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Endogenous DKK1 and FRZB Regulate Chondrogenesis and Hypertrophy in Three-Dimensional Cultures of Human Chondrocytes and Human Mesenchymal Stem Cells

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Hypertrophic differentiation occurs during in vitro chondrogenesis of mesenchymal stem cells (MSCs), decreasing the quality of the cartilage construct. Previously we identified WNT pathway antagonists Dickkopf 1 homolog (DKK1) and frizzled-related protein (FRZB) as key factors in blocking hypertrophic differentiation of human MSCs (hMSCs). In this study, we investigated the role of endogenously expressed DKK1 and FRZB in chondrogenesis of hMSC and chondrocyte redifferentiation and in preventing cell hypertrophy using three relevant human cell based systems, isolated hMSCs, isolated primary human chondrocytes (hChs), and cocultures of hMSCs with hChs for which we specifically designed neutralizing nano-antibodies. We selected and tested variable domain of single chain heavy chain only antibodies (VHH) for their ability to neutralize the function of DKK1 or FRZB. In the presence of DKK1 and FRZB neutralizing VHH, glycosaminoglycan and collagen type II staining were significantly reduced in monocultured hMSCs and monocultured chondrocytes. Furthermore, in cocultures, cells in pellets showed hypertrophic differentiation. In conclusion, endogenous expression of the WNT antagonists DKK1 and FRZB is necessary for multiple steps during chondrogenesis: first DKK1 and FRZB are indispensable for the initial steps of chondrogenic differentiation of hMSCs, second they are necessary for chondrocyte redifferentiation, and finally in preventing hypertrophic differentiation of articular chondrocytes.

Keywords: cell signaling, chondrogenesis, differentiation, MSC

Introduction

Articular cartilage repair is a challenge due to very limited capacity of self-repair after damage. Autologous chondrocyte implantation (ACI) has become the golden standard treatment for large-size cartilage defects [1]. However, ACI leads to donor-site morbidity and is dependent on 2D expansion of isolated chondrocytes, which are prone to undergo dedifferentiation during proliferation [2]. To overcome this drawback, a (partial) replacement of chondrocytes with mesenchymal stem cells (MSCs) is an option. Although the application of MSCs in cartilage repair is promising, the use of MSCs in cartilage engineering still has some drawbacks. One of the problems with using MSCs for cartilage regeneration is the expression of hypertrophic markers during in vitro chondrogenesis followed by formation of transient calcifying cartilage [3].

So maintaining the chondrogenic phenotype during in vitro expansion and avoiding hypertrophy of chondrogenically differentiating MSCs remain a big challenge in these cell-based strategies [4]. Successful chondrogenesis of human MSCs (hMSCs) is regulated by various signaling pathways, including FGF, TGF-β, WNT/β-catenin, Notch, and hypoxia [5]. Articular chondrocytes produce a stable nonmineralizing cartilage [6], and coculture of MSCs and articular cartilage reduces hypertrophy [7] and enhances functional properties of engineered cartilage [8].

WNT/β-catenin signaling plays a pivotal role in articular cartilage [9], where enhanced signaling activity results in cartilage destruction through chondrocyte hypertrophic

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differentiation and subsequent development of an osteoarthritis (OA)-like phenotype [10]. Decreased WNT activity also results in cartilage destruction through the induction of apoptosis [11]. Therefore, maintaining strict control over WNT/β-catenin signaling in articular cartilage is of the highest importance.

We previously found that the WNT antagonists Dickkopf 1 homolog (DKK1) and frizzled-related protein (FRZB) are highly enriched in articular cartilage compared to epiphyseal growth plate cartilage [12]. These antagonists constitute a key part of the natural mechanism that balances WNT/β-catenin signaling activity. DKK1 antagonizes WNT signaling pathway by binding to the frizzled coreceptors' low-density lipoprotein receptor–related protein 5 (LRP-5) and LRP-6 [13,14]. Secreted frizzled-related proteins (sFRP) antagonize WNT signaling through interactions with WNTs and/or through the formation of nonfunctional complexes with the frizzled receptors [15–17], resulting in the inhibition of both canonical and noncanonical pathways [18]. DKK1 increases hMSC proliferation and promotes entry into the cell cycle in vitro [19]. In addition, we have previously shown that in chondrogenically differentiating hMSC pellet cultures and in explanted mouse tibiae, exogenous addition of DKK1 or FRZB prevented terminal hypertrophic differentiation of chondrocytes, supporting their role in articular cartilage homeostasis [12].

WNT pathway activity repressed the expression of cartilage-specific extracellular matrix (ECM) molecules and might be involved in chondrocyte dedifferentiation during in vitro expansion of primary human chondrocytes (hChs) [20,21]. Healthy articular chondrocytes keep their differentiated state and do not undergo dedifferentiation suggesting that endogenous WNT/β-catenin signaling is blocked by an unknown mechanism. DKK1 and FRZB are natural antagonists and are highly expressed by healthy hChs [12]. We previously found that when cultured under prolonged passaging, chondrocytes lose their phenotype and that this is accompanied by changes in expression of DKK1 and FRZB [22]. This indicates that the combined endogenous expression of DKK1 and FRZB plays a role in chondrocyte dedifferentiation.

So the correct regulation of the WNT signaling plays an important role in the maintenance of the chondrogenic potential, as well as in suppression of endochondral ossification [23]. However, the mechanism by which WNT signaling is regulated in vivo is as yet unknown. It has been reported that FRZB knockout mice have no cartilage phenotypic changes, but are more susceptible to OA in mechanical injury [24]. DKK1 knockout mice show no change in cartilage degeneration; only osteophyte formation is increased upon mechanical loading [24]. This suggests the functional redundancy of DKK1 and FRZB because both of them are capable of inhibiting the WNT/β-catenin pathway, one can compensate for the loss of the other. We propose that the combined endogenous expression of DKK1 and FRZB plays a determining role in both the chondrogenesis of hMSCs and prevention of hypertrophic differentiation in vivo.

There is ample evidence that the correct modulation of WNT activity is necessary and sufficient for the formation and maintenance of healthy articular cartilage. However, the exact mechanisms regulating the amplitude of WNT activity in articular cartilage in vivo are as yet unknown and are the subject of this project. We hypothesized that endogenous expression of DKK1 and FRZB is both necessary and sufficient for articular cartilage formation and prevention of hypertrophic differentiation. Specifically, we hypothesized that simultaneously blocking DKK1 and FRZB would negatively influence the chondrogenic potential of hMSCs and dedifferentiated chondrocytes and that blocking endogenous DKK1 and FRZB leads to hypertrophic differentiation. To prove our hypotheses, we have evaluated the combined role of endogenous levels of DKK1 and FRZB by blocking their activity using neutralizing VHH antibodies in three relevant human cell based systems: isolated hMSCs, isolated primary hChs, and cocultures of hMSCs with hChs.

Materials and Methods

Selection of anti-DKK1 VHH from immune VHH library

Two llamas were immunized with recombinant human DKK1 (R&D Systems) as described in reference 25. The immunization protocol was approved by the Utrecht University Ethics Committee (DEC: 2007.III.01.013). Total RNA was extracted from peripheral blood lymphocytes; cDNA was synthesized. VHH were subsequently cloned in phagemid pUR8100, which was derived from pHEN [26] described by reference 27. VHH binding to DKK1 was selected by panning in two rounds of selection as described [25]. Screening of the selected VHH for binding to DKK1 led to the identification of 14 different VHH. The amino acid sequences of the VHH show different clones from different families (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd), of which two (G5 and H7) were recurrent and, therefore, used for further analysis.

To test the affinity of the anti-DKK1 VHH, sequences were subcloned into the expression plasmid pMEK219 containing C terminal Myc and His tags. VHH were subsequently produced from *Escherichia coli* by induction of the lac promoter with 1 mM isopropyl β-D-1-thiogalactopyranoside [28,29] and were purified from the periplasmic fraction through the C terminal His-tag by cobalt affinity chromatography (TALON His-Tag Purification Resin; Clontech). The size and purity of the VHH were assessed by SDS-PAGE [30,31].

Purified VHH were tested by enzyme-linked immunosorbent assay (ELISA). Plates were coated with DKK1 (60 nM) and blocked with 4% skimmed milk in phosphate-buffered saline (MPBS), then incubated with a concentration range of VHH (0–7 μM). Unbound VHH were washed with PBS-Tween (PBST), and bound VHH were detected by incubation with mAb anti-myc (9E10) and a horseradish peroxidase-conjugated anti-mouse.

To assess the biological activity of the anti-DKK1 VHH, KS483-4C3 mouse progenitor cells were used as a model for osteogenic differentiation [32]. Cells were seeded at 10,000 cells/cm^2 (day 0). At day 4, cells were cultured for another 3 days with ascorbic acid (50 μg/mL; Sigma Aldrich) and stimulated with BMP6 (100 ng/mL; R&D Systems) in the presence or absence of DKK1 (300 ng/mL; R&D Systems) with a concentration series of VHH G5 or H7 (0–70 nM). At day 7, alkaline phosphatase (ALP) activity was evaluated by CDP-Star Kit (Roche). Luminescence was measured using Vector Microplate Luminometer (Promega). The luminescence units were corrected for DNA content. DNA concentration was determined using the CyQUANT Cell Proliferation Assay (Invitrogen).

Selection of anti-FRZB from a nonimmunized llama VHH library

VHH binding to FRZB (R&D Systems) was selected from nonimmunized llama VHH-phage display library [33], kindly provided by BAC B.V. (Thermo Fisher) in two panning rounds [33]. Selection and screening were as described for the anti-DKK1 VHH, with the exception of applying more phages for the first round of selection [33]. Screening of the FRZB binders led to identification of five VHH candidates. The amino acid sequences of the VHH are indicated in Supplementary Fig. S2. Anti-FRZB VHH were cloned in the expression plasmid pMEK222 containing C terminal FLAG and His tags. Production and purification of the VHH were as described for the anti-DKK1 VHH. Apparent affinity of the purified FRZB VHH was measured with ELISA as described for anti-DKK1, with the exception of detecting bound VHH with mAb M1 directed against FLAG instead of mAb 9E10.

Cell culture and expansion

Human primary chondrocytes were obtained from relatively healthy looking full thickness cartilage, dissected from knee biopsies of three patients [mean ± standard deviation (SD) age 60 ± 3 years] undergoing total knee replacement, as described previously [34]. To isolate cells, the cartilage was digested in chondrocyte proliferation medium containing collagenase type II (0.15%; Worthington) for 20–22 h. Subsequently, the hChs were expanded at a density of 3,000 cells/cm^2 in chondrocyte proliferation medium until the monolayer reached 80% confluency. Chondrocyte proliferation medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× nonessential amino acids, 0.2 mM ascorbic acid 2-phosphate (AsAP), 0.4 mM proline, 100 U/mL penicillin, and 100 µg/mL streptomycin. The hChs were used in passage two unless otherwise stated. The hMSCs were isolated from human bone marrow aspirates as described previously [34] and cultured in MSC proliferation medium (α-MEM supplemented with 10% FBS, 1% L-glutamax, 0.2 mM ascorbic acid, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 ng/mL bFGF).

Pellet cultures and chondrogenic differentiation

To get enough cell pellets for analysis, micropatterned agarose chips were used in this experiment. Micropatterned agarose chips were prepared at a concentration of 4% w/v by replica molding as described previously [35]. Polydimethylsiloxane stamps were used to routinely replicate the microstructures. For monocultures, 250,000 cells of hChs or hMSCs were seeded into 1,585 microwells of micropatterned agarose chips. This resulted in roughly 160 cells/pellet.

For cocultures, 250,000 cells were seeded at a ratio of hMSC/hChs = 80/20. Cells were suspended in chondrogenic differentiation medium [DMEM supplemented with 50 µg/mL ITS-premix, 50 µg/mL of AsAP, 100 µg/mL of sodium pyruvate, 10 ng/mL of TGF-β3, 10^{-7} M of dexamethasone (DEX), 100 U/mL of penicillin, and 100 µg/mL of streptomycin] and pipetted into the agarose wells. Subsequently, the agarose chips were centrifuged for 5 min at 500 *g* to form pellets. Cell pellets were incubated in the microwells at 37°C in a humid atmosphere with 5% CO_2 for a period of 4 weeks before analysis. The medium was supplemented with 5 µg/mL of VHH anti-DKK1 (D-H7) and VHH anti-FRZB (TSF-1F7). Nonspecific goat IgG (5 µg/mL) was used as a negative control. The medium, with antibody supplements, was refreshed twice per week.

Total RNA extraction and quantitative polymerase chain reaction

Cell pellets were pooled and RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany). The concentration and purity of RNA samples were determined using the NanoDrop 2000 (Thermo scientific, Wilmington, United States). Total mRNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative polymerase chain reaction (qPCR) was performed using the SYBR Green SensiMix (Bioline). PCR was carried out using the Bio-Rad CFX96 (Bio-Rad) under the following conditions: cDNA was denatured for 5 min at 95°C, followed by 39 cycles consisting of 15 s at 95°C, 15 s at 60°C, and 30 s at 72°C. For each reaction, a melting curve was generated to test primer dimer formation and nonspecific priming. Primer sequences are listed in Supplementary Table S1.

Histological analysis

Cell pellets were collected after 4 weeks incubation and fixed with 10% phosphate-buffered formalin (pH = 7) for 15 min at room temperature, dehydrated with graded ethanol, and embedded in paraffin using routine procedures. Five micrometer thick sections were cut using a microtome (Shandon). Before staining, the slides were deparaffinized in xylene and rehydrated with graded ethanol.

Alcian blue and safranin O staining. Slides were either stained for sulfated glycosaminoglycans (GAGs) with a 0.5% w/v solution of Alcian blue (pH = 1, adjusted with HCl) for 30 min. The samples were then counterstained with nuclear fast red (0.1% w/v of nuclear fast red in 5% aluminum sulfate) for 5 min to visualize nuclei [36] or stained for sulfated GAG with a 0.1% solution of safranin O for 5 min (Sigma Aldrich).

Alizarin red S staining. Slides were stained with a 2% w/v solution of Alizarin red S (pH = 4.2, adjusted with ammonium hydroxide) to assess the presence of mineralization. Light microscopy was used to visualize red mineralized nodules as described [36].

Immunohistochemistry

Immunohistochemical staining of collagen type II was performed using 5 µm sections. Slides were deparaffinized in xylene and rehydrated with graded ethanol. Samples were preincubated with 5 µg/mL proteinase K (Sigma Aldrich) for 10 min at room temperature followed by 1 mg/mL hyaluronidase (Sigma Aldrich) for 40 min at 37°C. Samples were blocked in 1.5% normal goat blocking serum in PBS for 1 h. Rabbit polyclonal collagen type II antibody (Abcam; ab34712) was diluted 1:200 in PBS containing 1.5% blocking serum (Santa Cruz Biotechnology) and incubated overnight at 4°C. Nonimmune controls underwent the same procedure without primary antibody incubation. The target protein was detected by incubation in rabbit ABC staining system (sc-2018; Santa Cruz) according to the manufacturer's protocol and imaged using a NanoZoomer.

Immunofluorescent staining

Immunofluorescent (IF) staining of collagen type II and collagen type X was performed using 5 µm sections. Slides were deparaffinized in xylene and rehydrated with graded ethanol. Samples were preincubated with 5 µg/mL proteinase K (Sigma Aldrich) for 10 min at room temperature followed by 1 mg/mL hyaluronidase (Sigma Aldrich) for 40 min at 37°C. Samples were blocked in 5% BSA in PBS for 1 h, then incubated with rabbit anti-collagen II antibody (ab34712; Abcam), which was diluted 1:100 in 5% BSA in PBS and mouse collagen type X (2031501005; Quartett LifeSpan), which was diluted 1:50 overnight at 4°C. Cells were rinsed with PBST thrice, 5 min/time. Then Alexa®Fluor 546-labeled goat anti-rabbit or anti-mouse antibody in 5% BSA in PBS was added and incubated for 2 h at room temperature. Samples were rinsed with PBS and added mounting medium with DAPI. Slides were viewed by BD pathway confocal microscopy.

Apoptosis assay

Apoptosis was detected in paraffin-embedded pellets using The DeadEnd™ Colorimetric TUNEL assay (Promega) following the manufacturer's procedure. Apoptotic nuclei were stained dark brown.

Statistical analysis

For the experiments using primary human cells (hMSCs and hChs), samples were obtained from three hCh donors and two hMSC donors. Each experiment was performed in triplicate. Statistical differences between two groups were analyzed by two-tailed Student's *t*-tests or one-way ANOVA. $P < 0.05$ was considered statistically significant and indicated with an asterisk. Data are expressed as the mean ± SD.

Results

The camelidae family expresses a special class of antibodies that are devoid of light chains [37]. These antibodies are called single domain antibodies engineered from heavy chain camelid antibodies (VHH), which are formed by two protein chains. Compared with conventional antibodies, using VHH has several advantages, including small size (15 kDa) and high binding specificity and affinity [38], simple and easy to clone, and subsequently to genetically modify, they can be produced in bacteria and yeast [38,39]. Moreover, VHH antibodies easily penetrate into 3D cell pellets. Selection of functional VHH is of the utmost importance to determine the quality of the data and is not trivial. We therefore describe the selection of the VHH against DKK1 and the VHH against FRZB in the sections below.

Selection of anti-DKK1 VHH

Anti-DKK1 VHH were selected from llama VHH-phage display libraries immunized with recombinant DKK1. The phage display library is constructed from RNA of peripheral blood mononuclear cells isolated from the immunized llama. Selection led to the identification of two VHH, G5 and H7, which display apparent affinities (Kd) of 5.9×10^{-8} M and 1.1×10^{-7} M, respectively (Fig. 1A). The ability of the two VHH to modulate DKK1 biological activity was tested in vitro in murine KS483-4C3 mesenchymal precursor cells, which were induced to express ALP after treatment with BMP6. BMP-induced ALP expression is mediated through canonical WNT-signaling [10]. Indeed cotreatment of cells with BMP6 and DKK1 effectively abrogated ALP induction (Fig. 1B). This inhibition could be reversed by the addition of VHH G5 or H7 in a dose-dependent manner (Fig. 1C, D). Both VHH showed effective neutralization of DKK1 in this bioassay.

Selection of anti-FRZB VHH

VHH against FRZB were selected from a nonimmune llama VHH-phage display library. Two rounds of panning selection led to the selection of eight candidate VHH. After subcloning into pMEK222 and sequence determination, five VHH with different sequences were successfully expressed and purified. ELISA was used to rank the different VHH according to the apparent affinities (Fig. 1E).

Functional analysis of anti-DKK1 and anti-FRZB VHH

Functional assays were performed to choose the most efficient VHH out of two DKK1 VHH and five FRZB VHH in blocking the function of DKK1 or FRZB in human primary chondrocytes, respectively. We found that H7 was most efficient in blocking DKK1, and TSF-1F7 was most effective for blocking FRZB (Supplementary Fig. S3A, B). Although TSF-1F7 has the lowest affinity to FRZB based on ELISA, it has the best neutralizing efficiency in cells compared to the other four FRZB VHH. This might be due to specific binding of TSF-1F7 to the biological domain of endogenous FRZB thereby blocking its function, while the other four FRZB VHH bind other FRZB domains.

VHH against DKK1 and FRZB function as neutralizing antibodies as measured by restored WNT signaling

Dose–response experiments were performed to determine the optimum concentration of the VHH in blocking DKK1 and FRZB. A concentration of 5 µg/mL of neutralizing VHH completely reversed the blocking effect of DKK1 or FRZB on WNT3A-induced *AXIN2* mRNA expression as determined by qPCR analysis (Fig. 2A, B). This concentration of 5 µg/mL was used in all subsequent experiments. At the same concentration, a nonspecific IgG control did not have any effect on the expression of the examined marker genes (Fig. 2C–E).

Blocking DKK1 and FRZB inhibits both chondrogenesis of hMSCs and chondrocyte redifferentiation

We have found that the endogenous expression levels of DKK1 and FRZB in hMSCs are low (Supplementary Fig. S4), while their expression increases during hMSC chondrogenesis (Supplementary Fig. S5). This suggests that DKK1 and FRZB are involved in chondrogenesis of hMSCs. To prove this, neutralizing VHH were added to block DKK1 and FRZB in monoculture hMSC pellets during chondrogenic induction.

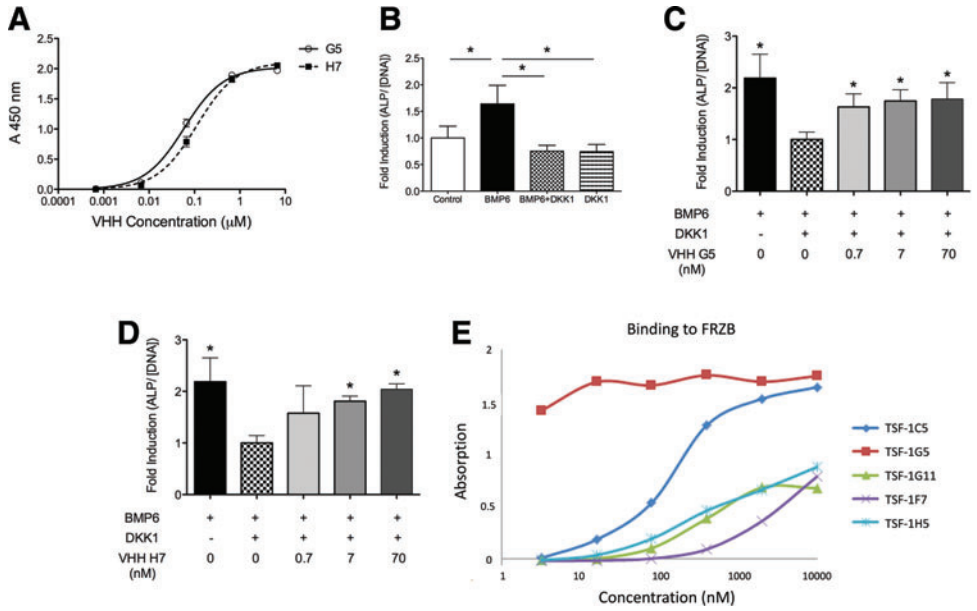

FIG. 1. Selection and analysis of anti-DKK1 and anti-FRZB VHH, **(A)** VHH bind to DKK1 and neutralize DKK1 activity. Concentration range of VHH G5 and H7 was incubated in wells coated with DKK1 (60 nM) or PBS (negative control). After several washes, bound VHH were detected with a mouse anti-VHH serum and a donkey anti-rabbit antibody coupled to a peroxidase. The amount of converted HRP (absorbance at 450 nm; A450 nm) is proportional to the amount of bound VHH. Error bars represent SD ($n=3$). **(B)** Addition of BMP6 stimulates ALP expression in KS483-4C3 cells. The coincubation of BMP6 with DKK1 reversed the ALP expression back to basal levels. The addition of DKK1 to the standard cell culture did not show any significant difference in ALP expression compared with control. The ALP activity is normalized by the total DNA content of KS483 cells after 7 days of culture and expressed as fold induction relative to control. KS483-4C3 cells were stimulated with BMP6, DKK1, and VHH G5 **(C)** or VHH H7 **(D)** in a concentration range of 0–70 nM. Coincubation of VHH with DKK1 and BMP6 reversed DKK1-mediated inhibition of BMP6-induced ALP activity in a dose-dependent manner demonstrating effective neutralization of DKK1 activity by the VHH G5 and H7. ALP activity was measured and expressed as relative enzyme activity corrected for DNA and expressed as fold induction relative to BMP6/DKK1/VHH (+/+/–). $*P<0.05$. ($n=3$). **(E)** The apparent affinities of different FRZB VHH. Plates were coated with FRZB (60 nM) and blocked with 4% skimmed milk in PBS (MPBS), then incubated with a concentration range of different VHH (0–7 μM). Unbound VHH were washed with PBS-Tween and bound VHH were detected by incubation with mAb M1 and a HRP-conjugated anti-mouse. ALP, alkaline phosphatase; DKK1, Dickkopf 1 homolog; FRZB, frizzled-related protein; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; SD, standard deviation. Color images available online at www.liebertpub.com/scd

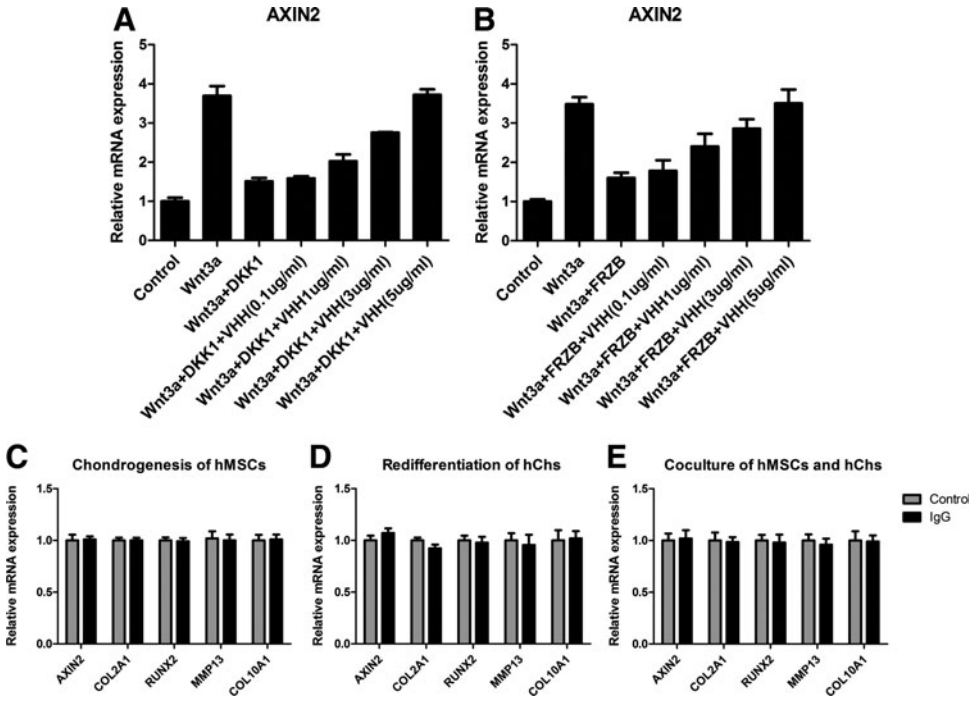

FIG. 2. VHH against DKK1 **(A)** or FRZB **(B)** can be used for restoring WNT signaling in hChs. A concentration of 5 μg/mL DKK1 or FRZB neutralizing antibodies restores WNT activation to its original levels **(A, B)**. The control IgG antibody did not interfere with gene expression in hMSCs **(C)**, hChs **(D)**, and cocultures of hChs and hMSCs **(E)** of the investigated genes compared to control cells receiving no treatment. hChs, human chondrocytes; hMSCs, human mesenchymal stem cells.

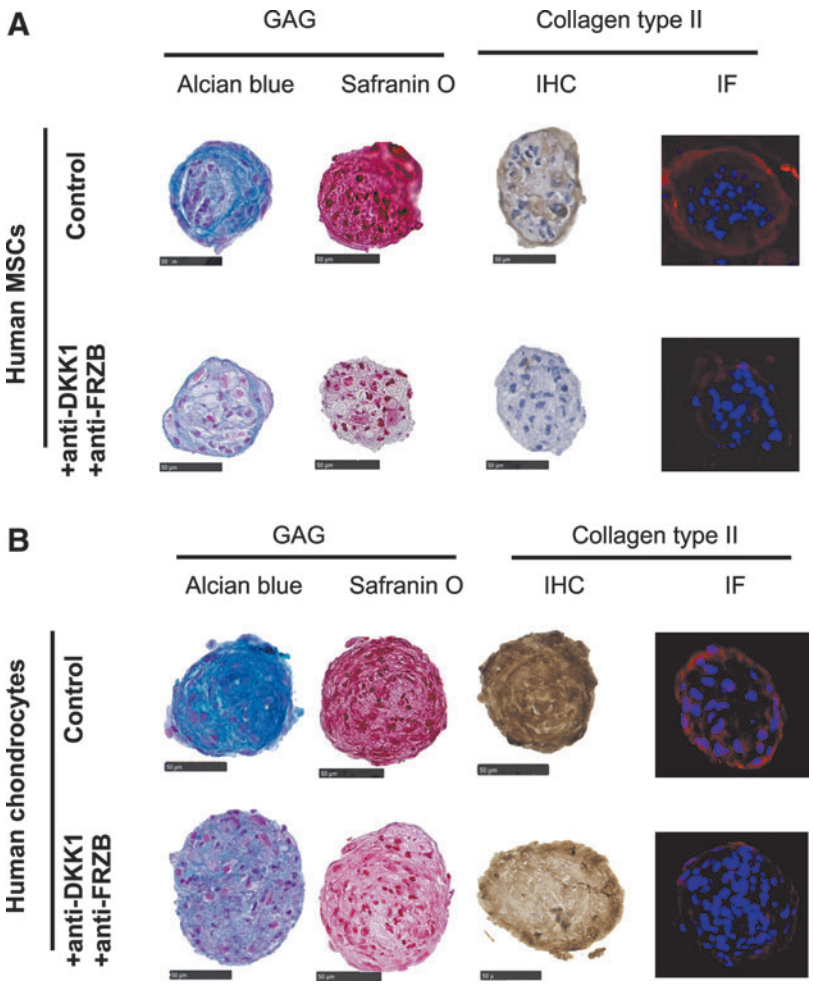

FIG. 3. Blocking DKK1 and FRZB inhibits both chondrogenic differentiation of hMSCs and chondrocyte redifferentiation. Histological analysis of GAGs using Alcian blue and safranin O staining. Collagen type II was measured by IHC and immunofluorescence staining on mid-sagittal sections of the chondrogenically differentiating hMSCs **(A)** and the chondrocyte **(B)** micromasses. GAG, glycosaminoglycan; IHC, immunohistochemistry. Color images available online at www.liebertpub.com/scd

The effect of anti-DKK1 and anti-FRZB treatment on chondrogenesis of hMSCs was measured after 4 weeks. We found a significant loss of GAG deposition and the expression of collagen type II in pellets with DKK1 and FRZB blockade compared to the controls. Immunofluorescence further confirmed immunohistochemistry (IHC) result showing the loss of collagen type II (Fig. 3A).

We hypothesized that endogenous expression of DKK1 and FRZB is essential for maintaining the chondrocyte phenotype and that DKK1 and FRZB are necessary for chondrocyte redifferentiation. To test this hypothesis, cells were dedifferentiated during expansion culturing for two passages after which pellet cultures were made to initiate redifferentiation in chondrogenic differentiation medium containing TGF-β and DEX. Neutralizing VHH against DKK1 and FRZB were added to the pellet cultures. The effect of anti-DKK1 and anti-FRZB treatment on chondrocyte redifferentiation was measured after 4 weeks. Based on Alcian blue staining, successful redifferentiation of hChs took at least 3 weeks in 3D cultures (Supplementary Fig. S6). Alcian blue and safranin O showed that GAG deposition was absent in pellets in which DKK1 and FRZB were blocked (Fig. 3B). In addition, blocking DKK1 and FRZB resulted in reduced amounts of collagen type II protein deposition, measured by IHC and immunofluorescence (Fig. 3B).

Blocking DKK1 and FRZB results in expression of hypertrophic markers and cellular hypertrophy in pellet cocultures

To investigate if endogenous expression of DKK1 and FRZB prevents hypertrophy, neutralizing VHH against DKK1 and FRZB were added to coculture pellets of hMSCs and hChs. Coculture pellets were cultured for 4 weeks in chondrogenic differentiation medium. Gene expression in the cocultures was measured by qPCR. Control experiments using a nonspecific IgG did not affect gene expression (refer to Fig. 2E). Results showed a 1.5-fold increase in mRNA levels of *AXIN2* after the addition of the neutralizing VHH, suggesting the activation of WNT signaling after blocking of DKK1 and FRZB (Fig. 4A). Furthermore, blocking DKK1 and FRZB resulted in upregulation of the hypertrophic marker *COL10A1* (Fig. 4B) and the master regulator of bone formation *RUNX2*. *MMP13* expression was marginally increased, although significant as shown by *t*-test.

Alcian blue and safranin O staining indicated that coculturing of hMSCs and hChs in chondrogenic differentiation medium containing neutralizing VHH showed no obvious difference in GAG production (Fig. 4C). IHC and IF results showed that collagen type II expression had no difference between control and cultures treated with

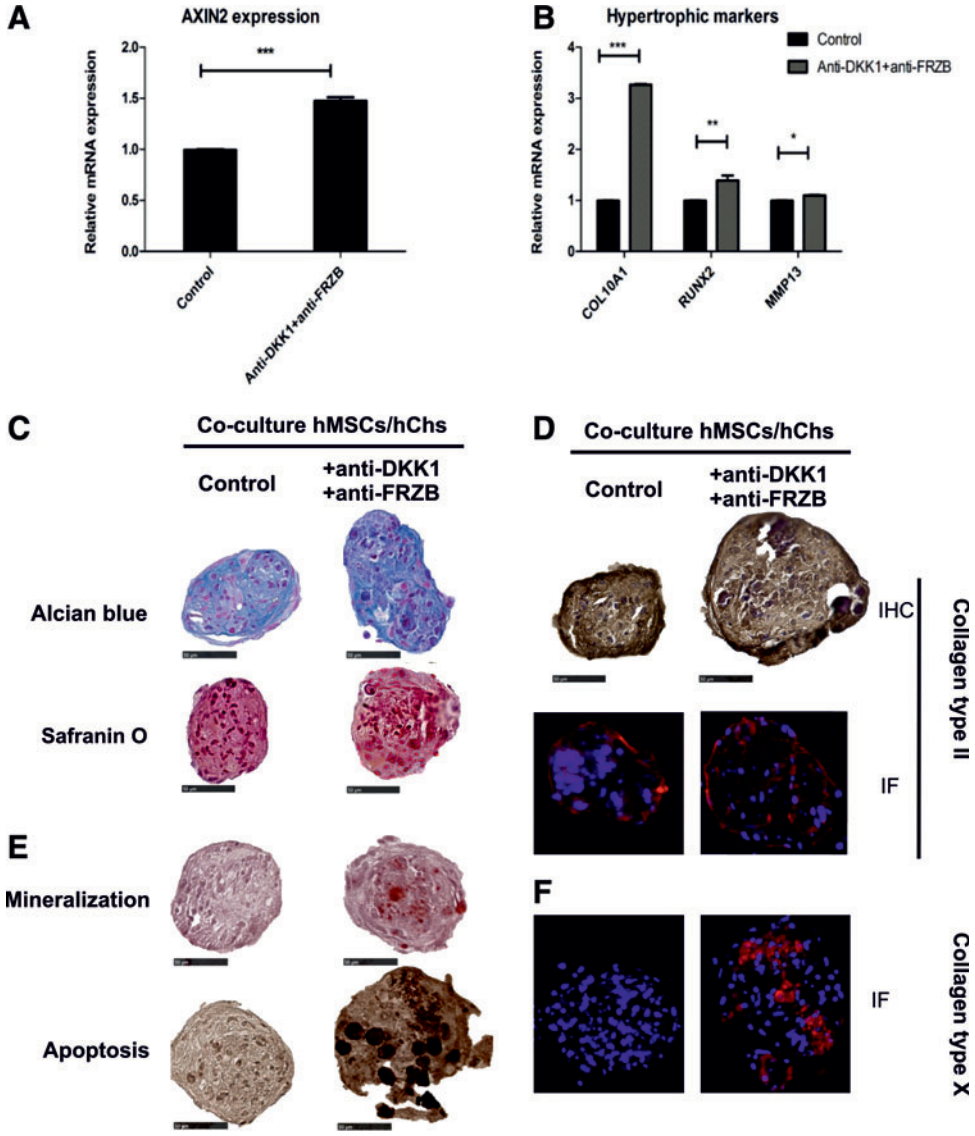

FIG. 4. The effect of neutralizing antibodies against DKK1 and FRZB on 3D coculture pellets of hMSCs and hChs after 4 weeks of culture. Gene expression was determined by quantitative real-time polymerase chain reaction analysis **(A, B)**. The expression of target gene was standardized to *GAPDH* expression. Bars show the mean ± SD of triplicate cultures. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ versus control. **(C)** Paraffin sections of coculture pellets stained for glycosaminoglycan by Alcian blue and safranin O. **(D)** The expression of type II collagen was visualized by IHC and immunofluorescence (scale bar 50 μm). **(E)** Alizarin red for matrix mineralization (visible as *dark red* regions) and TUNEL assay for apoptosis (apoptotic nuclei were stained *dark brown*). **(F)** Collagen type X was measured by immunofluorescence. Data are based on eight samples. For each experiment, a representative section is shown. Color images available online at www.liebertpub.com/scd

neutralizing VHH (Fig. 4D). In vivo and in vitro, during the process of hypertrophic differentiation, cells enlarge, terminally differentiate, mineralize, and ultimately undergo apoptosis [40,41]. To elucidate the effects of blocking DKK1 and FRZB on (matrix) mineralization and apoptosis, we used Alizarin red S staining to evaluate mineralization and a TUNEL assay to detect the apoptosis of cells. Alizarin red S staining indicated that loss of DKK1 and FRZB increased matrix mineralization; the positive staining was especially present in the center of the pellets (Fig. 4E). The TUNEL assay showed that apoptotic cells were present in both the control and the experimental group (Fig. 4E). The number of apoptotic cells was increased in the VHH-treated group. In addition, the coculture pellets treated with neutralizing VHH highly expressed collagen type X (Fig. 4F) and were less compact and more sensitive to tissue handling.

Together, these data suggest that blocking DKK1 and FRZB increases hypertrophic differentiation by increasing hypertrophic marker expression, promotion of cartilage mineralization, and subsequent apoptosis in these cultures.

Discussion

In this article, we proved that (1) endogenous expression levels of DKK1 and FRZB are necessary for chondrogenesis of hMSCs, (2) endogenous levels of DKK1 and FRZB play a stimulating role in chondrocyte redifferentiation, and (3) endogenous DKK1 and FRZB are able to inhibit hypertrophic differentiation in a coculture system.

Chondrogenesis is defined by a series of steps that can be described as MSC condensation, cell proliferation, and differentiation into chondroprogenitors followed by secretion of ECM components and further maturation into chondrocytes [40] (Fig. 5). WNT/β-catenin signaling plays an important role in multiple steps [42] particularly in the initiation of chondrogenesis and inducing hypertrophic differentiation. Both in vivo and in vitro experiments reveal that low levels of β-catenin activity are required for the initiation of chondrogenesis in the first phase [43,44], while high level of β-catenin activity blocks the express of cartilage marker by inhibiting the activity of SOX9 [42]. However, the

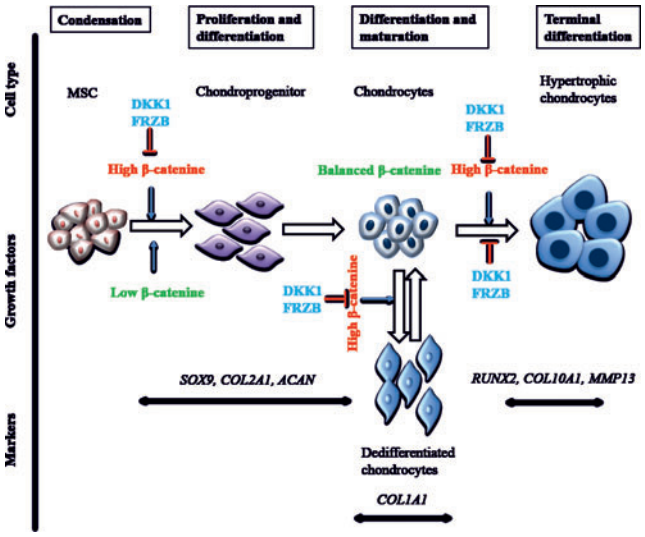

FIG. 5. Endogenously produced WNT antagonists DKK1 and FRZB determine chondrogenesis and chondrocyte terminal differentiation. Chondrogenesis is defined by a series of processes. In the first step of chondrogenesis, DKK1 and FRZB promote chondrogenesis and prevent chondrocyte dedifferentiation through inhibiting WNT/β-catenin signaling. In the second step, DKK1 and FRZB inhibit chondrocyte terminal differentiation by blocking WNT/β-catenin signaling. The expression of typical markers of the various stages of chondrogenesis is indicated at the *bottom*. Color images available online at www.liebertpub.com/scd

endogenous mechanism regulating these processes in vivo has not been described. We hypothesized that endogenous expression of DKK1 and FRZB is sufficient to block or repress WNT signaling resulting in initiation of chondrogenesis and prevention of hypertrophic differentiation. Our results show that inhibition of DKK1 and FRZB during chondrogenesis of hMSCs resulted in loss of GAG deposition and collagen type II expression, implying that these factors are indeed necessary for chondrogenic induction of hMSCs.

Primary hChs are enzymatically released from the cartilage matrix for autologous cell-based therapeutic interventions (ACI) [1] and research purposes. However, long expansion time and multiple passaging in monolayer culture, which are generally required to get enough amounts of chondrocytes, lead to dedifferentiation of the isolated chondrocytes. Ryu et al. have reported that β-catenin expression levels are low in normal chondrocytes and significantly increased as cells undergo dedifferentiation in serial monolayer cultures. They found that overexpression of β-catenin using a β-catenin activator leads to dedifferentiation of chondrocytes and that transcriptional activation of β-catenin is sufficient to cause the loss of chondrocyte phenotype in rabbit cells [45]. In addition, chondrocytes from *APC* knockout mice, which display activation of canonical WNT/β-catenin signaling, show loss of normal phenotype and dedifferentiation, further proving the role of β-catenin signaling in chondrocyte dedifferentiation [44].

We proved our hypothesis that DKK1 and FRZB are able to prevent chondrocyte dedifferentiation by blocking WNT/β-catenin signaling. Our results showed that antibody-based inhibition of DKK1 and FRZB led to a significant decrease in GAG deposition and the loss of collagen type II, indicating that removal of DKK1 and FRZB releases the break on WNT signaling resulting in impaired redifferentiation. We have previously shown that the expression of DKK1 increased (marginally), while FRZB expression decreased during the dedifferentiation process [22].

During dedifferentiation, the small increase in DKK1 might not be sufficient to compensate for the loss of FRZB, which not only blocks canonical but also noncanonical WNT pathways. This is in contrast to DKK1 that only blocks canonical WNT signaling. Loss of FRZB during the dedifferentiation could therefore switch the fine balance between the canonical and the noncanonical WNT pathways. Our data suggest that endogenous expression of DKK1 and FRZB prevents chondrocyte dedifferentiation and that combined blocking of these factors during chondrogenesis prevents redifferentiation.

Loss-of-function and gain-of-function studies indicate that WNT/β-catenin signaling is involved in endochondral ossification. Overexpression of WNT/β-catenin signaling induces chondrocyte hypertrophy [42,46] while blocking WNT signaling using a WNT inhibitor during the last steps of chondrogenic differentiation prevents hypertrophic differentiation of the cells [23]. We proved our hypothesis that the endogenous WNT inhibitors DKK1 and FRZB play a determining role in preventing terminal chondrocyte differentiation by regulating WNT/β-catenin signaling.

In contrast to our monoculture experiments, we found that blocking of DKK1 and FRZB in coculture pellets of hMSCs and hChs did not inhibit cartilage-specific matrix formation and collagen type II production. The discrepancy between monocultures and cocultures suggests that in cocultures other mechanisms are operational that compensate for the loss of DKK1 and FRZB. We have previously shown that MSCs in cocultures secrete trophic factors that enhance cartilage formation and that these factors are not expressed in monocultures of either MSCs or chondrocytes [47]. This implies that in addition to DKK1 and FRZB expression, alternative factors that are specifically present in cocultures compensate for the loss of DKK1 and FRZB, by directly or indirectly regulating WNT activity.

Since we observed GAG and collagen II expression in the cocultures, indicative of cartilage formation, we used the cocultures to study the role of DKK1 and FRZB on terminal chondrocyte differentiation. We observed an increase in hypertrophic markers and matrix mineralization. Matrix mineralization is normally observed in endochondral bone formation [48]. It has been shown that premature induction of hypertrophy during in vitro chondrogenesis of hMSCs correlates with calcification after ectopic transplantion in SCID mice [49]. Both in vivo and in vitro studies have concluded that hypertrophic chondrocytes do not only express hypertrophic markers but also undergo apoptosis [41,49,50]. Similarly, our results showed that the number of apoptotic cells increased in coculture pellets with neutralizing VHH.

In conclusion, as far as we know, our study is the first one to show novel evidence on the combined role of endogenously produced DKK1 and FRZB in at least three stages of chondrocyte differentiation: (1) first, in the initial stage, during onset of chondrogenesis in MSCs, (2) in redifferentiating chondrocytes, and (3) during the hypertrophic differentiation phase (Fig. 5). This study may help us to further improve the clinical cell based cartilage repair strategies.

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Author Disclosure Statement

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