile changes in gene expression of Wnts and Wnt-related molecules by RA treatment, we carried out a PCR array using the RT2 Profiler PCR array for the Wnt signaling pathway (Superarray, Frederick, MD) following the manufacturer’s protocol. The average threshold cycle value (Ct value) was calculated from 4-fold reactions and normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. These experiments were repeated three times independently.

**Immunoblot and Co-immunoprecipitation**—Cellular levels of β-catenin were analyzed by immunoblotting. Cell lysates containing equal amounts of proteins were separated on 10% SDS-polyacrylamide gels and electrotransferred onto Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.1% Tween 20 and subjected to immunoblotting with antibodies against dephosphorylated β-catenin (Alexis, Lausen, Switzerland) or α-tubulin (Sigma), followed by incubation with secondary antibody conjugated with horseradish peroxidase. Protein bands were detected by means of Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). For co-immunoprecipitation, 3 × 10⁶ cells COS7 cells were plated into a 100-mm dish and transfected with HA-tagged RARγ or deletion mutants of RARγ1. After 48 h, cultures were washed twice with ice-cold phosphate-buffered saline and lysed in 1% Nonidet P-40 lysis buffer (20 mM HEPES, pH 7.5, 10 mM EGTA, 2.5 mM MgCl₂, 40 mM β-glycerophosphate, 1% Nonidet P-40, 2 mM α-NaO₄, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 1 mM dithiothreitol). Cell lysates were incubated on ice for 20 min and then centrifuged at 150,000 rpm for 10 min, and supernatants were kept at −80°C until use. Cell lysates were incubated with IgG beads (Roche Applied Science) at 4°C overnight and then incubated with anti-HA antibody (Roche Applied Science) for 1 h at 4°C, followed by incubation with IgG beads at 4°C for 1 h. Bound proteins were released by incubation with 3× SDS sample buffer for 5 min at 70°C. Immunoprecipitated and co-immunoprecipitated proteins were analyzed by immunoblotting.

**Immunofluorescence Staining**—Cells were plated at 30 × 10⁴/well onto 12-mm round glass coverslips (Hecht Assistent, Sondheim, Germany) coated with 1% gelatin solution in a 24-well plate. After 24 h, cells were fixed with 10% neutralized formalin, permeabilized with 0.5% Triton X-100, and incubated with primary β-catenin antibody (BD Biosciences) overnight at 4°C, followed by incubation with Alexafluor 594 dye-conjugated secondary antibody (Invitrogen) for 30 min. Cultures were also stained with 0.1 mg/ml 4′,6-diamidino-2-phenylindole (Sigma) for nuclear staining. Samples were mounted with GEL/MOUNT (Biomed, Foster City, CA) and analyzed under a fluorescent microscope (Nikon Eclipse TE2000-U). Images were captured and analyzed using ImagePro Plus 5.0 software.

**Statistical Analysis**—One-way analysis of variance followed by Boneferroni/Dunn post hoc multiple comparison tests (Prism 5, GraphPad Software Inc., La Jolla, CA) was used to determine statistical significance between groups. p values less than 0.01 were considered significant (*, p < 0.01 as indicated by brackets).

**RESULTS**

**Wnt/β-Catenin Signaling Is Modulated by Retinoid Agonists and Antagonists**—We first tested whether Wnt/β-catenin signaling in chondrocytes is affected by retinoid agonists and/or antagonists. Chondrocytes freshly isolated from neonatal mouse epiphyseal cartilage were transfected with the Wnt/β-
catenin reporter plasmid Super 8x TOPFlash and treated with different concentrations of (i) RA, which is the most potent natural retinoid agonist; (ii) the retinoid panantagonist AGN/VTP 194310 (ANT), which blocks the function of all RARs; or (iii) vehicle (EtOH). Reporter activity was dose-dependently increased by RA treatment (Fig. 1A) but was greatly inhibited by retinoid antagonist treatment (Fig. 1B). Transfection of control Super 8x FOPFlash reporter plasmid that encodes mutated LEF/TCF binding sites elicited no response (Fig. 1A, FOPFlash).

Transfection of control Super 8x FOPFlash reporter plasmid that encodes mutated LEF/TCF binding sites elicited no response (Fig. 1A, FOPFlash). The effects of RA and retinoid antagonist on Wnt/β-catenin signaling were also examined by monitoring β-catenin nuclear accumulation, a well recognized trait of Wnt/β-catenin signaling activation, using immunocytochemistry. Nuclear accumulation of β-catenin was indeed increased by RA treatment and decreased by retinoid antagonist treatment compared with controls (Fig. 1C).

To determine how rapidly RA and retinoid antagonist change β-catenin phosphorylation and stabilization and Wnt/β-catenin signaling activity, the levels of unphosphorylated β-catenin was examined at 3 or 24 h of RA treatment. Immunoblot analysis revealed that RA treatment did not significantly increase β-catenin content by 3 h (Fig. 1D, 3h, lane 2), although Wnt3a treatment had done so with or without RA treatment (Fig. 1D, 3h, lanes 4 and 5). RA treatment did increase β-catenin content by 24 h (Fig. 1D, 24h, lane 2) and further enhanced it during Wnt3a co-treatment (Fig. 1D, 24h, lane 5). Interestingly, treatment with retinoid antagonist inhibited β-catenin accumulation during Wnt3a treatment (Fig. 1D, 24h, lane 6).

The effects of RA and retinoid antagonist on Wnt/β-catenin signaling were also validated in chondrocyte cultures isolated from TOPGAL Wnt/β-catenin reporter transgenic mice that express β-galactosidase linked to LEF/TCF binding site-containing promoter ele-
FIGURE 2. RA enhances and retinoid antagonist inhibits Wnt/β-catenin signaling effects on matrix accumulation and gene expression of aggrecan, Mmp13, and Mmp3. Epiphyseal chondrocytes were treated with EtOH (A and D), 300 nM RA (B and E), or 100 nM ANT (C and F) plus 100 ng/ml rWnt3a (D–F) or vehicle for 48 h. Cultures were stained with Alcian blue (A–F), and staining intensity was measured by image analysis (G). Total RNA was prepared from those cultures and subjected to semiquantitative real-time PCR analysis of aggrecan (H), Mmp13 (I), or Mmp3 (J) transcript levels. Values represent averages and S.D. obtained from three independent samples. *, p < 0.01.

Wnt/β-catenin signaling induction by Wnt3a in these cultures (Fig. 1G, closed column), whereas RA enhanced Wnt3a effects in parallel chondrocyte cultures (Fig. 1F, closed column), suggesting that interactions and effects of retinoid and Wnt/β-catenin signaling pathways differ in mesenchymal prechondrogenic cells versus differentiated chondrocytes. To verify this conclusion, freshly isolated limb bud cells were grown at high cell density and treated with BMP-2, a combination that greatly stimulates their chondrogenic differentiation (40). Once the cultures had differentiated and produced cartilage nodules, the cells were dissociated by collagenase treatment, replated, and processed for Wnt/β-catenin reporter assays. The cells now exhibited responses to RA and retinoid antagonist similar to those of epiphyseal chondrocytes. Wnt3a treatment stimulated reporter activity (Fig. 1H, open column), and co-treatment with RA further stimulated it (Fig. 1H, closed column), whereas retinoid antagonist treatment decreased reporter activity (Fig. 1H, striped column). These results indicate that the responses to Wnt/β-catenin and retinoid signaling pathways change during chondrogenic cell differentiation.

Retinoid Agonists and Antagonists Alter Wnt/β-Catenin Signaling Effects on Proteoglycan Metabolism—To determine whether modulation of Wnt/β-catenin signaling by retinoid agonists and antagonists alters chondrocyte phenotypic function, we treated chondrocyte cultures with RA or antagonist without or with Wnt3a co-treatment and then monitored proteoglycan accumulation by Alcian blue staining (Figs. 2, A–F). Treatment with RA or Wnt3a alone decreased proteoglycan content compared with controls (Fig. 2, A, B, and D). Notably, treatment with retinoid antagonist increased proteoglycan content in the absence or presence of Wnt3a (Fig. 2, C and F, respectively). Computer-assisted quantification of staining intensity confirmed visual assessments (Fig. 2G). To relate proteoglycan content to proteoglycan synthesis and degradation, we examined gene expression of aggrecan and matrix proteases. Both RA and Wnt3a inhibited gene expression of aggrecan (Fig. 2H) and up-regulated expression of Mmp13 and Mmp3 (Fig. 2, I and J), and co-treatment with RA and Wnt3a enhanced these effects (Fig. 2, H–J). In good agreement with Alcian blue staining data, retinoid antagonist treatment stimu-
Inhibition of RA-stimulated Wnt/β-catenin signaling by DKK-1. A and B, epiphyseal chondrocytes were treated with or without 300 nM RA for 24 h, and RNA from each population was subjected to PCR array analysis. Values represent expression levels of Wnt proteins (A) and receptors/co-receptors (B) relative to glyceraldehyde-3-phosphate dehydrogenase. Wnt genes whose expression level was below 0.01 are not listed.

C, chondrocyte cultures were transfected with Super 8x TOPFlash plasmid and then treated with EtOH, 300 nM RA, 20 ng/ml Wnt3a, or 0.5 μg/ml BIO with (closed column) or without (open column) 2 μg/ml DKK-1. DKK-1 was added 30 min before treatment with Wnt3a. Reporter luciferase activity was measured 24 h after Wnt3a treatment. Values represent averages and S.D. obtained from three independent samples. *, p < 0.01.

D–I, cultures were treated with EtOH (D and G) or 300 nM RA with (E and H) or without (E and H) 2 μg/ml DKK-1 for 24 h and stained with β-catenin antibodies to examine the β-catenin nuclear levels (D–F). G–J are the phase contrast images corresponding to D–F, respectively.

Up-regulation of Wnt Proteins, Receptors, and Co-receptors by RA—To determine how RA stimulates Wnt/β-catenin signaling, we examined the gene expression levels of Wnt proteins, receptors, and modulators. PCR array analysis clearly revealed that RA treatment significantly enhanced gene expression of Wnt2b and Wnt5a in cultured chondrocytes and slightly increased Wnt4, Wnt5b, and Wnt9a, whereas expression levels of Wnt4, Wnt5b, and Wnt9a were very low (Fig. 3A). Interestingly, we also found strong up-regulation of Fzd-8, Lrp-5, and Lrp-6 by RA treatment (Fig. 3B). Among the Wnts that were up-regulated by RA, Wnt2b, Wnt4, and Wnt9a are known to stimulate Wnt/β-catenin signaling (3, 8, 41); in addition, Fzd-7 and Fzd-8 transmit both β-catenin-independent canonical and non-canonical signals, and Lrp5 and Lrp6 are essential co-receptors for Wnt/β-catenin signaling. To determine the significance and implications of these changes in gene expression, we treated chondrocyte cultures with Dickkopf-related protein 1 (DKK-1), an extracellular inhibitor of the Wnt/β-catenin signaling pathway that binds co-receptor LRP5/6 and disturbs Wnt signal transduction through Frizzled receptors (42). Treatment with DKK-1 completely abolished the increase in Wnt/β-catenin signaling induced by Wnt3a, as revealed by reporter activity (Fig. 3C, Wnt3a). When the cultures were treated with the GSK3β inhibitor BIO, reporter activity was strongly increased (Fig. 4A, BIO), reflecting the well known fact that inhibition of GSK3β favors β-catenin accumulation and signaling. Indeed, DKK-1 treatment had no effects on BIO-induced stimulation of reporter activity (Fig. 3C, BIO), verifying the fact that DKK-1 blocks Wnt/β-catenin signaling at the cell surface receptor level and not the cytoplasmic β-catenin stabilization level. The inhibition of RA action by DKK-1 was also tested by examination of β-catenin nuclear accumulation (Fig. 3, D–I). RA treatment increased the number of chondrocytes displaying strong β-catenin nuclear staining (Fig. 3E), whereas control cultures displayed weak staining (Fig. 3F). DKK-1 co-treatment strongly counteracted the effects of RA treatment (Fig. 3F). Together, the above data suggest that RA stimulates Wnt/β-catenin signaling mainly by modulating expression of Wnt receptors and co-receptors.

Inhibition of Wnt/β-catenin Signaling by RARγ—Previous studies indicated that nuclear receptor proteins interact with β-catenin and can positively or negatively regulate β-catenin transcriptional activity. RARs was found to physically interact with β-catenin and inhibit β-catenin signaling in retinoid agonist-treated cells (16). We previously found that RARγ is the major RAR expressed in growth plate chondrocytes (23, 43). Thus, we asked whether RARγ is the main modulator of Wnt/β-catenin signaling in chondrocytes. Chondrocytes were co-transfected with a Wnt/β-catenin reporter plasmid and expression vectors encoding RARα, RARβ, RARγ, or empty vector as a control. The cells were maintained in retinoid-free medium.
increased Wnt/β-catenin reporter activity in control cultures (Fig. 4A), but this response was reduced by nearly 50% by RARγ overexpression (Fig. 4A). Overexpression of RARα or RARβ was far less effective (Fig. 4A).

To test whether endogenous RARγ acts in a similar manner, we measured Wnt reporter activity during siRNA-mediated silencing of RARγ. Wnt/β-catenin reporter activity was dramatically increased in RARγ siRNA-treated cultures with or without Wnt3a co-treatment (Fig. 4B), whereas silencing of RARα or RARβ had minimal effects (Fig. 4B). Effectiveness and specificity of siRNA on RAR expression were confirmed by semiquantitative PCR (Fig. 4C).

RARγ Associates with β-Catenin and Inhibits β-Catenin Association with LEF—Next, we examined the mechanism by which RARγ inhibits Wnt/β-catenin signaling. Because RARα was found to associate with β-catenin during RA treatment and to affect β-catenin transcriptional activity (16), we asked whether RARγ also associates with β-catenin. COS7 cell cultures were transfected with HA-tagged RARγ and treated with BIO to increase β-catenin levels; some of the cultures were co-treated with RA. Cell homogenates were immunoprecipitated with anti-HA antibodies (IP: HA in Fig. 5) or control IgGs (IP: IgG), followed by immunoblot with anti-β-catenin (WB: β-catenin) or anti-HA antibodies (WB: HA). In cultures not receiving RA, RARγ associated with β-catenin as indicated by co-immunoprecipitation with anti-HA antibodies (Fig. 5A, lane 2), but not control IgGs (Fig. 5A, lane 1). Interestingly, in cultures treated with RA, the RARγ-β-catenin complexes were undetectable (Fig. 5A, lane 3), suggesting that complex formation occurred only in retinoid-free cultures and involved unliganded RARγ. Note that BIO treatment was necessary to observe RARγ-β-catenin complex formation (Fig. 5C, lanes 2 and 3), indicating that detection of such complexes in untreated cultures may require more sensitive methods.

To examine the domains of RARγ that are important for association with β-catenin, we tested RARγ mutants lacking the N-terminal domain (AF-1) or C-terminal domain (AF-2) (Fig. 5B) and carried out similar co-immunoprecipitation experiments. Deletion of AF-1 completely hampered the ability of RARγ to associate with β-catenin (Fig. 5C, lane 4), whereas deletion of AF-2 domain had no effect (Fig. 5C, lane 5).

Next, we asked whether the association of RARγ with β-catenin affects β-catenin interactions with LEF/TCF. We carried out two-hybrid assays using a constitutively active form of β-catenin linked to Gal4 (Gal4-CA-β-catenin; αα in Fig. 6) and LEF1 linked to VP16 (VP16-LEF; LEF in Fig. 6) (16). When chondrocytes were transfected with these two plasmids together with a Gal4-sensitive luciferase reporter plasmid, luciferase activity was markedly increased as expected (Fig. 6A, column 4), reflecting interactions of Gal4-CA-β-catenin with Gal4 binding sites on the luciferase VP16 reporter and transcriptional trans-activation. Companion cells transfected with control VP16 empty vector (con) or a dominant-negative form of LEF fused to VP16 (dnLEF) exhibited background luciferase activity (Fig. 6A, columns 1–3 and 5), verifying trans-activation activity of Gal4-CA-β-catenin. Chondrocytes were then transfected with Gal4-CA-β-catenin and VP16-LEF plasmids plus plasmids encoding full-length RARγ (γfull in Fig. 6) or γΔAF1

**Figure 4. Effects of RARs on Wnt/β-catenin signaling activity.** A, epiphyseal chondrocytes were transfected with Super 8x TOPFlash and RARα, RARβ, or RARγ expression plasmids and were then treated with 100 ng/ml Wnt3a for 24 h after transfection; luciferase activity was measured after an additional 24 h. B, chondrocyte cultures were transfected with siRNA for RARα, RARβ, or RARγ or control random siRNA (Con) and Super 8x TOPFlash plasmid and treated with 100 ng/ml Wnt3a for 24 h after transfection; luciferase activity was measured after an additional 24 h. C, total RNAs were prepared from cultures transfected with siRNA for RARα, RARβ, or RARγ or control random siRNA and subjected to semiquantitative real-time PCR analysis of RARα, RARβ, and RARγ transcript levels. Values are means ± S.D. obtained from three independent samples. *, p < 0.01; **, p < 0.05.

conditions in which RARs are largely unliganded. Overexpression of RARα, RARβ, and RARγ was comparable in each culture (data not shown). Treatment with Wnt3a strongly
or \( \gamma \Delta AF2 \) deletion mutants. RAR\( \gamma \) overexpression diminished reporter activity elicited by Gal4-CA-\( \beta \)-catenin and VP16-LEF (Fig. 6A, column 6). In contrast, expression of \( \gamma \Delta AF1 \) deletion mutant did not inhibit reporter activity but actually enhanced it (Fig. 6A, column 7), and expression of \( \gamma \Delta AF2 \) partially decreased reporter activity moderately (Fig. 6A, column 8).

These results indicate that RAR\( \gamma \) interferes with association of \( \beta \)-catenin with LEF and that both AF1 and AF2 domains are required for inhibition. Thus, we asked whether the AF1 and AF2 domains are actually involved in inhibition of Wnt/\( \beta \)-catenin signaling. We found that both deletion mutants failed to inhibit Wnt3a-induced reporter activity (Fig. 6B, columns 7 and 8), whereas full-length RAR\( \gamma \) did so (Fig. 6B, column 6).

**DISCUSSION**

In this study, we show that the Wnt/\( \beta \)-catenin and retinoid signaling pathways interact and affect matrix homeostasis and phenotypic expression in chondrocytes. We find that RA stimulates Wnt/\( \beta \)-catenin signaling, increases gene expression of Wnt proteins and receptors, and enhances the inhibitory effects of Wnt/\( \beta \)-catenin signaling on matrix accumulation (Fig. 7A). In contrast, overexpression of RAR\( \gamma \) inhibits Wnt/\( \beta \)-catenin signaling in chondrocyte cultures maintained under retinoid-free conditions, and silencing of endogenous RAR\( \gamma \) expression strongly stimulates \( \beta \)-catenin transcriptional activity (Fig. 7B). Furthermore, we present evidence that RAR\( \gamma \)
interacts with β-catenin in retinoid-free cultures but does not do so in RA-treated cultures. Treatment with retinoid antagonist counteracts the ability of Wnt3a to inhibit matrix accumulation and aggrecan expression. Taken together, our findings suggest that RARγ can regulate Wnt/β-catenin signaling in chondrocytes positively or negatively depending on retinoid ligand availability. RARγ enhances Wnt/β-catenin signaling in retinoid-free conditions but inhibits it in RA-treated cells (Fig. 7).

Regulation in Chondrocyte Differentiation and Maturation—Studies have indicated that unliganded RARα represses transcriptional activity of target genes and promotes chondrogenic differentiation of mesenchymal cells in the developing limb (44, 45). Our findings indicate that RARγ interacts with β-catenin in retinoid-free cultures and inhibits the antichondrogenic effects of Wnt/β-catenin signaling. We recently reported that conditional ablation of RARγ and RARβ genes in cartilage leads to growth retardation (43). In these compound mutants, the prehypertrophic zone of growth plate displayed lower amounts of proteoglycans compared with wild type growth plates. Gene silencing of RARγ also inhibited proteoglycan synthesis in chondrocyte cultures maintained under retinoid-free conditions (43). Analysis of RARE reporter mice previously showed that retinoid signaling is undetectable in upper zones of growth plate but increases in the hypertrophic zone (46). Together with findings in this study, it is likely that unliganded RARγ decreases Wnt/β-catenin signaling in upper growth plate zones and sustains strong proteoglycan expression and accumulation typical of those zones. The molecules participating in these mechanisms could include direct and indirect targets of Wnt/β-catenin signaling, such as aggrecan, MMPs, twist-1, and other matrix and regulatory genes (13, 47).

It was suggested recently that Wnt/β-catenin signaling is involved in pathologies of articular cartilage. Genetic studies showed that mutation of FRZB, a Wnt antagonist, is linked to incidence of hip arthritis in certain patient cohorts (48, 49). Increased Wnt/β-catenin signaling activity has been documented in human osteoarthritic cartilage (50, 51) and OA animal models (6). In addition, acute experimental activation of Wnt/β-catenin signaling stimulates catabolic action and cartilage matrix degradation (6) and induces degeneration of articular cartilage (51). Thus, increased Wnt/β-catenin signaling could enhance articular cartilage degeneration, and inhibition of this pathway could represent a new way to prevent or treat joint degenerative diseases. Our results suggest that a retinoid antagonist may inhibit Wnt/β-catenin signaling at the level of Wnt proteins and receptors. Indeed, systemic application of a retinoid antagonist was found to decrease the degree of joint degenerative changes in collagen- or bacteria-induced arthritis (52).

Stimulation of Wnt/β-Catenin Signaling by Retinoid Signaling—Retinoid signaling has been found to be a potent inhibitor for Wnt/β-catenin signaling in colon and breast cancer cells (14–17). Such inhibition is believed to occur through physical interaction of β-catenin with RARα and to occur preferentially when retinoid ligands are present in the tissues (15–17). Our results show that retinoid signaling also inhibits Wnt/β-catenin signaling in mesenchymal cell cultures but stimulates it in chondrocyte cultures, indicating that interrelations between these two signaling pathways are differentiation-dependent and change during the transition from undifferentiated mesenchymal cells to differentiated chondrocytes. This transition is known to involve changes in RAR expression; mesenchymal cells mostly express RARα and RARβ, whereas chondrocytes mainly express RARγ (43, 53). Thus, our data and previous studies (16) suggest that RARα and RARγ may have opposite effects on Wnt/β-catenin signaling in prechondrogenic cells and chondrocytes, respectively.

Further insights into the unique roles of retinoid and Wnt/β-catenin signaling pathways in chondrocytes come from our global gene expression analyses. We observe that RA treatment up-regulates gene expression of several Wnt proteins, receptors, and co-receptors. Among the Wnt genes up-regulated by RA, Wnt2b, Wnt4, and Wnt9a all stimulate the β-catenin signaling pathway in several cell types (3, 8, 41), whereas Wnt5a can stimulate the non-canonical pathway (3) and inhibits β-catenin signaling by directing β-catenin for degradation by the ubiquitin proteasome system (8). However, Wnt5a has been reported to have the ability to activate β-catenin signaling in the
presence of appropriate Frizzled receptors (54). In our chondrocyte cultures, Wnt5a showed no effect on Wnt reporter activity in absence or presence of Wnt3a, but activity significantly increased in cells co-treated with RA and Wnt3a (data not shown). Thus, Wnt5a could also participate in stimulation of Wnt/β-catenin signaling by RA. Stimulation of Wnt2b and Wnt5a gene expression was observed within 6 h after RA treatment (data not shown), and these genes have several candidate sites for RAR/RXR binding in their 3-kb upstream promoter regions as determined by in silico analysis, suggesting that these genes might be direct targets of retinoid signaling. In addition to Wnt genes, we also observe up-regulation of Fzd-7, Fzd-8, Lrp-5, and Lrp-6. These Wnt receptors and co-receptors could also enhance responsiveness to Wnt proteins (3, 55). These changes are probably responsible for enhancement of Wnt3a action by RA treatment.

**Inhibition of Wnt/β-Catenin Signaling by RARγ**—In cells maintained in retinoid-free conditions, RARα associates with co-repressors, such as N-CoR and R2/SMRT, and inhibits gene transcription (33, 56, 57). Our findings indicate that in chondrocytes maintained in similar retinoid-free conditions, RARγ also functions as a repressor of β-catenin-LEF/TCF complex action. The mechanisms of this inhibition may involve competition of β-catenin recruitment/binding to LEF/TCF proteins. In addition, we observe that treatment with a retinoid antagonist decreases Wnt/Catenin Signaling by RA, modulating cytosolic β-catenin degradation. There has been increased attention to novel non-genomic mechanism of signal transduction through nuclear hormone receptors (14, 58). For example, RARα interacts with the subunits of phosphatidylinositol 3-kinase and activates the phosphatidylinositol 3-kinase/Akt pathway, depending on ligand availability in tumor cells (58). Interestingly, activation of the Akt pathway has also been reported to stimulate Wnt/β-catenin pathway in several systems (59, 60). Thus, RARγ might affect the Akt pathway or other signaling pathways that are directly or indirectly involved in β-catenin stabilization.

The finding that the inhibition of Wnt/β-catenin signaling by RARγ is reversed by treatment with RA differs with previous reports on RARα action in tumor cells (14–17). The difference could be explained by the diversity of RAR isoforms involved. For example, we showed earlier that inhibition of Wnt/β-catenin signaling by RARα is ligand-dependent (16), whereas we show here that inhibition of Wnt/β-catenin signaling by RARγ is ligand-independent and involves AF1 and AF2 domains that differ significantly from analogous domains in other RARs. Our results suggest that the AF1 domain of RARγ is necessary for interaction with β-catenin and inhibition of β-catenin transcriptional activity and that the AF2 domain is not involved in protein complex formation but is required for inhibition of β-catenin signaling activity. It is also likely that regulation of Wnt/β-catenin signaling by RA could vary according to cell type. For example, RA inhibits β-catenin signaling in colon and breast cancer cells (15–17), whereas RA stimulates signaling in ES cells (19) and neuronal cells (21) just as we show here in chondrocytes. These different types of cells probably respond differently to RA in terms of induction or repression of Wnt proteins and receptors and other coactivators and corepressors.

In sum, the findings in our study indicate that the Wnt/β-catenin and retinoid signaling pathways are tightly connected and cooperatively affect chondrocyte function and phenotype. This cross-regulation could be quite important in the regulation of chondrogenesis and proper progression of endochondral ossification during skeletal growth. It will be interesting to determine whether the cross-talks and interactions between these two signaling pathways may be altered in pathologies of permanent cartilage, such as joint disease and disc degeneration, and whether modulation of these interactions could be targeted for therapy.

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