α-CATENIN INHIBITS β-CATENIN-TCF/LEF TRANSCRIPTIONAL ACTIVITY AND COLLAGEN TYPE II EXPRESSION IN ARTICULAR CHONDROCYTES THROUGH FORMATION OF A GLI3R/α-CATENIN/β-CATENIN TERNARY COMPLEX *

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Running head: α-Catenin and Gli3R in β-catenin signaling

Chondrocytes, a unique cell type in cartilage tissue, are responsible for the regulation of anabolic and catabolic homeostasis in cartilage-specific extracellular matrix synthesis. Activation of Wnt/β-catenin signaling induces dedifferentiation of articular chondrocytes, resulting in suppression of type II collagen expression. We have previously shown that α-catenin inhibits β-catenin-Tcf/Lef (T-cell factor/lymphoid-enhancing factor) transcriptional activity in articular chondrocytes with a concomitant recovery of type II collagen expression. In the current study, we elucidated the mechanism underlying this inhibition of β-catenin-Tcf/Lef transcriptional activity by α-catenin, showing that it requires direct interaction between α-catenin and β-catenin. We further showed that it involves recruitment of Gli3R, the short transcription-repressing form of the transcription factor Gli3, to β-catenin by α-catenin. The resulting inhibition of β-catenin transcriptional activity leads to increased expression of type II collagen. Gli3R and α-catenin actions are co-dependent: both are necessary for the observed inhibitory effects on β-catenin transcriptional activity. Reducing Gli3R expression levels through activation of Indian Hedgehog (Ihh) signaling is also sufficient to activate β-catenin transcriptional activity, suggesting that the ternary complex, Gli3R/α-catenin/β-catenin, mediates Ihh-dependent activation of Wnt/β-catenin signaling in articular chondrocytes. Collectively, this study shows that α-catenin functions as a nuclear factor that recruits the transcriptional repressor Gli3R to β-catenin to inhibit β-catenin transcriptional activity and prevent dedifferentiation of articular chondrocytes. Finally, osteoarthritic cartilage showed elevated levels of β-catenin and decreased levels of α-catenin and Gli3R, suggesting that decreased levels of α-catenin and Gli3R levels contribute to increased β-catenin transcriptional activity during osteoarthritic cartilage destruction.

The capacity of chondrocytes, a unique cell type in cartilage tissue, to express collagen type II (COL2A1) is important for the maintenance of cartilage homeostasis. In pathological conditions, such as osteoarthritis (OA), chondrocytes lose their differentiated phenotype, characterized by the loss of cartilage-specific extracellular matrix synthesis, through a process called dedifferentiation (1). We have previously shown that Wnt signaling regulates the phenotype of articular chondrocytes (2-5). For instance, activation of canonical Wnt/β-catenin signaling is sufficient to induce dedifferentiation of articular chondrocytes (2-4). Our previous studies also indicated that α-catenin inhibits the transcriptional activity of β-catenin-Tcf/Lef (T-cell factor/lymphoid-enhancing factor), and thereby blocks β-catenin-mediated inhibition of collagen type II expression in articular chondrocytes (5).
α-Catenin is well known to regulate cell-cell adhesion and actin cytoskeletal dynamics through specific molecular-assembly processes. Monomeric α-catenin forms a ternary complex of cadherin/α-catenin/β-catenin that stabilizes cadherin-mediated cell-cell adhesion, whereas dimeric α-catenin suppresses actin polymerization by inhibiting the Arp2/3 actin-organizing complex (6, 7). In addition, α-catenin is localized to both the cytosol and nucleus, where it regulates various signaling pathways, including Ras, mitogen-activated protein kinase, nuclear factor-κ, Hedgehog (Hh), and canonical Wnt pathways (8, 9). In the canonical Wnt signaling pathway, nuclear accumulation of β-catenin activates the transcription factor, Tcf/Lef. β-Catenin exerts its function as a co-activator by recruiting multiple transcriptional activators to the β-catenin-Tcf/Lef complex. α-Catenin appears to regulate canonical Wnt pathways by inhibiting the transcriptional activity of β-catenin-Tcf/Lef, a mechanism in which α-catenin acts as an antagonistic nuclear factor (8, 9). Indeed, our previous study indicates that α-catenin is associated with β-catenin in the cytosol and the nucleus of articular chondrocytes, and inhibits β-catenin-Tcf/Lef transcriptional activity (5). It has also been shown that overexpression of neuronal α-catenin inhibits Wnt/β-catenin signaling in Xenopus embryos (10). Additionally, α-catenin is localized to nuclei of the SW480 colon cancer cell line, and ectopic expression of α-catenin inhibits β-catenin-Tcf/Lef transcriptional activity (11), supporting an antagonistic function of α-catenin in β-catenin-Tcf/Lef transcriptional activity.

In addition to the regulation of β-catenin signaling, α-catenin is known to regulate Hh signaling. For instance, targeted deletion of epithelial α-catenin in neural progenitor cells results in abnormal activation of the Hh pathway (12). Hh signaling is regulated by the proteasomal degradation of Gli transcription factors. In the absence of Hh ligand, the Hh receptor complex Patched1 (Ptc1)/Smoothened (Smo) promotes proteasomal degradation of the Gli transcription factors, Gli2 and Gli3, resulting in increased production of the corresponding repressor forms, Gli2R and Gli3R. Stimulation of the Ptc1/Smo complex by Hh ligand inhibits this proteasomal degradation, resulting in a decrease in GliR levels (13). The Hh signaling pathway also crosstalks with the Wnt/β-catenin signaling pathway. For instance, Gli3R physically interacts with β-catenin, and Gli3R overexpression in chick neural tubes inhibits Wnt/β-catenin signaling (14). Moreover, a recent study demonstrated that Hh signaling acts as an upstream activator of the canonical Wnt signaling pathway to control cell-cycle progression in the developing chick neural tube (15). Therefore, it is possible that α-catenin and GliR cooperate in the regulation of β-catenin-Tcf/Lef transcriptional activity.

On the basis of our previous finding that α-catenin functions as an antagonistic nuclear factor to inhibit transcriptional activity of β-catenin-Tcf/Lef (5), we here sought to characterize the mechanisms underlying α-catenin regulation of Wnt/β-catenin signaling in articular chondrocytes. α-Catenin does not contain DNA-binding domains and also shows no homology with members of the transducin-like enhancer of split (TLE)/Groucho family of transcriptional co-repressors that compete with β-catenin for binding to Tcf/Lef transcription factors (16). We therefore hypothesized that α-catenin may recruit transcriptional repressor(s) to the β-catenin-Tcf/Lef transcriptional machinery, focusing our attention on Gli3R as a potential transcriptional repressor. Additionally, it has been shown that activation of β-catenin signaling (17) or Hh signaling (18) in chondrocytes causes OA cartilage destruction. We therefore explored possible function of α-catenin and Gli3R in OA pathogenesis. Here, we report that nuclear α-catenin antagonizes β-catenin-Tcf/Lef transcriptional activity by recruiting Gli3R to β-catenin, and thereby increases expression of type II collagen in articular chondrocytes. We additionally report that OA cartilage shows elevated levels of β-catenin and decreased levels of α-catenin and Gli3R, suggesting that the decrease of α-catenin and Gli3R levels ensures the increase of β-catenin transcriptional activity during OA cartilage destruction.
EXPERIMENTAL PROCEDURES

Antibodies – Rabbit anti-α-catenin (H-297), rabbit anti-HA probe (Y-11), goat anti-lamin B (C-20), rabbit anti-Gli3 (H-280), and mouse anti-c-Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-β-catenin and mouse anti-Erk antibodies were from BD Transduction Lab (San Jose, CA). Rabbit anti-DYKDDDDK (FLAG tag) antibody was purchased from Cell Signaling (Danvers, MA). Mouse anti-collagen type II (6B3) was from Millipore (Billerica, MA). Mouse anti-Gli3 (2C9) and rabbit anti-α-catenin (C2081) antibodies were purchased from Sigma (St. Louis, MO). Secondary antibodies were from Jackson ImmunoResearch Lab (West Grove, PA) or Invitrogen (Carlsbad, CA).

Plasmids – Human α-E-catenin cDNA was subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen) by polymerase chain reaction (PCR). The 5’ forward primer was designed to introduce a FLAG tag at the 5’ end of α-catenin. Site-directed mutagenesis was employed to remove a β-catenin binding motif (117-143) from FLAG-tagged α-catenin using a Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The oligonucleotide primers were as follows: forward primer, 5’-ctgctgcaggagagttcgcagatgatccctgcatggcagatgtctacaattacttgtcagctg-3’; reverse primer, 5’-cagctgaacaagtaatttgtagacatctgcagggatcatctgcgaactctcctgcagcag-3’. The cDNA encoding HA-tagged Gli3R, consisting of amino acids 1-645, was obtained by PCR from human Gli3 cDNA (a gift from B. Wang, Weill Cornell Medical College, NY). A 5’ primer containing an HA tag and a 3’ primer corresponding to the C-terminal end of Gli3R were used for PCR. All plasmid constructs were confirmed by DNA sequencing.

Primary culture of articular chondrocytes – Articular chondrocytes from rabbit cartilage were prepared from 2-week-old New Zealand White rabbits, as described previously (19). Mouse articular chondrocytes were isolated from femoral heads, femoral condyles, and tibial plateaus of each mouse, as described previously (20, 21). Chondrocytes were maintained as a monolayer in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin.

β-Catenin reporter gene assay – β-Catenin-Tcf/Lef transcriptional activity was determined using a reporter gene assay, as described previously (5). Briefly, rabbit or mouse articular chondrocytes were transfected with 1 μg of a Tcf/Lef reporter construct – either a TOPFlash with a site for optimal Lef-binding or a FOPFlash (negative control) with a mutated Lef-binding site (Upstate Biotechnology Inc., Lake Placid, NY) – and 0.3 μg of pCMV-β-galactosidase using Metafectene (Biontex, San Diego, CA) or Lipofectamine 2000 (Invitrogen). In some experiments, mouse articular chondrocytes transfected with plasmid constructs were treated for 18 h at 37°C with recombinant mouse Ihh amino terminal peptide (C28II; R&D Systems, Minneapolis, MN) in DMEM containing 0.5% fetal bovine serum. Luciferase activity was expressed relative to β-galactosidase activity to normalize for transfection efficiency.

siRNA knockdown of α-catenin and Gli3 – Two complementary oligonucleotides (sense, 5’-gtacctcggacctgtttgagagatcattcaagagatgtactccgaaagcaggtcctttttggaaa-3’; antisense, 5’-agcttctccaaaaagggacctgctttcggagtacatctcttgaatgtactccgaaagcaggtcctttttggaaa-3’) were used to knockdown α-catenin in rabbit chondrocytes (5). A scrambled α-catenin siRNA (5’-gtacctcggacctgtttgagagatcattcaagagatgtactccgaaagcaggtcctttttggaaa-3’) was used as a negative control. Rabbit chondrocytes were transfected with annealed α-catenin siRNA using Lipofectamine 2000. ON-TARGETplus SMARTpool Gli3 siRNA (Thermo Scientific, Lafayette, CO) was used to knockdown Gli3 in mouse chondrocytes, whereas an ON-TARGET non-targeting pool was used as a negative control. Mouse Gli3 ON-TARGETplus SMART pool siRNA was introduced into mouse chondrocytes using Dharmafect 1 (Thermo Scientific).

Immunoprecipitation and immunoblotting – Proteins were immunoprecipitated from nuclear fractions or whole-cell lysates. Nuclear fractions were prepared from primary cultures of rabbit
articular chondrocytes. Briefly, cells were incubated on ice for 15 min with lysis buffer I (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol) containing Complete-Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and phosphatase inhibitors (5 mM NaF, 1 mM Na3VO4). Cell lysates were centrifuged at 15,000 × g at 4°C for 30 s, and pellets were resuspended in lysis buffer II (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) containing protease inhibitor cocktail and phosphatase inhibitors. The cell lysates were centrifuged at 15,000 × g for 15 min, and the supernatant (nuclear fraction) was diluted with 1.7 volumes of lysis buffer II without NaCl. Nuclear fractions (~200 µg proteins) were incubated with rabbit anti-Gli3 antibody (H-280), mouse anti-β-catenin antibody (BD Transduction Lab), or normal rabbit IgG at 4°C overnight. Thereafter, they were incubated with magnetic beads conjugated with protein G (Dynalbeads Protein G, Invitrogen) at 4°C for 1 h, and then washed with washing buffer (20 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1% Triton X-100, 5 mM NaF, 1 mM Na3VO4). For immunoprecipitation from whole-cell lysates, rabbit articular chondrocytes transfected with FLAG-tagged wild-type (WT) α-catenin or a Δ117-143 α-catenin mutant were incubated for 30 min on ice in mild lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor cocktail and phosphatase inhibitors. Cell lysates were centrifuged at 15,000 × g for 10 min, and the cleared cell lysate was incubated with anti-FLAG antibody overnight at 4°C. Immune complex proteins captured by Dynalbeads Protein G (Invitrogen) were washed and eluted in SDS sample buffer, fractionated by SDS-PAGE, transferred to membranes, and immunoblotted with the appropriate antibodies.

GST-fusion protein pull-down assays – Human αE-catenin cDNA (full length or fragments) was subcloned into pGEX-4T vector. GST-α-catenin fusion proteins were expressed in Escherichia Coli BL21, immobilized on glutathione-sepharose 4B beads (GE Healthcare, Piscataway, NJ), and incubated at 4°C for 2 h in GST-binding buffer (20 mM HEPES pH 7.9, 50 mM KCl, 2.5 mM MgCl2, 0.2% NP-40, 10% glycerol, 1.5% goat serum). Mouse articular chondrocytes were incubated on ice for 10 min in RIPA buffer (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% SDS, 1% deoxycholate, 1% NP-40) containing protease inhibitor cocktail and phosphatase inhibitors. Cell lysates (~1 mg) were added to the equilibrated GST-α-catenin bound on glutathione-sepharose 4B beads. After incubation at 4°C overnight, the beads were washed with GST washing buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM NaF, 1 mM Na3VO4), resuspended in SDS sample buffer, fractionated by SDS-PAGE, transferred to membranes, and immunoblotted with the appropriate antibodies.

Immunofluorescence microscopy – Because of antibody cross-reactivity issues, mouse chondrocytes were used for immunofluorescence microscopy. The cells were fixed in 3.5% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. The fixed chondrocytes were blocked with 1% BSA for 1 h at room temperature and incubated at 4°C overnight with the appropriate primary antibodies. After washing with PBS, the cells were incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 594-conjugated goat anti-mouse IgG) at room temperature for 1 h and washed with PBS. Fluorescence images were obtained using a confocal laser-scanning microscope (FV1000; Olympus).

Experimental OA and histology of OA cartilage – Human OA cartilage was sourced from individuals (age 51–72 years) undergoing arthroplasty for OA knee joints. The Wonkwang University Hospital Institutional Review Board approved the use of these materials, and all individuals provided informed, written consent before the operative procedure. Cartilage destruction was scored according to the International Cartilage Repair Society (ICRS).
grading system. Human OA cartilage samples were frozen, sectioned at a thickness of 9 μm, fixed in paraformaldehyde, and stained with alcian blue using a standard protocol (21). Experimental OA in mice was induced by destabilization of the medial meniscus (DMM) surgery (21, 22). Sham-operated animals were used as controls for DMM surgery. Mice were sacrificed 8 weeks after DMM surgery for histological analyses. Cartilage destruction was examined using safranin-O staining and scored using Mankin’s method, as described previously (21, 23). Briefly, knee joint was fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA (pH 7.4) for 14 days at 4 °C, and embedded in paraffin (Leica Microsystems, St Louis, MO). The paraffin blocks were sectioned (5 μm), deparaffinized in xylene, hydrated with graded ethanol, and stained with safranin O using a standard protocol. For immunostaining of β-catenin, α-catenin and Gli3 proteins, control and OA cartilage tissues were fixed in 4% paraformaldehyde, decalcified in paraformaldehyde, decalcified, embedded in paraffin, and sectioned at 5-μm thickness. Proteins (α-catenin, β-catenin, and Gli3) were detected using standard immunohistochemistry and immunofluorescence microscopy (23).

Statistical analysis — Data are expressed as means ± SEMs. Statistical significance was evaluated by ANOVA and post hoc tests by using PASW statistics 18 (SPSS, Chicago, IL). Significance was accepted at the 0.05 level of probability (P < 0.05).

RESULTS

Direct binding of α-catenin to β-catenin is necessary for the inhibitory effects of α-catenin on β-catenin-Tcf/Lef transcriptional activity — We have previously shown that α-catenin inhibits β-catenin-Tcf/Lef transcriptional activity with a concomitant modulation of collagen type II expression in articular chondrocytes (5). To establish the mechanism underlying this inhibitory effect of α-catenin, we first examined whether direct binding of α-catenin to β-catenin is required. Initially, we co-transfected articular chondrocytes with S37A β-catenin (a stable mutant that escapes proteosomal degradation) and FLAG-tagged WT α-catenin (FLAG-α-catenin) or mutant α-catenin lacking part of the β-catenin-binding motif (FLAG-Δ117-143 α-catenin), followed by immunoprecipitation with an anti-FLAG antibody and western blotting for β-catenin. As shown in Figure 1A, β-catenin was co-immunoprecipitated with WT α-catenin, but not with the Δ117-143 α-catenin mutant, indicating that deletion of a 27-amino-acid segment of α-catenin that forms part of the β-catenin binding motif (24) was sufficient to inhibit α-catenin binding to β-catenin. Next, using the TOPFlash reporter assay system, we evaluated the functional implications of this interaction. Consistent with our previous report (5), transfection of S37A β-catenin induced the transcriptional activity of β-catenin-Tcf/Lef, an effect that was inhibited by ectopic expression of WT α-catenin. However, exogenously expressed Δ117-143 α-catenin did not affect the transcriptional activity of β-catenin-Tcf/Lef (Fig. 1B), indicating that direct binding to β-catenin is required for the inhibitory effects of α-catenin. Consistent with the regulation of β-catenin transcriptional activity, ectopic expression of α-catenin blocked β-catenin-induced down regulation of collagen type II expression, whereas the α-catenin Δ117-143 mutant did not (Fig. 1B). Intriguingly, overexpression of the α-catenin Δ117-143 mutant promoted a β-catenin-dependent reduction in collagen type II expression. Ectopically expressed S37A β-catenin is mainly localized to the nucleus in subconfluent chondrocytes, and its distribution was not affected by the ectopic expression of WT or mutant α-catenin (Fig. 1C), suggesting that the inhibitory effects of α-catenin are not due to its modulation of β-catenin nuclear localization.

Gli3R inhibits β-catenin-Tcf/Lef transcriptional activity in articular chondrocytes — Next, we tested our hypothesis that α-catenin may recruit Gli3R as a transcriptional repressor to inhibit the β-catenin-Tcf/Lef transcriptional machinery. We first performed immunoprecipitation assays using primary cultures of articular chondrocytes transfected with
empty vector or S37A β-catenin. Gli3R was co-immunoprecipitated with both endogenous and transfected β-catenin (Fig. 2A), whereas neither Gli3R nor β-catenin was detected in normal IgG (negative control) immunoprecipitates (data not shown), indicating the formation of a complex between β-catenin and Gli3R. Interestingly, overexpression of S37A β-catenin markedly elevated Gli3R protein levels (Fig. 2A). Because Wnt/β-catenin activity is known to increase Gli3 expression in embryonic chick neural tubes (15), it is likely that the increase in Gli3R levels caused by S37A β-catenin transfection is due to increased expression of Gli3. We also tested whether ectopic expression of HA-tagged Gli3R inhibits β-catenin-Tcf/Lef transcriptional activity using the TOPFlash assay system. As shown in Fig. 2B, the transcriptional activity of β-catenin-Tcf/Lef induced by S37A β-catenin was markedly inhibited in a dose-dependent manner by transfected HA-Gli3R. The inhibitory effects of S37A β-catenin on collagen type II expression, shown in Fig. 1B, were also blocked by overexpression of HA-Gli3R (Fig. 2C). Similar to the effects of α-catenin, ectopic expression of HA-Gli3R did not alter the nuclear localization of ectopically expressed β-catenin in chondrocytes (Fig. 2D), suggesting that the association of Gli3R with β-catenin in the nucleus, rather than changes in β-catenin distribution, plays an important role in the inhibition of β-catenin-Tcf/Lef transcriptional activity.

The inhibitory actions of α-catenin and Gli3R on β-catenin transcriptional activity are mutually dependent – Because conditional ablation of α-catenin in neuronal progenitor cells increases the expression levels of Hh pathway target genes and Gli3R inhibits β-catenin-Tcf/Lef transcriptional activity (12, 14), we next examined whether the inhibitory effects of α-catenin on β-catenin transcriptional activity are mediated by Gli3R. This was validated by siRNA-mediated knockdown of Gli3 in mouse chondrocytes transfected with FLAG-tagged α-catenin and S37A β-catenin. Because proteasomal degradation of Gli3 produces Gli3R, we targeted Gli3 to reduce Gli3R levels. Treatment of Gli3 siRNA did not alter the basal transcriptional activity of β-catenin. However, Gli3 siRNA increased the transcriptional activity in S37A β-catenin transfected samples (Fig. 3A). TOPFlash reporter assays revealed that the inhibitory effects of α-catenin on β-catenin transcriptional activity were abolished by Gli3R knockdown. Consistent with this increase in β-catenin transcriptional activity, the loss of Gli3R inhibited the recovery of collagen type II expression (Fig. 3A). The full-length Gli3 activator form was barely detectable in articular chondrocytes (data not shown). Thus, it is likely that Gli3R plays a major role in α-catenin-dependent inhibition of β-catenin transcriptional activity. Gli3R is known to associate with β-catenin, leading to inhibition of β-catenin-Tcf/Lef transcriptional activity (14). Additionally, the above results indicate that Gli3R is necessary for the inhibitory effects of α-catenin on β-catenin transcriptional activity. We therefore performed the reciprocal experiments, examining whether Gli3R-dependent inhibition of β-catenin transcriptional activity requires α-catenin. Using an siRNA that is effective against rabbit α-catenin (5), we knocked down α-catenin expression and examined β-catenin-Tcf/Lef transcriptional activity. As shown in Fig. 3B, the inhibitory effect of overexpressed HA-Gli3R on β-catenin-Tcf/Lef transcriptional activity was markedly reversed by the knockdown of α-catenin expression, in association with the concomitant suppression of type II collagen expression. α-Catenin siRNA did not alter the basal transcriptional activity of β-catenin but increased the transcriptional activity in S37A β-catenin transfected cells (Fig. 3B). These results suggest that α-catenin links Gli3R to the β-catenin-Tcf/Lef transcriptional machinery in articular chondrocytes.

α-Catenin mediates formation of a ternary complex with Gli3R and β-catenin – Next, we determined whether α-catenin binds to Gli3R and thus forms a ternary complex with β-catenin in articular chondrocytes. Proteins from nuclear fractions of articular chondrocytes were immunoprecipitated with an anti-Gli3 antibody (H-280) recognizing amino acids 1-280 of Gli3. Thus, this antibody precipitates both Gli3 and
Gli3R. However, most of the precipitated protein was Gli3R; full-length Gli3 was barely detectable in Gli3 immunoprecipitates. Immunoprecipitation analyses showed that both α-catenin and β-catenin were co-precipitated with Gli3R (Fig. 4A). Additionally, both α-catenin and Gli3R were co-precipitated with β-catenin, indicating that α-catenin, β-catenin, and Gli3R form a ternary complex in the nuclei of articular chondrocytes. Because there is no evidence that α-catenin directly interacts with Gli3R, we performed pull-down assays using a GST-α-catenin fusion protein. To identify the Gli3R binding domain of α-catenin, we generated GST-α-catenin fusion proteins carrying vinculin homology domain 1 (VH1), VH2 or VH3 (Fig. 4B), and incubated these GST-α-catenin proteins with articular chondrocyte lysates prepared in RIPA buffer, which disrupts most protein-protein interactions. GST pull-down assay revealed that α-catenin containing an N-terminal VH1 domain (1-290) is responsible for binding to Gli3R (Fig. 4C), suggesting that α-catenin provides the link between Gli3R and β-catenin that results in inhibition of β-catenin transcriptional activity. However, the N-terminal domain of α-catenin also binds to β-catenin, raising the possibility that Gli3R may compete with α-catenin for β-catenin binding. To determine if binding of α-catenin to β-catenin is independent of Gli3R, we assayed anti-FLAG immunoprecipitates of lysates from articular chondrocytes expressing FLAG-tagged WT or Δ117-143 α-catenin for the presence of Gli3R (Fig. 4D). Gli3R was detected in both FLAG-α-catenin WT and Δ117-143 mutant immunoprecipitates. However, Gli3R was more enriched in FLAG-α-catenin Δ117-143 mutant immunoprecipitates, suggesting that binding of α-catenin to β-catenin partially hinders the interaction between α-catenin and Gli3R (Fig. 4D).

Stimulation of Ihh/Gli3R signaling results in dedifferentiation of articular chondrocytes via activation of β-catenin-Tcf/Lef transcriptional activity – It has been shown that Ihh signaling regulates chondrocytes hypertrophy by activating canonical Wnt/β-catenin signaling and controlling the expression of parathyroid hormone-related protein (PTHrP) during endochondral ossification (25). Thus, a decrease in Gli3R expression levels attributable to stimulation of Ihh signaling is likely to be responsible for the activation of β-catenin transcriptional activity in articular chondrocytes. To test this possibility, we treated articular chondrocytes with recombinant Ihh protein and assayed for β-catenin-Tcf/Lef transcriptional activity using the TOPFlash system. Consistent with the observations of others (25), we found that stimulation of Ihh signaling induced a small (1.8 fold), but statistically significant, increase in β-catenin-Tcf/Lef transcriptional activity (Fig. 5A). Stimulation of Ihh signaling also reduced expression levels of type II collagen. Ectopic expression of a dominant-negative form of TCF4 (AN-TCF4) inhibited Ihh-dependent activation of β-catenin-Tcf/Lef transcriptional activity (Fig. 5A), indicating that the canonical Wnt/β-catenin signaling pathway is a downstream target of Ihh signaling. Upon activation of Ihh signaling, Gli2 and Gli3 become activated and Gli repressor levels are decreased (26). To determine if a reduction in Gli3R activity is sufficient for Ihh-dependent activation of β-catenin-Tcf/Lef transcription, we treated articular chondrocytes overexpressing HA-tagged Gli3R with Ihh recombinant proteins and assayed for promoter-reporter activity using the TOPFlash system. Ectopically expressed HA-Gli3R suppressed Ihh-dependent activation of β-catenin-Tcf/Lef transcriptional activity and reversed the Ihh signaling-induced decrease in the levels of type II collagen (Fig. 5B). These data suggest that Ihh/Gli3R signaling plays a major role in the activation of Wnt/β-catenin signaling in articular chondrocytes.

Increase of β-catenin and decrease of α-catenin and Gli3R in OA cartilage – Next, we examined in vivo functional significance of α-catenin and Gli3R in regulating β-catenin transcriptional activity. For this purpose, we determined protein expression levels of β-catenin, α-catenin and Gli3 in human OA cartilage and mouse experimental OA cartilage. We first examined protein levels in OA-affected human
cartilage obtained from individuals undergoing arthroplasty. Human OA cartilage damage was confirmed by alcian blue staining. β-Catenin protein levels, as determined by immunohistochemistry, in damaged regions of human OA cartilage were markedly increased compared to undamaged regions (Fig. 6A). α-Catenin expression levels were similar between damaged and undamaged part of OA cartilage. However, some OA chondrocytes in the damaged part did not express α-catenin protein. In contrast, Gli3R expression was markedly reduced in damaged regions of human OA cartilage compared to undamaged regions (Fig. 6A).

Experimental OA was developed by DMM surgery in mice (22). Consistent with our previous reports (21, 23), DMM caused OA cartilage destruction as determined by Makin’s method and safranin-O staining of articular cartilage (Fig. 6B). We also compared α-catenin, β-catenin, and Gli3 protein levels of DMM-induced OA cartilage and sham-operated control cartilage. Consistent with previous reports (17), β-catenin protein levels were increased in OA chondrocytes (Fig. 6B), suggesting the increase of β-catenin transcriptional activity. In contrast, levels α-catenin and Gli3R were markedly decreased in OA chondrocytes (Fig. 6B). Because in vitro data indicate that α-catenin and Gli3R inhibits β-catenin transcriptional activity, the decrease of α-catenin and Gli3R levels is likely to ensure the increase of β-catenin transcriptional activity in OA chondrocytes.

DISCUSSION

Nuclear accumulation of β-catenin and subsequent stimulation of β-catenin-Tcf/Lef transcriptional activity causes dedifferentiation of articular chondrocytes, characterized by decreased type II collagen expression and initiation of type I collagen expression. We have previously shown that both α-catenin and β-catenin accumulate during dedifferentiation of chondrocytes through escape from proteasomal degradation. We have also previously shown that formation of an α-catenin–β-catenin complex inhibits β-catenin transcriptional activity, thus reversing β-catenin-mediated suppression of collagen type II expression and restoring collagen type II levels (5). A question left unanswered by our previous studies was, what is (are) the mechanism(s) by which α-catenin inhibits β-catenin-Tcf/Lef transcriptional activity? α-Catenin does not contain DNA-binding domains or a homology domain of the TLE/Groucho family of transcriptional co-repressors, which compete with β-catenin for binding to Tcf/Lef transcription factors (16). We thus tested the hypothesis that α-catenin may recruit some transcriptional repressor(s) to the β-catenin-Tcf/Lef transcriptional machinery, focusing on Gli3R as a potential transcriptional repressor. It is possible that interaction of Gli3R/α-catenin and Gli3R/β-catenin could be separate events. However, because β-catenin immunoprecipitates contain Gli3R and α-catenin, it is highly likely that α-catenin forms a ternary complex with Gli3R and β-catenin in articular chondrocytes, resulting in inhibition of β-catenin-Tcf/Lef transcription. Furthermore, we found that Gli3R and α-catenin are mutually dependent: both are required for the observed inhibitory effect on β-catenin-Tcf/Lef transcription. Our results imply that a decrease in Gli3R expression levels, presumably caused by stimulation of Ihh signaling, enables β-catenin to activate Tcf/Lef transcription in articular chondrocytes. Our current results are consistent with the previous observation that β-catenin physically interacts with Gli3R and ectopic expression of Gli3R inhibits Wnt/β-catenin signaling (12, 14). Additionally, catenins appear to possess the nuclear signaling activity necessary to regulate Wnt target gene expression (27).

Nuclear accumulation of α-catenin is observed in certain colon cancer cell lines, including SW480 and SW620 (11, 28). Additionally, nuclear-targeted, ectopic expression of α-catenin inhibits β-catenin-Tcf/Lef transcription in SW480 cells, suggesting that nuclear α-catenin suppresses β-catenin-Tcf/Lef transcription (11). Nevertheless, α-catenin lacks DNA-binding motifs; however, it does contain three regions with strong homology to the actin-binding protein vinculin. A number of α-catenin binding partners are cytoskeletal proteins, but an
interaction of α-catenin with β-catenin-associated co-regulators involved in Tcf/Lef transcription has not been reported. Here, we demonstrated that α-catenin recruits the transcriptional repressor Gli3R to β-catenin in the nuclei of articular chondrocytes. The α-catenin–Gli3R interaction is reminiscent of the p120 catenin–Kaiso interaction in terms of controlling β-catenin-Tcf/Lef transcription. p120 catenin bound to the cytoplasmic domain of cadherin controls cadherin stability and turnover (29, 30). Kaiso is a member of the BTB/POZ (Broad complex, Tramtrak, Bric à brac/Pox virus and zinc finger) family and has been identified as a p120-binding protein (31). Overexpression of Kaiso suppresses β-catenin-transcriptional activity in Xenopus embryos, and the morpholino-directed depletion of p120 promotes this Kaiso-dependent repression. In contrast, ectopic expression of p120 elevates β-catenin transcriptional activity by relieving the Kaiso-dependent repression (32). Thus, there are some discrepancies between α-catenin and p120 in the regulation of β-catenin transcriptional activity. Nevertheless, both α-catenin and p120 modulate the activity of transcriptional repressors associated with the β-catenin-Tcf/Lef transcriptional machinery.

Gli3R requires the Ski co-repressor in order to recruit histone deacetylase complexes and exert its transcriptional repressor activity (33). The deacetylation of histone proteins plays an important role in reorganizing chromosomal domains into a transcriptionally silent state. In canonical Wnt/β-catenin signaling, the repressive activity of Tcf/Lef results from its interaction with the Groucho/TLE/histone deacetylase 1 complex. Nuclear β-catenin displaces the Groucho/TLE-HDAC complex, leading to its replacement by p300/CBP acetyltransferase and subsequent transactivation of β-catenin-Tcf/Lef, presumably through histone acetylation and subsequent chromatin remodeling (34). Therefore, it would be interesting to examine if a Gli3R-associated histone deacetylase enhances the repressive activity of Groucho/TLE and thereby inhibits β-catenin-Tcf/Lef transcriptional activity.

Activation of the Ihh pathway in developing cartilage promotes chondrocyte hypertrophy—a critical step for endochondral bone formation—potentially through upregulation of canonical Wnt/β-catenin signaling (25). However, the identity of the molecular mechanisms underlying the Ihh-dependent activation of Wnt/β-catenin signaling in chondrocytes has remained unclear. The current study provides evidence that an α-catenin/Gli3R complex could serve as a linker connecting Ihh signaling to Wnt/β-catenin signaling in chondrocytes.

It has been shown that β-catenin and Hh signaling plays critical roles in OA pathogenesis. For instance, chondrocyte-specific activation of β-catenin signaling in mice causes OA cartilage destruction (17). In addition, higher levels of Hh signaling in chondrocytes cause a more severe OA phenotype, whereas genetic inhibition of Hh signaling reduces the severity of OA in mice (18). We found in this study that β-catenin levels are increased in OA cartilage, whereas levels of α-catenin and Gli3R are markedly decreased in degenerating articular cartilage. Based on our in vitro results, the decrease of α-catenin and Gli3R levels leads to increased β-catenin transcriptional activity, which in turn causes OA cartilage destruction.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: DMEM, Dulbecco’s Modified Eagle Medium; DMM, destabilization of the medial meniscus; GliR, repressor form Gli; GST, glutathione S-transferase; Hh, Hedgehog; Ihh, Indian Hedgehog; OA, osteoarthritis; RT-PCR, reverse transcription-polymerase chain reaction; Tcf/Lef, T-cell factor/Lymphoid enhancing factor; VH, vinculin homology domain; WT, wild-type.

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FIGURE LEGENDS

FIGURE 1. α-Catenin inhibits β-catenin transcriptional activity in articular chondrocytes via direct interaction with β-catenin. A, Rabbit articular chondrocytes transfected with FLAG-tagged WT α-catenin (FLAG-α-catenin) or a mutant α-catenin (FLAG-∆α-catenin) lacking the β-catenin-binding motif (114-143) were immunoprecipitated with anti-FLAG antibody and blotted with the indicated antibodies. FLAG-tagged ∆α-catenin did not associate with β-catenin. B, Articular chondrocytes were co-transfected with the indicated plasmid constructs (in μg) and incubated for 24 h. The transcriptional activity of β-catenin was determined by a reporter gene assay (TOPFlash; left panel). Expression levels of β-catenin, FLAG-tagged α-catenin, collagen type II (Coll-II), and extracellular signal-regulated protein kinase (ERK), a loading control, were determined by western blotting (right panel). (**P < 0.005 and *P < 0.05; n = 6). C, Articular chondrocytes were transfected with FLAG-tagged α-catenin and S37A β-catenin. Following incubation for 24 h, FLAG-tagged α-catenin and β-catenin were detected by immunofluorescence confocal microscopy.

FIGURE 2. Gli3R binding to β-catenin inhibits β-catenin transcriptional activity in articular chondrocytes. A, Whole-cell lysates of rabbit chondrocytes transfected with S37A β-catenin were immunoprecipitated with an anti-β-catenin antibody and examined for co-precipitation of Gli3R. B and C, Articular chondrocytes were transfected with the indicated amounts (μg) of S37A β-catenin and HA-tagged Gli3R expression vectors. After incubating cells for 24 h, the transcriptional activity of β-catenin was determined by TOPFLash assay. (**P < 0.001 and *P < 0.01; n = 5) (B). Expression levels of the indicated proteins were determined by western blotting (C). D, Articular chondrocytes were transfected with HA-Gli3R and S37A β-catenin. Following incubation for 24 h, HA-Gli3R and β-catenin were detected by immunofluorescence confocal microscopy.

FIGURE 3. The inhibitory actions of α-catenin and Gli3R on β-catenin transcriptional activity are mutually dependent. A, Mouse articular chondrocytes were transfected with S37A β-catenin (0.5 μg), FLAG-α-catenin (3 μg), and control siRNA (100 nM) or mouse Gli3 siRNA (100 nM), and incubated for 24 h. The transcriptional activity of β-catenin was determined by reporter gene assay. Expression levels of the indicated proteins were determined by western blotting. B, Rabbit articular chondrocytes were transfected with S37A β-catenin (1 μg), HA-Gli3 (in μg), and control siRNA (50 nM) or rabbit α-catenin siRNA (50 nM), and incubated for 24 h. β-Catenin transcriptional activity was determined by TOPFLash assay. Expression levels of the indicated proteins were determined by western blotting. Values represent means ± SEMs (*P < 0.05 and **P <0.001; n = 6).
FIGURE 4. Formation of an α-catenin/β-catenin/Gli3R ternary complex in articular chondrocytes. A, Nuclear fractions of rabbit chondrocytes were immunoprecipitated with rabbit and mouse IgG, rabbit anti-Gli3, mouse anti-β-catenin antibodies and immunoblotted with the designated primary antibodies (upper and middle panels). Aliquots of nuclear extracts were also immunoblotted with anti-α-catenin, anti-β-catenin, and anti-Gli3R antibodies. An antibody specific to the nuclear protein lamin B was used to verify the efficiency of fractionation (lower panel). B, Diagram of the GST-α-catenin constructs used in GST-pull-down assays (upper panel). Purified GST-α-catenin fusion proteins were resolved by SDS-PAGE and stained with Coomassie Blue (lower panel). C, GST-pull-down assay to show the interaction between GST-α-catenin fusion proteins and Gli3R. The indicated GST-α-catenin proteins bound to glutathione-coated beads were incubated with chondrocyte lysates prepared in RIPA buffer. Proteins bound to the beads were immunoblotted with an anti-GST antibody and an anti-Gli3 antibody recognizing the N-terminal motif (1-111) of Gli3. D, Chondrocytes were transfected with FLAG-tagged WT or mutant α-catenin and immunoprecipitated with anti-FLAG antibodies. The resulting immunocomplexes were analyzed by western blotting with the indicated antibodies.

FIGURE 5. The stimulatory effect of Ihh on β-catenin transcriptional activity requires down regulation of Gli3R. A and B, Mouse articular chondrocytes were transfected with the indicated plasmid constructs (µg) and treated with mouse Ihh amino-terminal peptides in the presence of 0.5% fetal bovine serum. The cells were then lysed and used for β-catenin reporter gene assays (upper panels). Values represent means ± SEMs (**P < 0.001; n = 7). Expression levels of the indicated proteins were detected by western blotting (lower panels).

FIGURE 6. Increase of β-catenin and decrease of α-catenin and Gli3 in experimental OA cartilage. A, Alcian blue staining of undamaged and damaged regions of human OA cartilage. β-Catenin protein was detected by immunohistochemical staining. α-Catenin and Gli3 were detected by immunofluorescence miscoscopy (n = 8). B, Experimental OA was induced by DMM surgery in C57Bl/6 mice. Cartilage destruction was scored by Mankin’s method from sham-operated control cartilage and DMM-induced OA cartilage. Values represent means ± SEMs (**P <0.001; n = 10). B, Safranin-O staining in sham-operated control cartilage and DMM-induced OA cartilage. β-Catenin protein was detected by immunohistochemical staining. α-Catenin and Gli3 were detected by immunofluorescence miscoscopy. Nuclei was detected by DAPI staining. Scale bars: 30 μm.
Figure 4

A

IgG | Gli3R  
---|---
α-catenin | IP
β-catenin | Gli3R

IgG | β-catenin
---|---
α-catenin | IP
β-catenin | Gli3R

IgG | Input
---|---
α-catenin | Gli3R
β-catenin | Gli3R

IgG | LaminB
---|---

B

α-catenin | 
---|---

GST  | 
---|---

Input | GST only
---|---
GST 1-906 | GST 1-290
GST 290-651 | GST 651-906

C

Input | Input
---|---
GST | GST
GST only | GST 1-906
GST 1-290 | GST 290-651
GST 651-906 | GST 651-906

IB: Gli3R

D

Input | EV
---|---
WT | Gli3R
Δ117-143 | Gli3R

IB: FLAG

β-catenin | IB
---|---

Input | Input
---|---
Gli3R | Gli3R
ERK | ERK

IB: GST
Figure 5

A

B

Luciferase activity (Fold increase)

Ihh (ng/ml)

Myc-ΔTCF4

Gli3R

Coll-II

Erk

Luciferase activity (Fold increase)

Ihh (ng/ml)

HA-Gli3R

HA-Gli3R

Coll-II

Erk
Figure 6

A) 

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B) 

Mankin score

<table>
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** indicates significant difference (p < 0.01).
α-Catenin inhibits β-catenin-Tcf/Lef transcriptional activity and collagen type II expression in articular chondrocytes through formation of a Gli3R/α-catenin/β-catenin ternary complex
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