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Roles of β-catenin signaling in phenotypic expression and proliferation of articular cartilage superficial zone cells

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Abstract

The superficial zone (SFZ) of articular cartilage has unique structural and biomechanical features, is thought to promote self-renewal of articular cartilage and is thus important for joint long-term function, but the mechanisms regulating its properties remain unclear. Previous studies revealed that Wnt/β-catenin signaling is continuously active in SFZ indicating that it may be essential for SFZ function. Thus, we examined whether Wnt/β-catenin signaling regulates proliferation and phenotypic expression in SFZ cells. Using transgenic mice we found that acute activation of Wnt/β-catenin signaling increases SFZ thickness, *Proteoglycan 4* (*Prg4*, also called *lubricin*) expression and the number of slow-cell cycle cells, while conditional ablation of *β-catenin* causes the opposite. We developed a novel method to isolate SFZ cell-rich populations from the epiphyseal articular cartilage of neonatal mice, and found that the SFZ cells in culture exhibit a fibroblastic cytoarchitecture and higher *Prg4* and *Ets-related gene (Erg)* expression and lower a*ggrecan* expression compared to chondrocyte cultures. Gene array analyses indicated that SFZ cells have distinct gene expression profiles compared to underlying articular chondrocytes. Treatment of Wnt3a strongly stimulated SFZ cell proliferation and maintained strong expression of *Prg4* and *Erg*, while ablation of *β-catenin* strongly impaired proliferation and phenotypic expression. When the cells were transplanted into athymic mice, they formed *Prg4*- and *aggrecan*-expressing cartilaginous masses attesting to their autonomous phenotypic capacity. Ablation of *β-catenin* caused a rapid loss of *Prg4* gene expression and strong increases in expression of *aggrecan* and *collagen 10*, the latter being a trait of hypertrophic chondrocytes. Together, the data reveal that Wnt/β-catenin signaling is a key regulator of SFZ cell phenotype and proliferation and may be as important for articular cartilage long-term function.

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Keywords

articular cartilage; β-catenin; chondrocytes; superficial zone; Wnt signaling

INTRODUCTION

Articular cartilage is an essential component of limb diarthrodial joints and sustains unhindered movement of long bones. When articular cartilage is damaged structurally and/or functionally by trauma, overuse or inflammation, it is usually unable to fully recover and heal. The tissue is also prone to progressive degeneration during natural aging and other congenital or acquired conditions. Such insufficient recovery abilities and progressive deterioration can lead to severe cartilage damage in joint diseases such as osteoarthritis and rheumatoid arthritis (1–3). Thus, it remains very important to understand why articular cartilage has poor repair capacity and whether it possesses mechanisms and cells potentially able to support repair and self-renewal that could be targeted therapeutically. It is well established that articular cartilage contains several zones characterized by distinct structure, composition and biomechanical properties (4–6). The superficial zone (SFZ) is composed of elongated cells that produce high levels of unique molecules including *tenascin-C*, the *Ets-related gene transcription factor* (*Erg*) and *Prg4,* but relatively low levels of aggrecan (6–10). This combination of phenotypic traits is thought to be important for SFZ function and overall joint mechanics and lubrication (11–14). Interest in superficial zone biology has been re-invigorated by recent studies suggesting that it may contain cells with stem or progenitor capacity (15, 16). Dowthwaite et al. isolated SFZ cells from postnatal bovine articular cartilage using differential adhesion to fibronectin-coated substrates (15). They found that the cells have progenitor characteristics based on high colony formation capacity and *Notch 1* expression and can acquire and express a chondrogenic phenotype over passage number. Thus, SFZ cells may normally act in self-renewal and maintenance of articular cartilage.

In addition to the above gene products, SFZ cells express specific signaling molecules that include members of the Wnt, bone morphogenetic protein and transforming growth factor families and their receptors and modulators (6, 17). However, it remains unclear whether and how these and/or other signaling factors regulate the nature and phenotype of SFZ. Elucidation of the roles of these pathways would be important since it could lead to therapeutic strategies to promote articular cartilage repair and restore structural and functional homeostasis. We reported recently that Wnt/β-catenin signaling is particularly high in SFZ cells, and that ablation of β-catenin leads to loss of SFZ cells while transient signaling stimulation induces SFZ thickening (18). These findings indicate that Wnt/β-catenin signaling may be a critical regulator of behavior and function of SFZ cells in addition to its important roles in cartilage development and function (6, 19–22). To gain a better understanding of the roles of Wnt/β-catenin signaling in SFZ function, we determined the cellular and molecular changes induced by activation and inactivation of Wnt/β-catenin signaling in transgenic mice and SFZ mouse cell cultures. Our data do indicate that Wnt/β-catenin signaling is required for proliferation and phenotypic expression of SFZ cells.

Materials and Methods

Transgenic mice

All studies were conducted with approval by the IACUC. We recently described the creation of *Col11-CA-βcatER* mice that harbor an N-terminally-truncated β-catenin linked to a modified estrogen receptor ligand-binding domain (ER) under the control of *collagen 11* promoter/enhancer (23). To activate Wnt/β–catenin signaling, 2-week-old *Col11-CA-βcatER* transgenic mice were injected with tamoxifen for 7 days with doses up to 200 μg/20 μl/mouse as described previously (23). Control mice received the same volume of vehicle cocktail (10% ethanol and 90% corn oil). Three wild type and 3 transgenic mice were sacrificed two weeks after the last tamoxifen or vehicle administration. Hind limbs were dissected and subjected to histopathological analyses. *Col2CreER;β–cateninfl/fl* mice: mice conditionally deficient in *β–catenin* were created by mating *β–catenin* floxed mice (*β–catenin*$^{fl/fl}$) possessing loxP sites in introns 1 and 6 in the β–catenin gene (6.129-Ctnnb1tmKem/KnwJ line purchased from the Jackson Laboratory, Bar Habor, ME) with *Col2a1-CreER* mice (kindly provided by Dr. S. Mackem, NIDCR) (24). To ablate the *β–catenin* gene, we injected tamoxifen at a dose of 100 μg/10 μl volume/mouse per day on postnatal day 5, 6 and 7 (P5 to P7). Mice (3 *β–catenin*$^{fl/fl}$*;Col2a1-CreER* mice and 3 *β–catenin*$^{fl/fl}$ mice) then were sacrificed at 7 weeks of age. Efficiency of Cre recombinase activity was confirmed by analysis of compound *Col2a1-CreER*/*Rosa R26R* transgenic-reporter mice following a similar regimen of tamoxifen injections. *CagCreE;β–catenin*$^{fl/fl}$ mice were mated with: *β–catenin*$^{fl/fl}$ mice that have a tamoxifen-inducible Cre-mediated recombinase system driven by the chick β-actin promoter/enhancer coupled to the cytomegarovirus immediate-early enhancer (*CagCreER*; B6.Cg-Tg(CAG-cre/Esr1*) 5Amc/J purchased from the Jackson Laboratory). Ablation of *β–catenin* was induced by treatment with 1 μM 4-hydroxytamoxifen (Sigma-Aldrich, St. Louis, MO) in culture. *Gdf5Cre;Rosa26R* mice: *Gdf-5-Cre* transgenic mice were described previously (25) and line B was used in the present study. Mice were mated with Rosa R26R Cre-inducible *LacZ (β–galactosidase*) mice in which the reporter *β–galactosidase* gene is silent and becomes irreversibly expressed after Cre-recombinase removal of a floxed silencer within its constitutive promoter reporter mice (26).

Histological examination and in situ hybridization

Knee joints were dissected after perfusion fixation with 4% (v/v) paraformaldehyde, decalcified with EDTA for 3–5 days and embedded in paraffin. Sections (6 μm thick) were subjected to staining with hematoxylin and eosin (H&E) or Bromodeoxyuridine (BrdU) immunostaining. Sections were also used to examine *Prg4* expression by in situ hybridization using ^{35}S-labeled riboprobes of a 2,605 bp mouse *Prg4* gene fragment (41–2646; AB034730). Frozen sections of knee joints were prepared from P5 mouse and stained with alpha 5 integrin antibodies (1:200) (CD49e, BD Biosciences, San Jose, CA).

Transplants were fixed with 4% paraformaldehyde and embedded in paraffin or OTC compound. Sections were subjected to staining with H&E or alcian blue (pH 1.0). Detection of β-galactosidase activity was preformed using X-gal substrate (Millipore, Billerica, MA) and β-galactosidase stain base solution (Millipore). Immunostainig for collagen 2 was

performed by incubation with anti-bovine collagen 2 polyclonal antibodies (1:200, Cosmo Bio USA, Inc., Carisbad, CA) followed by incubation with Alexa fluor 594-labeled anti-rabbit IgGs (1:200, Invitrogen). Parallel control sections were incubated with preimmune rabbit IgGs (5 μg/ml, Vector Laboratories, Inc., Burlingame, CA).

BrdU labeling and detection

Mice received a daily intraperitoneal injection of BrdU (25 mg/kg, Invitrogen, San Diego, CA) 5 times starting at P11. Longitudinal long bone sections were incubated with anti-BrdU antibodies (1:200, Roche Diagnostics, Indianapolis, IN) followed by incubation with Alexofluor 488-anti mouse immunoglobulin (IgG). In these experiments we used 3 mice per group. Three slides per sample were subjected to BrdU staining, and 3 independent regions per slide were examined. Similar results were obtained in all examined samples.

Superficial zone (SFZ) cell and chondrocyte cultures

The proximal end of femur and distal end of tibia were dissected from neonatal mouse (P3–P5) knee joints, and ligaments and tendons were carefully excised from their attachment sites by a surgical knife with the aid of a stereomicroscope. Tissues were incubated with 0.25% trypsin (Invitrogen) (w/v) for 1 h followed by 1.5 hr digestion with 173U/ml type 1 collagenase (Worthington Biochemical Corp., Lakewood, NJ). Dissociated cells (500,000 cells/100 mm dish) were seeded on culture dishes pre-coated with 0.1% plasma fibronectin solution (GEMINI Bio-Products, West Sacramento) for 2 hr followed by blocking with 3% BSA for 30 min. The unattached cells were washed out with Dulbecco modified Eagle medium (DMEM) twice 20 min after plating, and the attached cells were maintained in DMEM containing 10% (w/v) fetal bovine serum (GEMINI). SFZ cells were subcultured at a 1:10 ratio into uncoated culture dishes when subconfluent. The SFZ cells were transferred to pellet culture (200,000 cells/pellet) after 6 passages. Chondrocytes were isolated by additional overnight collagenase digestion of residual epiphyseal cartilage tissue as previously described (27). Cultures were treated with: recombinant mouse Wnt3a (rWnt3a) (Chemicon, Temecula, CA, USA); conditioned medium containing Wnt3a (27); Wnt/β-catenin signaling inhibitors (recombinant mouse Dkk-1 (R&D systems); IWR-1-endo (Santa Cruz Biotechnology Inc.); KN-93 (an effective inhibitor of Ca2+/calmodulin-dependent protein kinase II, Santa Cruz Biotechnology Inc.); or SP600125 (a selective inhibitor of c-Jun N-terminal kinases, Santa Cruz Biotechnology Inc.).

Transfection and Wnt reporter assays

SFZ cells were plated at an initial density of 2×10^4/well into 96-well plates that had been pre-coated with a Wnt/β-catenin reporter plasmid (Super 8× TOPFlash, Addgene Inc. Cambridge MA) in the presence of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 hrs, cultures were treated with rWnt3a (100 ng/ml) and/or recombinant mouse Dkk-1 (100 ng/ml, IWR-1 (10 μM), 6BIO (1 μg/ml, Enzo life Sciences International Inc., Plymouth meeting PA), and luciferase activity was measured using Bright-Glo assay kit (Promega Corp., Madison, WI). Super 8× TOPFlash encodes 7 copies of LEF/TCF binding sites linked to firefly luciferase and reflects Wnt/β-catenin signaling activity.

RNA isolation and gene expression assays

Total RNA was isolated using TRIzol reagent (Invitrogen) following manufacturer's protocol and reverse-transcribed into cDNA. The resulting cDNA was reverse transcribed and subjected to polymerase chain reaction (PCR) or quantitative PCR assays. Real-time PCR was performed with an Applied Biosystems 7900HT Sequence Detection Systems running SDS 2.1 software using SYBR green (Applied Biosystems, Foster City, CA). Average threshold cycle value (Ct value) was calculated from triplicate reactions. 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 a standard curve and normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*). Primers are designed to amplify sequences common among known splice variants of each gene. Primer sequences used for real-time PCR amplification were as follows: 5′-TCT GGA AAT GAC AAC CCC AAG CAC A-3′ and 5TGG CGG TAA CAG TGA CCC TGG AAC T-3′ for 5463–5939 of mouse *Aggrecan* (NM_007424), 5′-CTT GTG GAC AAT CCT CAG GTT TCT GTT C-3′ and 5′-TCG GTC ACC ATC AAT GCC ATC TAT G-3′ for 822–1040 of mouse *Collagen 9 (a1)* (NM_007740), 5′-TGC TGC TAA TGT TCT TGA CCC TGG TTC-3′and 5′-ATG CCT TGT TCT CCT CTT ACT GGA ATC C-3′ for 717–876 of mouse *collagen 10 (a1)* (NM_009925), 5′-TGG AGT GCT GTC CTG ATT TCA AGA G-3′and 5′-GGT GAT TTG GGT GAG CGT TTG GTA-3′for 201–456 of mouse *Prg4* (NM_021400), 5′-CAC CAT CTC CAC CAC GCA GAA T-3′ and 5′-TGC TGA ATG TTG CCA CCT CTC TTG-3′ for 34–310 of mouse *Prg4* (NM_021400), 5′-TGG GAT TGG TTC TGC TGT CAA G-3′ and 5′-CAT TTC TTC CGT GGA TGC CTT CAC-3′ for mouse *tenascin C* (NM-011607), 5′-CCA GCG TCC TCA GTT AGA TCC TTA CCA-3′and 5′-TCA TGT TGG GCT TGC TCT TCC TCT C-3′for 1123–1349 of mouse *Erg* (NM_133659), 5′-CCA GGA GAA CCC CAA GAT GCA CAA-3′and 5′-TCA TGC TGT AGC TGC CGT TGC TC-3′for 597–937 of mouse *SOX 2* (NM_011443), 5′-GGC GTT CGC TTT GGA AAG GTG TTC-3′and 5′-CTC GAA CCA CAT CCT TCT CT-3′for 554–866 of mouse *Oct4* (NM_013633), 5′-GGT GTT CCT GGT CCT CGT TT-3′and 5′-CAA AGG AGG TGA CAA TGC TGG-3′for 648–1306 of mouse *CD105* (NM_007932), and 5′-AAG CCC ATC ACC ATC TTC CAG GAG-3′, 5′-ATG AGC CCT TCC ACA ATG CCA AAG-3′ for 258–568 of *glyceraldehyde 3-phosphate dehydrogenase* (NM_008084), using proper filters to visualize fluorescent markers. *PRG4* gene expression was also determined by the Taqman gene expression assay using pre-designed Taqman probe and primers (Assay ID: Mm00502413_m1). The primer set amplifies part of exon 10–12 of *Prg4* (NM_021400.3) which region is common among all known *Prg4* isoforms.

First strand cDNA was synthesized from 1 μg of total RNA with 1 μM of random 6 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 Go-taq (Sigma) by incubation at 95°C for 3 min, 25–30 cycles (27 for *Prg4*; 30 for *Erg* and *CD105*; 25 for *Gapdh*) of 95 °C for 30 sec and 60 °C for 30 sec followed by incubation at 72°C for 10 min. Primer sequences for PCR amplification are described above. Primer sequences for *Prg4* are 5′-TGG AGT GCT GTC CTG ATT TCA AGA G-3′and 5′-GGT GAT TTG GGT GAG CGT TTG GTA-3′for 201–456 of mouse *Prg4* (NM_021400).

To analyze gene expression of Wnts, we carried out PCR analysis using RT2 Profiler PCR array for Wnt signaling pathway (Superarray, Frederick, MD) following the manufacturer's protocol. Average threshold cycle value (Ct value) was calculated from 4-fold reactions and normalized to that of housekeeping gene GAPDH. These experiments were repeated twice independently.

Gene arrays

SFZ cells and chondrocytes were isolated from compound transgenic mice (5 mice per experiment) of *Gdf5Cre;Rosa26-EYFP* (B6.Cg-Gt(ROSA)26Sortm3.1(CAG-EYFP)Hze/J, The Jackson Laboratories) as described above. Pure populations of SFZ cells and articular chondrocytes were isolated as enhanced yellow fluorescent protein (EYFP)-positive and negative cells by flow cytometry in our central facility respectively, and were immediately used for total RNA preparation without expansion. Two independent RNA samples were prepared for SFZ cells and articular chondrocytes.

Total RNAs were isolated from using a RNeasy Mini Kit and a RNeasy MinElute Cleanup kit (QIAGEN Inc., Valencia, CA). DNase-treated RNA was ethanol precipitated and quantified on a NanoDrop ND-1000 spectrophotometer, followed by RNA quality assessment by analysis on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA). RNA amplification and labeling were performed by the WT-Ovation Pico RNA amplification system (NuGen Technologies, Inc.) as described previously (28). Each Affymetrix gene chip mouse exon 1.0 ST array (Affymetrix, Santa Clara, CA) was hybridized with fragmented and biotin-labeled target for 18 hrs. Arrays then were washed and stained using Genechip Fluidic Station 450, and hybridization signals were amplified using antibody amplification with goat IgG (Sigma-Aldrich) and anti-streptavidin biotinylated antibody (Vector Laboratories, Burlingame, CA, USA). Chips were scanned on an Affymetrix Gene Chip Scanner 3000, using Command Console Software. Background correction and normalization were done using Robust Multichip Average (RMA) with Genespring V 10.0 software (Agilent, Palo Alto, CA, USA). Volcano plot was used to identify differentially expressed gene list using the unpaired 2-sample student t-test as well as p-value less than or equal to 0.05 and fold change more than or equal to 2. The differentially expressed gene list was loaded into Ingenuity Pathway Analysis (IPA) 5.0 software (http://www.ingenuity.com) to perform biological network and functional analyses (29).

Transplants

SFZ cells were harvested and mixed with a collagen solution (3 mg/ml, Cellmatrix, Nitta Gelatin Inc., Osaka, Japan) at the concentration of 6.0–8.0 × 10^6/ml following the manufacturer protocol. The cell mixture (250 μl/mouse) was subcutaneously injected in athymic mice (CD-1 Nude Mouse, Charles River Laboratories International, Inc., Wilmington, MA). Transplanted cells were collected one week later and subjected to histological inspection or preparation of total RNA.

Statistical analysis

Student's t test was used to determine statistical significance between groups. P values less than 0.01 were considered significant (*$p < 0.01$ as indicated by bars).

Results

Modulation of articular cartilage superficial zone organization by β-catenin signaling

We previously created the transgenic mouse line *Col11-CA-βcatER* in which β-catenin signaling can be transiently activated in cartilage by tamoxifen administration, and we showed that signaling activation leads to significant changes in articular cartilage superficial zone (23). To better understand the nature of these changes and their underlying mechanisms, 2 week-old *Col11-CA-βcatER* mice received a daily injection of tamoxifen (or vehicle) for 7 days, and their limb joints were examined 2 weeks after the last injection. We found that their tibial superficial zone was considerably thicker and displayed a proportional increase in cell number (Figure 1B) compared to that in companion vehicle-treated transgenic or wild type mice in which it had its typical thin appearance and organization (Figure 1A). To determine whether such structural change was associated with phenotypic changes, we examined expression of *Prg4*, a key extracellular protein needed for joint lubrication and specifically expressed by SFZ cells (30–33). In controls *Prg4* expression was restricted to the thin superficial zone layer in both center (Figure 1C and 1D) and lateral side (Figure 1G and 1H) of articular cartilage and synovium (Figure 1G and 1H, syn). In tamoxifen-treated *Col11-CA-βcatER* mice however, *Prg4* expression encompassed the entire and thickened superficial zone tissue (Figure 1E, 1F, 1I and 1J) while it had not increased in synovium (Figure 1I and 1J, syn). Tissue architecture and *Prg4* expression were not affected in control wild type mice subjected to the same regiment of tamoxifen injection as previously reported (23).

In very good agreement, we observed the opposite responses when *β-catenin* was conditionally ablated in cartilage after tamoxifen injection of *Col2CreER;β-catenin* $^{fl/fl}$ mice. Thus, while control mice displayed a typical superficial zone with typical *Prg4* expression (Figure 1O, 1P, 1S and 1T), the cell number in surperficial zone was severely reduced and *Prg4* expression was barely detected in companion *β-catenin*-deficient mice (Figure 1Q, 1R, 1U and 1V). *Prg4* transcripts were normal in synovium in tamoxifen injected *Col2CreER;β-catenin* $^{fl/fl}$ mice (Figure 1U and 1V, arrows), indicating that the decrease in *Prg4* expression in the superficial zone was specific and reflected site-specific β-catenin deficiency.

The changes in superficial zone cellularity following activation or inactivation of β-catenin signaling indicate that this pathway may be very important in regulating local proliferative activity. In addition, the superficial zone is thought to contain slow cell-cycle cells that would sustain long-term self-renewal and maintenance of articular cartilage (34, 35). Thus, we examined whether modulation of Wnt/β-catenin signaling affected such slow cell-cycle population. To do so, we injected BrdU in *Col11-CA-βcatER* or *Col2CreER;β-catenin*$^{fl/fl}$ mice at postnatal stages and induced activation or inactivation of Wnt/β-catenin signaling by tamoxifen injections, respectively. Mice were then maintained for up to 7 weeks from the last injection, and their knee joints were analyzed for presence and distribution of BrdU-labeled cells. We found that in control mice the superficial zone (but not the deep zones) still contained BrdU-labeled cells after 6–8 weeks (Figure 1G and 1O, arrows), indicating that the SFZ contains long-lasting slow-cell cycle cells as previously reported (34). Activation of

Wnt/β-catenin signaling led to a significant increase in labeled cells in superficial zone (Figure 1H, arrows), while deletion of β-catenin clearly diminished the size of that cell population (Figure 1P). These findings indicate that Wnt/β–catenin signaling strongly influences the organization of articular cartilage and particularly its SFZ structure, and may regulate *Prg4* expression and the slow cell-cycle population in the SFZ.

Isolation and testing of SFZ cells

To be able to study more directly how modulations of the Wnt/β–catenin signaling affect SFZ cells, we set out to establish a method by which the cells could be effectively isolated from articular cartilage of postnatal mice and studied *in vitro*. To this end, we microsurgically isolated the epiphyseal cartilaginous ends of knee long bones of neonatal mice free of adjacent ligaments and other connective tissues, and subjected them to a 1 h trypsin incubation followed by a 1.5 h collagenase incubation. The trypsin digestion removed residual soft tissues around the epiphysis (Figure 2A), while the collagenase digestion rendered the articular cartilage superficial uneven (Figure 2C, arrows), indicating that it had removed SFZ cells. To confirm that this short collagenase incubation largely, if not exclusively, removed the superficial zone but not the entire articular cartilage, we used similar epiphyseal ends isolated from *Gdf5Cre;RosaR26R* mice in which the entire articular layers, but not secondary ossification center (SOC) and underlying growth plate cartilage, are β-galactosidase positive (18, 25). After the trypsin incubation, the epiphyseal pieces still contained β-galactosidase-positive cells and had a smooth contour (Figure 2B). After the collagenase digestion, the outer perimeter of the articular cartilage had became rough, but still contained numerous β-galactosidase positive chondrocytes (Figure 2C), indicating that the collagenase treatment released SFZ cells but had left the articular chondrocyte population behind and largely intact. To further purify the SFZ cells, we exploited the fact that SFZ cells strongly and selectively express α5 integrin (15) that we confirmed by immunostaining (Figure 2D). Thus, we allowed the collagenase-released cells to adhere to fibronectin-coated substrates for 15–20 min and removed unattached cells by washing. The attached cells, representing highly enriched SFZ cells, were then maintained in 10% FBS containing Dulbecco modified Eagle medium. To isolate chondrocytes, the residual cartilaginous tissue following the 1.5 h collagenase digestion was incubated in collagenase overnight, and the released chondrocytes were maintained under similar culture conditions.

Microscopic analysis showed that the SFZ cells displayed an elongated and fibroblastic cytoarchitecture (Figure 2E, SFZ), while chondrocytes were polygonal and epithelioid as to be expected (Figure 2D, CC). Total RNAs isolated from SFZ and chondrocyte cultures were processed for reverse transcribed-PCR analysis to assess the phenotypic characteristics of each population. The SFZ cells strongly expressed *Prg4*, *Ets*-related gene (*Erg*) *and tenascin C* all of which are abundant gene products in articular cartilage superficial (10, 18), while chondrocytes displayed much lower expression of these genes (Figure 2G). Expression of *collagen 9 a1* (*Col9a1*), *aggrecan* (*Acan*) and *matrilin-1* (*Mat-1*) was much lower in SFZ cells than chondrocytes (Figure 2H). Interestingly, SFZ cultures also expressed stem cell markers including *Sox2*, *CD34* and *CD105* (Figure 2I), but not *Oct4* (data not shown), indicating that they contain cells with stem or progenitor properties (15). We have also compared the gene expression profiles of Wnts in SFZ cells versus chondrocytes and found

significant differences in expression of Wnt2b, Wnt4, Wnt5a, Wnt11 and Wnt16 (Figure 2J). Expression of other Wnt family genes was very low in both SFZ cell and chondrocyte cultures. The data indicate that SFZ cells have unique phenotypic characteristics that distinguish them from underlying chondrocytes.

Gene Array Analysis

Next, we carried out genome-wide gene expression analyses to further characterize the phenotype and potentials of SFZ cells and compare them to those of articular chondrocytes. Populations of SFZ cells and chondrocytes were isolated from compound *Gdf5Cre;RosaR26R-EYFP* transgenic mice by differential enzymatic digestion as above. The populations were further sorted by flow cytometry to purify eYFP-positive SFZ cells and articular chondrocytes and immediately processed for RNA isolation without culturing. Gene array analyses showed that SFZ cells and articular chondrocytes expressed both predictable and unexpected sets of genes, and 170 genes showed over 2.0 fold statistical differences in gene expression levels at a threshold value set at 0.05 (Figure 3, Supplement Table 1). Thus, compared to chondrocytes the SFZ cells exhibited low expression of cartilage matrix genes including *aggrecan*, *collagen 2 a1*, *collagen 9 a1*, *collagen 11 a1* and *matrillin 1*, but higher expression of *tenascin C* and *CD44*. Interestingly, the cells strongly expressed also a number of genes that were previously linked to joint disorders and articular cartilage degeneration and including asporin (36), tumor necrosis factor alpha induced protein 6 (37), frizzled-related protein (38) and ERBB receptor feedback inhibitor 1 (39). Functional genome wide predictive analyses revealed that the genes differentially expressed by SFZ cells are highly associated with molecular networks of ‘Connective tissue disorders, Genetic disorders, Cellular assembly and Organization’ and ‘Cell-to-cell signaling and interaction, Tissue development and cancer’ (Supplement Figures 1 and 2).

Wnt/β-catenin signaling and SFZ cell function

To gain further insights into Wnt/β-catenin signaling roles in the regulation of SFZ cell behavior and phenotype, SFZ cells in primary culture as above were maintained in control conditions or treated with Wnt3a, a stimulator of Wnt/β-catenin signaling, for several days. In control cultures the cells had become polygonal and epithelioid (Figure 4A), whereas the Wnt3a-treated cells were elongated and had grown in number considerably (Figure 4B). To characterize such proliferative response, SFZ cultures were continuously treated with Wnt3a and cell numbers were determined at each passage. Wnt3a treatment did increase SFZ cell number during the first and second passage over control values, but this effect dwindled by the third passage (Figure 4E). To confirm that the effects of Wnt3a on cell proliferation were due to β-catenin-dependent pathways, the cultures were co-treated with Dkk-1, an inhibitor of Wnt/β-catenin signaling (40, 41) (Figure 4G, Wnt3a vs Wnt3a+Dkk-1). Co-treatment with Dkk-1 inhibited the Wnt3a-induced morphological changes (Figure 4D) and proliferation (Figure 4E, P2 Wnt3a vs Wnt3a+Dkk-1).

To test whether β-catenin signaling is actually required for proliferation, we isolated SFZ cells from *CagCreER;β-catenin*$^{fl/fl}$ mice and treated them with 4-hydroxytamoxifen (4OTH) for 2 days to ablate *β-catenin*; companion cells were treated with vehicle only. Cells were then replated and monitored over time. Compared to vehicle-treated controls (Figure 5A),

the β-catenin-deficient cells did not proliferate well and became flat and spread over the culture dish superficial (Figure 5B). Immunoblot analysis confirmed that the tamoxifen-treated cultures contained barely detectable levels of β-catenin (Figure 5E, lane 2), while vehicle-treated cells contained obvious amounts (Figure 5E, lane 1). Cell number quantification at first and third passage showed that β-catenin ablation had strongly and continuously inhibited cell proliferation (Figure 5F), and the inhibition of cell proliferation was not rescued by treatment with Wnt3a (data not shown). To exclude that the inhibition of cell proliferation was due to non-specific side effects of 4-hydroxytamoxifen (4OTH), we prepared SFZ cultures from β-catenin$^{fl/fl}$ mice and treated them with 4OHT. Treatment with 4OHT per se did not affect cell morphology (Figure 5D) and proliferation (Figure 5F, β-catfl/fl) as compared with vehicle-treated cultures (Figure 5C and 5F).

Next, we tested whether Wnt/β-catenin signaling affected gene expression of superficial zone gene markers *Prg4* and *Erg* over culture time and passage number. Cultures continuously treated with rWnt3a were able to maintain expression of those genes over several passages, while expression of those genes dwindled in control cultures over time (Figure 4F). Although Wnt3a treatment maintained expression of *Prg4* and *Erg*, it did not prevent the time-dependent loss of expression of the stem cell maker gene CD105 (Figure 4F). We co-treated SFZ cultures with Wnt3a and Dkk-1 or IWR-1 both of which inhibited Wnt 3a-stimulated β-catenin signaling pathway (Figure 4G), and found that Dkk-1 and IWR-1 suppressed Wnt3a action on maintenance of *Prg4* expression (Figure 4H). In addition, when we treated the cultures with BIO that is a GSK3β inhibitor and stimulates β-catenin signaling pathway (Figure 4G), we observed an increase in *Prg4* expression similar to that following Wnt3a treatment (Figure 4H). It has been shown that Wnt3a also activates non-canonical Wnt pathway, including the JNK and Ca2+/calmodulin pathways (41). However, inhibitors of these pathways did not affect and even enhanced *Prg4* expression in Wnt3a-treated SFZ culture (Figure 4H). In addition, deficiency of β-catenin in P1 SFZ culture did not change the levels of *Prg4*, *Erg*, *CD105* and *type 10 collagen* gene expression (Figure 5G), suggesting that β-catenin signaling may not directly regulate transactivation of these genes, though it is clearly required for cell proliferation and promotes maintenance of SFZ cell phenotype.

SFZ cell differentiation potentials

In a final set of experiments, we analyzed whether the SFZ cells are able to undergo chondrogenesis in *in vitro* pellet cultures or after transplantation *in vivo* and whether these potentials were modulated by Wnt/β-catenin signaling. SFZ cells were expanded in control and Wnt3a-containing cultures through six passages and were then maintained for an additional week in pellet cultures in the absence of Wnt3a. Both pellet cultures expressed similar levels of chondrogenic matrix genes such as *aggrecan*, but *Prg4* expression was much higher in Wnt3a-pretreated than control cultures (Figure 4H, *Prg4*).

To test SFZ cell differentiation potentials *in vivo*, SFZ cells isolated from *Gdf5Cre;RosaR26R* mice (to facilitate their monitoring *in vivo*) were cultured in presence or absence of Wnt3a for 7 days and transplanted subcutaneously in athymic mice. Both groups of SFZ cells formed ectopic solid tissue masses 1 week after transplantation (Figure 6A and

6C). Histological examination revealed that the control tissue was composed of small and round cells (Figure 6B, arrows) and was positive for alcian blue staining (Figure 6E and 6F) and type 2 collagen immunostaining (Figure 6I), while the Wnt3a-pretreated tissue contained more fibroblastic cells though it did stain with alcian blue (Figure 6G and 6H) and type 2 collagen antibodies (Figure 6K). Most of cells in both transplant tissues were positive to β-galactosidase staining (Figure 6M–6P), indicating that the ectopic tissues were largely composed of donor but not host cells. We isolated total RNA from the ectopic tissues and examined gene expression of markers of superficial zone cells and chondrocytes. Control tissues expressed *Prg4* and *aggrecan* and the Wnt3a-pretreated tissues expressed slightly higher levels of these genes (Figure 6Q and 6R). Interestingly, *collagen 10* was expressed in control tissue, but its level was much lower in Wnt3a-pretreated tissue (Figure 6S), indicating that the transplanted control cells had progressed toward hypertrophy while the Wnt3a-pretreated cells had retained an immature chondrocyte character.

In parallel experiments, we transplanted SFZ cells that lacked β-catenin. SFZ cells were isolated from *CagCreER;β–catenin*$^{fl/fl}$ mice, treated with 4-hydroxytamoxifen or vehicle as above, and then transplanted in athymic mice. Ectopic tissues formed by these β-catenin-deficient cells were histologically different from those formed by control cells (Figure 7A–7D). They displayed round and larger cells (Figure 7B) that were intensely stained by alcian blue pericellularly, whereas control tissues were only moderately stained (Figure 7C vs 7D). Interestingly the β-catenin-deficient tissues lacked a peripheral fibroblast layer (Figure 7B) that was evident in control tissues (Figure 7A, arrows). Gene expression analysis showed that the β-catenin-deficient tissues were characterized by strong expression of *aggrecan* and *collagen 10* (Figure 7F and 7G) and low expression of *Prg4* (Figure 7E) compared to control tissues. Thus deletion of β-catenin induced a loss of SFZ cell character and stimulated chondrogenic differentiation.

Discussion

We show here that Wnt/β-catenin signaling has essential roles in the regulation of SFZ organization and function *in vivo* and strongly supports proliferation and maintenance of SFZ cell phenotypic characteristics. The latter conclusion is based on direct analyses of SFZ cells made possible by our novel procedure by which the cells can be isolated from neonatal mouse articular cartilage and studied *in vitro*. Our gene array and *in vitro* studies reveal also that SFZ cells have expression and phenotypic profiles distinct from those of underlying articular chondrocytes, can maintain their *in vivo* articular SFZ characteristics in culture and have chondro-progenitor properties.

Superficial cells were previously isolated from bovine articular cartilage and found to exhibit chondro-progenitor properties, high colony formation efficiency and strong proliferative activity lasting over 45 cell population doubling (15). The SFZ cells we obtain from mouse epiphyseal ends actively proliferate until the 3rd passage and essentially stop proliferating by the 6th passage. What could be the reasons behind these apparent conflicting results? One likely possibility is that our cultures contain the overall articular cell superficial population that may include genuine superficial cells expressing *Prg4* and *Erg* as well progenitor or stem cells expressing genes such as CD105. We have not attempted yet to select for a

progenitor subpopulation as it was done in the bovine studies. Our interpretation is consistent with our findings that the mouse SFZ cultures express stem cell markers at 1st passage but not at later passages. Once transplanted into athymic mice, the mouse SFZ cells form ectopic cartilaginous tissue as revealed by morphology, gene expression and production of a highly sulfated proteoglycans matrix. This indicates that SFZ cells do have chondrogenic potentials, could serve as a source of articular chondrocytes and could play a key role in articular cartilage maintenance and appositional growth.

Several studies in a variety of systems have provided clear evidence for the importance of the SFZ in joint function (11, 14, 42, 43), and more recent work points to the likely roles of SFZ cells in self-renewal and maintenance of articular cartilage (15, 16). What has remained largely unclear is which signaling molecules and pathways regulate SFZ function, and our data indicate that the Wnt/β-catenin signaling represents one such critical pathway. We now show that acute activation of Wnt/β-catenin signaling *in vivo* increases cell number and thickness of superficial zone, and is accompanied by a proportional increase in *Prg4* expression, while β–catenin ablation elicits the opposite effects. We also show that SFZ cells *in vitro* maintain their phenotypic characteristics under chronic stimulation of Wnt/β-catenin signaling elicited by exogenous Wnt3a. A recent report has suggested that Wnt3a stimulates both canonical and non-canonical Wnt signaling in articular chondrocytes, and that the non-canonical pathway is important for inhibition of cartilage matrix gene expression while the canonical β-catenin-dependent pathway promotes cell proliferation (41). Our data indicate that stimulation of cell proliferation and maintenance of SFZ phenotype by Wnt3a treatment are dependent on β-catenin signaling. While untreated SFZ cell cultures displayed increases in collagen 2 and aggrecan expression over passage number, expression of these chondrocyte markers remained low in companion Wnt3a-treated cultures. Because co-treatment with Dkk-1 or IWR-1 did not alter these outcomes, it is very likely that Wnt3a inhibited cartilage gene matrix expression through a non-canonical pathways in SFZ cells as recently reported in articular chondrocyte cultures (41). In reciprocal experiments, we show that SFZ cells deficient in β-catenin lost proliferating activity and gave rise to ectopic cartilaginous tissue masses when transplanted. Thus it is clear from our experiments that β-catenin function is important to maintain SFZ cell phenotypic characteristics and to prevent SFZ cells from expressing an articular chondrocyte phenotype. It remains unclear whether specific Wnt proteins control β-catenin signaling in SFZ, whether other signaling molecules are involved and whether β-catenin may also be required for cell adhesion functions in the regulation of SFZ organization and function.

Our findings indicate that acute activation of Wnt/β-catenin signaling induces thickening of SFZ *in vivo* and SFZ cell proliferation *in vitro*. Thus, it is possible and plausible that a similar acute local stimulation of Wnt/β-catenin signaling could have therapeutic value and joint disease prevention applications. It could be used to transiently reactivate SFZ proliferation, stimulate phenotypic expression and encourage the cells to repopulate articular cartilage and restore function. These enticing possibilities have to be weighted against evidence that activation of Wnt/β-catenin signaling may cause articular cartilage degeneration and joint disease when chronic or ongoing for protracted time periods (19, 27, 44). Thus, a therapeutic strategy would be particularly useful in situations in which endogenous signaling is low and the joints are not in their terminal degenerative state. In

such cases, signaling could be activated for a short period during which SFZ cell proliferation and phenotypic expression could be stimulated without causing long-term damage to articular tissue.

In summary, our study has revealed that the function and organization of articular cartilage superficial zone are strongly influenced and regulated by Wnt/β-catenin signaling. This pathway could be critical for long-term maintenance of articular cartilage and joint function and has the potential to serve as a possible therapeutic target to stimulate superficial zone function and in turn joint regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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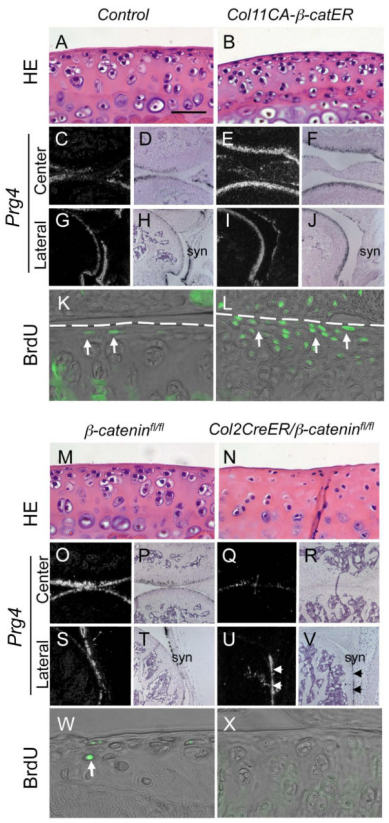

Figure 1.

Changes in articular cartilage organization, *Prg4* expression and slow-cell cycle cell number following β-catenin signaling modulation. A–L, 2 week-old *Col11-CA-β-catER* transgenic mice (B, E, F, I, J and L) and wild type littermates (A, C, D, G, H and K) received 7 daily peritoneal injections of tamoxifen (200 μg/20 μl/mouse) and were sacrificed at 7 weeks of age. M–X, *β-catenin*$^{fl/fl}$ (M, O, P, S, T and W) or *Col2CreER;β-catenin*$^{fl/fl}$ (N, Q, R, U, V and X) mice received 3 daily peritoneal injections of tamoxifen (100 μg/10 μl/mouse) starting at P5 and were sacrificed at 7 weeks. Some mice (K, L, W and X) also received a daily intraperitoneal injection of BrdU (25 mg/kg) 5 times starting from P11. Longitudinal sections of proximal tibial articular cartilage were stained with hematoxylin and eosin (HE; A, B, M and N) or processed for in situ hybridization analysis of *Prg4* (C, E, G, I, O, Q, S and U for dark field images and D, F, H, J, P, R, T and V) for bright field images). White arrows indicate BrdU-labeled cells (K, L and W). Bars represent: 10 μm for A, B, M and N; 80 μm for C–J and O–V; and 6.7 μm for K, L, W and X.

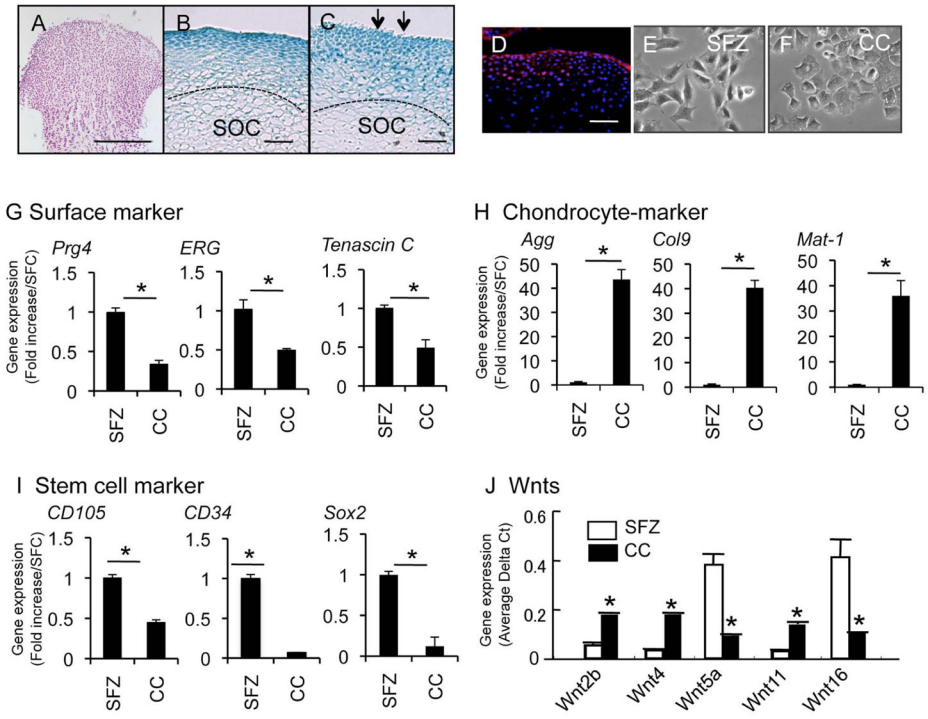

Figure 2.

Isolation of SFZ cells and gene expression analysis. A, Sections of mouse neonatal epiphyseal ends stained with H&E after incubation with 0.25% trypsin for 1 h. B and C, Sections of mouse neonatal epiphyseal ends from *Gdf5Cre;RosaR26R-LacZ* mice stained for β-galactosidase after incubation with 0.25% trypsin for 1 h (B) followed by 1.5 h digestion with 173U/ml collagenase (C). Dotted lines demarcate future secondary ossification center (SOC). D, Section of tibial epiphyseal end immunostained for α5 integrin; nuclei were counter-stained with DAPI (blue). Bars represent 200 μm for A and 20 μm for B–D. E and F, Phase contrast images of SFZ (E) and chondrocyte (F) cultures. Pictures were taken 48 h after plating. G–J, Total RNAs were prepared from SFZ or chondrocyte (CC) confluent cultures and subjected to quantitative PCR analysis for SFZ markers (G: *Prg4*, *Erg* and *Tenascin C*), chondrocytes marker (H: *aggrecan* (*Acan*), *collagen 9 a1* (*Col9a1*) and *matrilin-1* (*Mat-1*) or stem cell markers (I: *CD105*, *CD34* and *Sox 2*). Gene expression of Wnt family genes was examined by PCR array (RT2 Profiler PCR array for Wnt signaling pathway). *p<0.05.

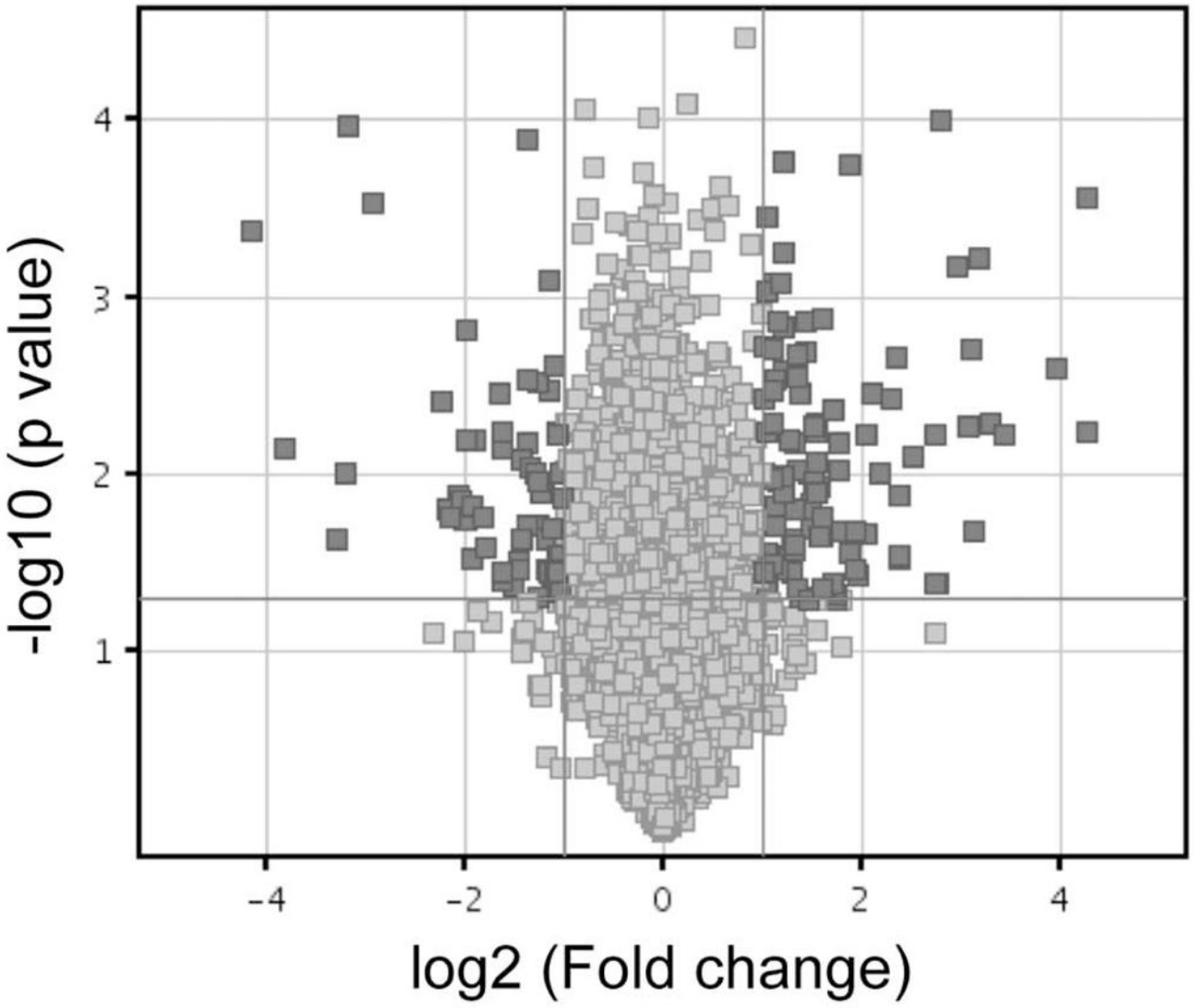

Figure 3.

Volcano plot of microarray data. Plot depicts differences in SFZ cell and articular chondrocyte expression patterns, and the dark gray plots represent more than or equal to 2.0 fold differentially expressed genes between the two groups at $p<0.05$.

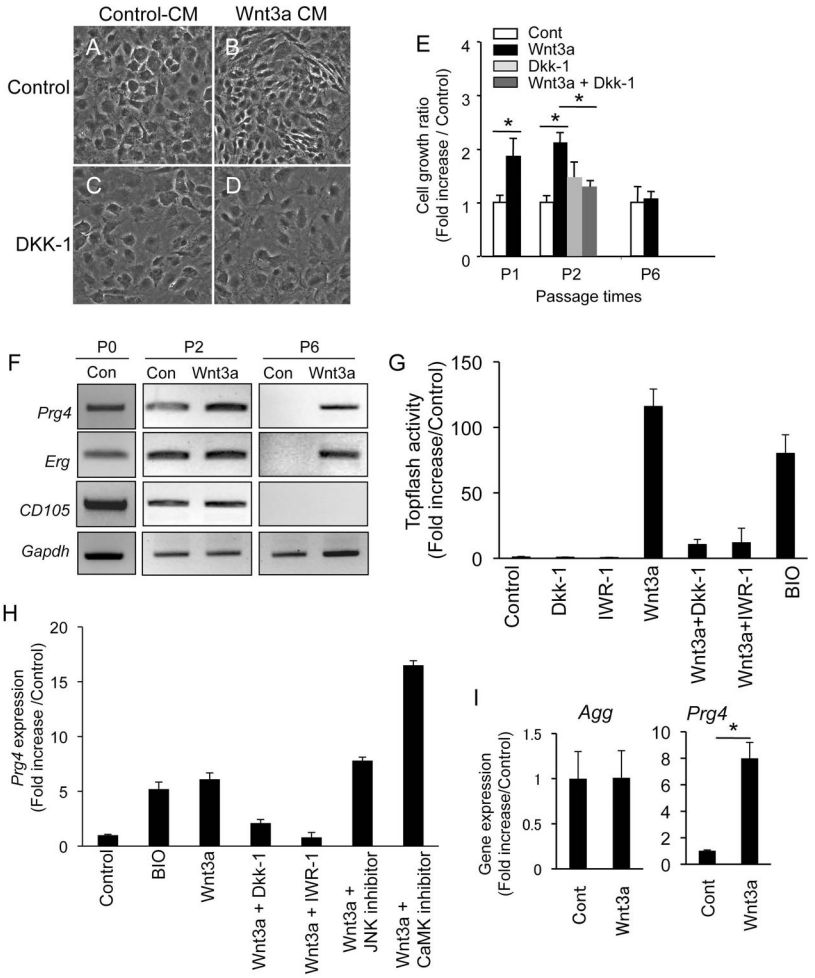

Figure 4.

Effects of Wnt3a on proliferation and gene expression in SFZ cultures. SFZ cells were isolated from epiphysial cartilage of wild type mice and serially passaged at the density of 4,200/cm^2 up to 6 times. A–F and I, The cultures were continuously treated with Wnt3a-containing conditioned medium (30%) or control conditioned medium after 1st passage in the presence or absence of 100 ng/ml of DKK-1. Phase contrast pictures were taken on day 4 in 2nd passage cultures (A–D). Cell numbers were counted at indicated passages; values are average and SD from three independent samples (E). Total RNAs were prepared from Wnt3a-treated or control SFZ cell cultures at indicated passages to analyze expression levels of *Prg4*, *Erg*, *CD105* and *Gapdh* by RT-PCR as described in Materials and Methods (F). Sixth passage cultures treated with or without Wnt3a were transferred into pellet cultures and cultured for 7 days. Total RNAs were then prepared to analyze expression level of *aggrecan* (*Acan*) or *Prg4* (I). G, The SFZ cells were transfected with Topflash Wnt reporter plasmid, treated with Wnt3a-containing conditioned medium (30%) or control conditioned medium in the presence or absence of 6BIO (BIO, 1 μg/ml), 100 ng/ml of DKK-1 or 10 μM IWR-1 for 24 h, and then the luciferase activity was measured. H, The cultures were continuously treated with Wnt3a-containing conditioned medium (30%) or control

conditioned medium after 1st passage in the presence or absence of 6BIO (BIO, 1 μg/ml), 100 ng/ml of DKK-1, 10 μM IWR-1, 10 μM SP600125 (JNK inhibitor) or 10 μM KN-93 (Ca2+/calmodulin-dependent protein kinase II inhibitor, CaMK inhibitor). Total RNAs were prepared from the SFZ cultures at passage 6 to analyze expression levels of *Prg4* by real time PCR. *$p<0.05$.

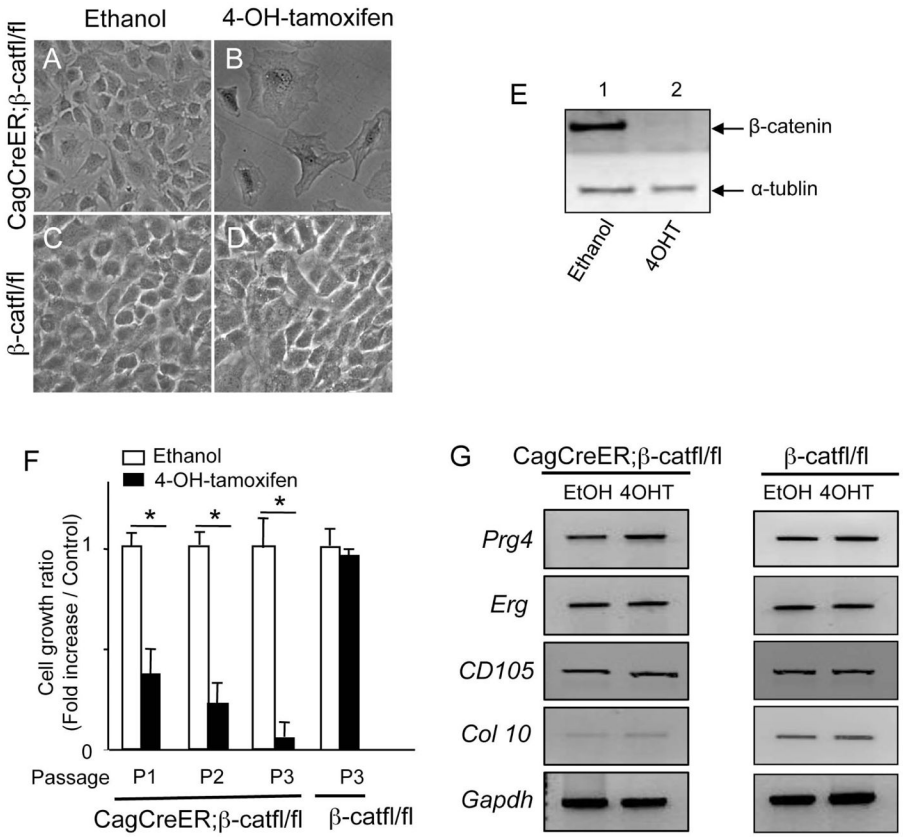

Figure 5.

Effects of β-catenin ablation on proliferation and gene expression in SFZ cultures. SFZ cells were isolated from epiphysial cartilage of *CagCreER;β-catenin*$^{fl/fl}$ or *β-catenin*$^{fl/fl}$ mice. Cultures were serially passaged at the density of 4,200/cm^2 up to 6 times. The *CagCreER/β-catenin*$^{fl/fl}$ or *β-catenin*$^{fl/fl}$ cell cultures were treated with 1 μM 4–hydroxytamoxifen (4-OH-tamoxifen) or vehicle (ethanol) for 2 days just after 1st passage and no further treatment was performed. Cultures were passaged two more times. A–D, Phase contrast pictures were taken on day 4 in 2nd passage cultures. E, Cell lysates prepared from 1st passage culture of *CagCreER/β-catenin*$^{fl/fl}$ SFZ cells were subjected to immunoblot analysis for β-catenin or α-tubulin. F, Cell numbers were counted at indicated passages; values are average and SD from three independent samples. H, Total RNAs were prepared from *CagCreER/β-catenin*$^{fl/fl}$ or *β-catenin*$^{fl/fl}$ cell cultures at P2 to analyze expression levels of *Prg4*, *Erg*, *CD105* and *Gapdh* by real time-PCR. *p<0.05.

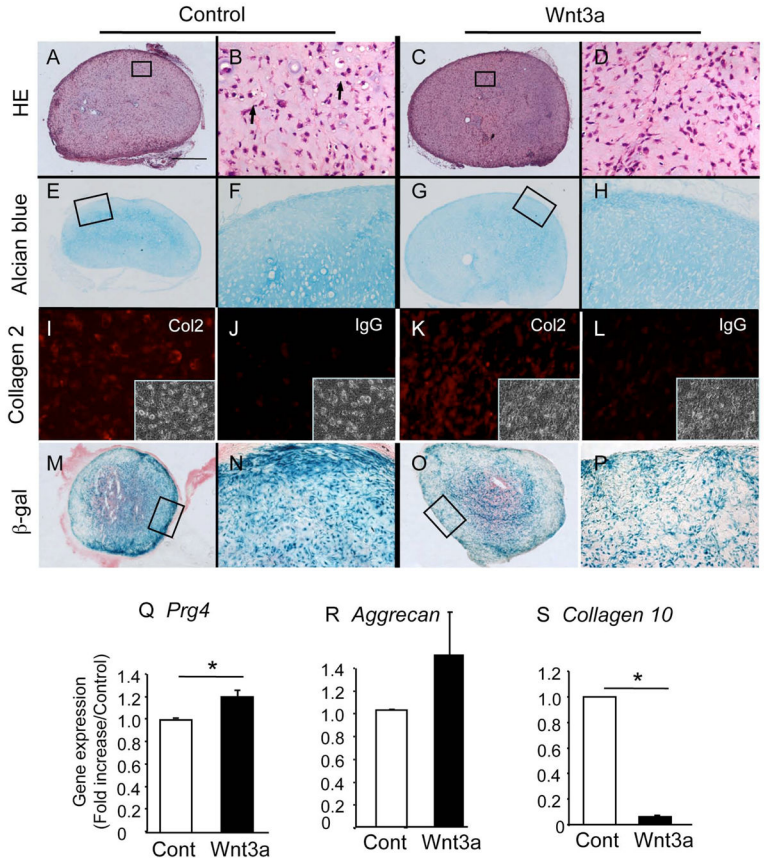

Figure 6.

Histology and gene expression of transplanted SFZ cells. Cells isolated from *Gdf5Cre;RosaR26R-LacZ* neonatal mice were subcultured at 1:10 ratio, treated with Wnt3a-containing conditioned medium (30%v/v) (C, D, G, H, K, L, O P) or control medium (A, B, E, F I, J, M, N) until confluent and then subcutaneously transplanted into athymic mice. Resulting ectopic tissue masses were harvested one week later and sections were stained with hematoxylin and eosin (A–D, HE) or alcian blue (E–H), or processed for collagen 2 immunostaining (I–L, I and K incubated with anti-collagen 2 antibody and J and L incubated with preimmune rabbit IgG) or for β-galactosidase staining (M–P, β-gal). Samples were also used for expression analysis for *Prg4* (Q), *aggrecan* (R) and *collagen 10* (S, *Col10*). B, D, F, H, N, and P are magnified images corresponding to squared areas in A, C, E, G, M and O, respectively. The bar represents 200 μm for A, C, E, G, M and O, and 50 μm for B, D, F, H, J–L, N and P. *p<0.05.

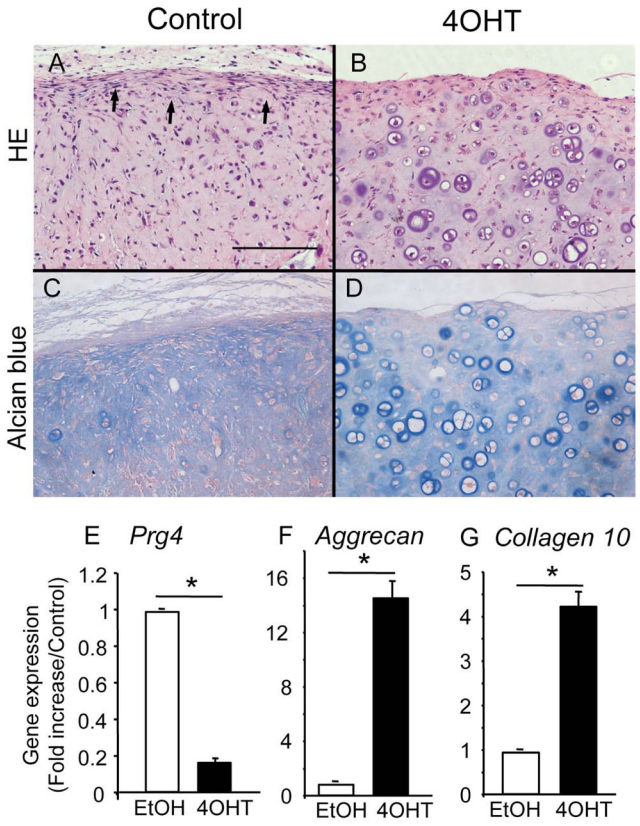

Figure 7.

Histology and gene expression of transplanted β-catenin-deficient SFZ cells. Cells isolated from *CagCreER/β-catenin*$^{fl/fl}$ neonatal mice were subcultured at 1:10 ratio, treated with ethanol (A, C) or 1 μM 4-hydroxytamoxifen (B, D, 4OHT) for 2 days, further cultured until confluent without 4-hydroxyytamoxifen and finally transplanted subcutaneously into athymic mice. Ectopic masses were harvested one week later and subjected to hematoxylin and eosin staining (A, B) and alcian blue staining (C, D)) or gene expression analysis for *Prg4* (E), *aggrecan* (F) and *collagen 10* (G). Arrows indicate elongated cells surrounding the control transplant tissue. Bar represents 50 μm. *p<0.05.