[Frontiers in Bioscience, Landmark, 18, 493-503, January 1, 2013]

WNT3A modulates chondrogenesis via canonical and non-canonical Wnt pathways in MSCs

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1. ABSTRACT

The multilineage commitment of mesenchymal stem cells (MSCs) is controlled via unknown mechanisms. In this study, we investigated the regulation of the differentiation of MSCs into chondrocytes via the Wnt signaling pathway. Overexpression of WNT3A in MSCs activated both the canonical and non-canonical Wnt pathways, which were responsible for different WNT3A-induced outcomes. WNT3A promoted MSC proliferation via the β-catenin-mediated canonical Wnt pathway, and inhibited chondrogenesis of MSCs via the calcium/calmodulin-dependent kinase II (CaMKII)-mediated non-canonical Wnt pathway. Interestingly, blockade of the canonical Wnt pathway by Dickkopf-related protein 1 exerted a synergistic effect on the inhibition of chondrogenesis of MSCs, while blockade of the non-canonical Wnt pathway by KN93 also exerted a synergistic effect on MSCs proliferation. These results suggest that the WNT3A-activated canonical and non-canonical pathways counteract each other in the setting of MSCs. This study provides evidence for the delicate regulation of the Wnt signaling cascade during chondrogenesis of MSCs, and suggests that genetic manipulation of the Wnt pathway may offer a powerful vehicle for modulating MSC differentiation in stem-cell-based cartilage repair.

2. INTRODUCTION

Articular cartilage is a durable but vulnerable tissue that plays an important role in frictionless joint motion. Unlike other connective tissues, cartilage does not contain blood vessels, and chondrocytes are bound within lacunae and are thus unable to migrate to damaged areas, making it difficult for cartilage to repair itself (1,2). The introduction of extra chondrocytes into articular cartilage can thus be used as a cell-based therapeutic technique to treat cartilage breakdown (3,4). Chondrogenesis is the process whereby cartilage is formed, and involves the differentiation of chondroblasts into chondrocytes, and their subsequent secretion of molecules to form the extracellular matrix (ECM) (1,2). However, the supply of mature chondrocytes from healthy articular cartilage is limited, and alternative sources of cells need to be considered. Stem cells fall into several categories according to their source: embryonic stem cells (ESCs), adult stem cells, and induced pluripotent stem cells (iPSCs). ESCs represent ideal candidates from the therapeutic perspective, but are ruled out because of ethical concerns. The shortfalls of iPSCs, including the low throughput of reprogrammed cells, the risk of mutations in genome insertion, and incomplete reprogramming, suggest that iPSCs are a poor substitute for ESCs (5–7). While ESCs and iPSCs are pluripotent, most adult stem cells are multipotent and are

classified according to their tissue origin, such as mesenchymal stem cells (MSCs), adipose-derived stem cells, and endothelial stem cells (5,8–10). The use of adult stem cells circumvents the controversy surrounding ESCs, and reduces the risk of transplant rejection, because adult stem cells can be harvested from the patient (11–13). MSCs have been isolated from placenta, adipose tissue, and bone marrow (14), and are able to differentiate into cells for several different tissues, such as cartilage, bone, muscle, and adipose tissue (15–18). Their ability to not only differentiate, but to also modulate the immune response makes MSCs an attractive prospect for clinical research and therapy (19). MSCs have been employed in hyaluronan and polymeric scaffolds to repair cartilage (15,20). Several strategies can be used to achieve this goal, but the rationale involves implanting MSCs at different stages of differentiation (undifferentiated, partially differentiated, and fully differentiated) together with scaffolds into diseased sites. Although these techniques have given rise to well-differentiated tissues, most studies have been performed in animal models, with limited numbers in humans (15), and no clinical MSC-based therapy is currently available.

The Wnt proteins are a group of secreted lipid-modified signaling proteins that activate various pathways in different types of cells. These pathways can be classified as canonical and non-canonical Wnt pathways (21–24), and play a variety of important roles in physiological processes such as embryonic development and cell differentiation (25–27). The interaction of extracellular Wnt ligands with their receptors initiates Wnt signaling. In the canonical Wnt pathway, some members of the Wnt family, such as Wnt1 and WNT3A, interact with Frizzled (FZD) receptors and their co-receptors, low-density lipoprotein receptor-related receptor 5/6 (LRP5/6), activating Dishevelled (DSH) family proteins, and leading to a change in the amount of nuclear β-catenin (23,24,28,29). DSH protects β-catenin from proteolytic degradation triggered by another complex comprising axin, glycogen synthase kinase-3 and the adenomatous polyposis coli protein. The blockade of β-catenin destruction raises the levels of cytoplasmic β-catenin, thus increasing the likelihood of some β-catenin translocating to the nucleus. This nuclear β-catenin then binds to T-cell factor (TCF)/lymphoid enhancer-binding factor transcription factors and promotes specific gene expression (30). The canonical and non-canonical pathways differ in terms of the specific ligands that activate each pathway, and whether or not β-catenin is activated. The canonical pathway employs β-catenin to transmit signals, while the non-canonical pathway utilizes calcium/calmodulin-dependent kinase II (CaMKII) or Rho to exert its effects. In the non-canonical Wnt pathway, Wnt4, WNT5A, and Wnt11 activate the heavily-investigated planar cell polarity (PCP) and the Wnt/Ca^{2+}/CaMKII pathways. WNT3A has been regarded as an activator of the canonical Wnt signaling pathway. Recent evidence showed that treatment of primary adult human articular chondrocytes (AHACs) with recombinant WNT3A resulted in the activation of the non-canonical Wnt/Ca^{2+}/CaMKII pathway (31). The PCP pathway is involved in the regulation of cytoskeletal structure, while the Wnt/Ca^{2+}/CaMKII pathway regulates intracellular Ca^{2+} levels. Ligand binding causes an increase in intracellular Ca^{2+}, which in turn activates CaMKII. CaMKII activates transforming growth factor (TGF)-β-activated kinase and Nemo-like kinase, which can interfere with TCF/β-catenin signaling in the canonical pathway (32). Both the canonical and non-canonical Wnt pathways are involved in skeletal development, chondrodysplasia in embryonic life, and in the postnatal development of osteoarthritis (33–36). Wnt pathways also play a role in the multilineage commitment of adult MSCs (37,38).

In this study, we investigated the ability of MSCs to differentiate into chondrocytes through modulation of the Wnt pathways. We increased WNT3A expression in MSCs by lentiviral infection and examined the effects on MSC proliferation. We also investigated the cytoplasmic pool of ß-catenin and Ca^{2+} mobility to differentiate between the canonical and non-canonical Wnt pathways, and used specific inhibitors of the canonical and non-canonical Wnt pathways to verify the Wnt signaling network in MSCs.

3. MATERIALS AND METHODS

3.1. Generation of vectors

WNT3A cDNA was inserted into the Mlu I and Cla I sites of the lentiviral vector, pLvx, which carries a GFP expressing cassette. The recombinant vectors were amplified in HEK293FT cells to generate high-titer preparations, and purified over three successive CsCl gradients. Following dialysis, the preparations were aliquoted and stored at -80°C. Viral titers were estimated by optical density and standard plaque assay. Using these methods, preparations of 10^7–10^8 particles/mL were obtained.

3.2. Rat MSC culture

Bone marrow stroma was extracted from the femurs and tibias of male Sprague-Dawley rats. Dispersed marrow plugs were centrifuged at 1000 rpm for 5 min, buoyant adipocytes were removed by vacuum aspiration, and the pellet resuspended and seeded into two 100-mm culture dishes per animal (approximately 10^8 nucleated cells/dish). The cells were maintained in 7 ml of growth medium (Dulbecco's modified Eagle's medium (DMEM), Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a 37°C, 5% CO_2 incubator for 14 days. Growth medium was replaced every 3 or 4 days.

3.3. Colorimetric tetrazolium salt analysis

The cells were infected for 24 h with WNT3A lentivirus or vehicle control, then cultured in 96-well plates at a density of 1×10^3 cells/well. A total of 20 µL of colorimetric tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-2H- tetrazolium bromide] solution was added to each well and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay reader (model 450; BioRad, Hercules, CA).

3.4. Gene expression analysis

Cells for all MSC chondrogenesis studies were harvested at day 21. The cells were rinsed once with phosphate buffered saline (PBS) and total RNA was

isolated using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. An aliquot of 500 ng of total RNA from each sample was reverse transcribed using a Thermoscript reverse transcription kit (Invitrogen, Carlsbad, CA) with oligo dT primer. Quantitative and semiquantitative polymerase chain reaction (PCR) was performed with hot-start DNA polymerase (Qiagen, Valencia, CA) in the presence of 0.1 × SYBR green (Sigma-Aldrich , St Louis, MO) and 0.2 × ROX dye (Invitrogen, Carlsbad, CA) using, respectively, a T7900 HD or a 96-well GeneAmp PCR system 9700 machine (Applied Biosystems, Foster City, CA).

3.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

After treatment, cells were washed once in ice-cold PBS containing phosphatase and protease inhibitors (Roche, Madison WI). Total cell extracts were obtained by scraping the cells into extraction buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.05% NP-40, pH 7.9) and leaving the lysates on ice for 30 min. The protein concentrations of the samples were determined by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA). The extracts were resolved by SDS-PAGE, and transferred to a nitrocellulose membrane (GE Healthcare, Uppsala, Sweden). β-catenin was detected using an anti-β-catenin rabbit monoclonal antibody (diluted 1:1000; Cell Signaling Technology, Danvers, MA), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using an anti-GAPDH mouse monoclonal antibody (diluted 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (diluted 1:2,000; Santa Cruz Biotechnology, Inc., ,Santa Cruz, CA). Antibodies were diluted in 5% nonfat milk/0.01% Tween 20 in PBS. In the case of CaMKII and phospho-CaMKII analysis, the membranes were blocked in 5% bovine serum albumin/0.01% Tween 20 in PBS, and the antibodies (1:2,000 anti-CaMKII Abcam, , Cambridge, MA; 1:1000 anti-pCaMKII and 1:1000 anti-lamin B Cell Signaling Technology, Danvers, MA) were diluted in the same buffer.

3.6. Ca^{2+}– mobilization assay

Ca^{2+}–mobilization was evaluated using a Fluo-4-Direct kit (Invitrogen, Carlsbad, CA), in accordance with the manufacturer's specifications. In brief, MSCs were labeled for 1 h (30 min at room temperature and 30 min at 37°C) with the Fluo-4-Direct calcium assay buffer containing Probenecid (Invitrogen, Carlsbad, CA), and were subjected to lentivirus infection as indicated for individual experiments. After treatment, the fluorescence emitted by the cells (excitation wavelength 494 nm, emission wavelength 516 nm) was recorded using a fluorimeter (Galaxy; Fluo-Star, BMG Labtech Inc., Stuttgart, Germany). The fluorescence was expressed as relative fluorescence units.

3.7. Chondrocyte differentiation and toluidine blue staining

Approximately 8–10×10^5 cells were placed in a 15-mL polypropylene tube (Falcon™ BD Biosciences, USA) and pelleted into micromasses by centrifugation at 450 *g* for 10 min. Pellets were cultured at 37°C in 5% CO_2 for 21 days in chondrogenic medium: DMEM (Invitrogen, Carlsbad, CA) supplemented with 10 ng/mL TGF-β3 (R&D Systems, UK), 100 nM dexamethasone, 50 µg/mL ascorbic acid, 40 µg/mL of L-proline (Sigma-Aldrich, , St Louis, MO), 100 µg/mL of sodium pyruvate (Sigma-Aldrich , St Louis, MO), 50 mg/mL of ITS+Premix (BD Biosciences) and 500 ng/mL of human recombinant BMP-2 (Peprotech, USA). Aggregates were embedded in 0.8% agarose for ease of handling and then fixed in 10% neutral-buffered formalin for 1 h at room temperature. After dehydration in graded alcohols, the aggregates were paraffin embedded and sectioned at 5 µm. Representative sections were stained using toluidine blue (Sigma-Aldrich , St Louis, MO) for the detection of matrix proteoglycans.

3.8. Lentiviral infection

Culture medium was removed before infection. The cells were washed gently with PBS and treated with the virus-containing medium combined with Polybrene (8 mg/mL) which was applied to subconfluent, proliferating MSCs. After 24 hours, culture medium was removed and fresh medium was added to the MSCs.

3.9. Subcellular fractionation

For subcellular fractionation, MSC aggregates were trypsinized and resuspended in ice-cold hypotonic buffer containing 10 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 2.5 mM sodium fluoride, 2 mM sodium orthovanadate and a cocktail of protease inhibitors. Cells were then homogenized on ice using a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant, consisting of the cytoplasmic fraction, was collected and diluted in Laemmli sample buffer, while the pellet consisting of the nuclear fraction was washed once in ice-cold hypotonic buffer and centrifuged at 1000 *g* for 10 min at 4°C.The nuclear pellet was lysed in RIPA lysis buffer and centrifuged at maximum speed at 4°C. The supernatant, consisting of the soluble nuclear fraction, was collected and diluted in Laemmli sample buffer. The cytoplasmic and soluble nuclear subcellular fractions were then analyzed by SDS-PAGE and western blotting.

3.10. Data analysis

All experiments were repeated three times with independent cultures and similar results were obtained. Statistical significance was determined by Student's t-test. *P* values <0.05 were considered significant.

4. RESULTS

4.1. Overexpression of WNT3A increases MSC proliferation and inhibits chondrogenesis

We inserted the WNT3A-expressing coding sequence into a green fluorescent protein (GFP)-expressing lentivirus vector, using the GFP lentivirus alone as a control. The efficiency of MSC infection was confirmed by fluorescence microscopy (Figure 1A), and overexpression of WNT3A was validated by western blotting (Figure 1B). The colorimetric tetrazolium salt (MTT) assay showed that WNT3A enhanced proliferation of MSCs: compared with

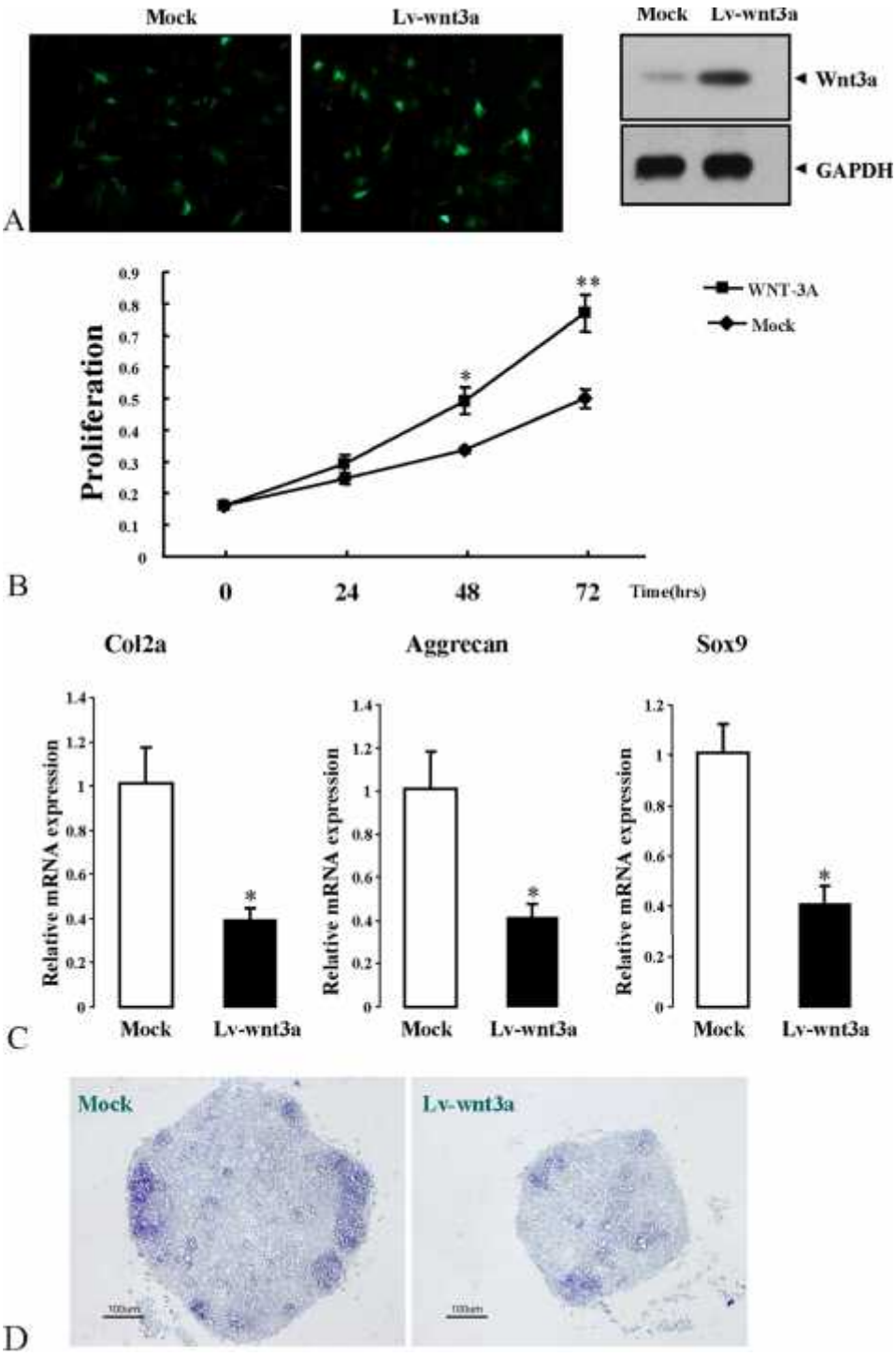

Figure 1. Overexpression of WNT3A increases MSC proliferation and inhibits chondrogenesis. (A) Infection efficiency of MSCs by lentiviruses was displayed by visualization of GFP expression under a fluorescence microscope. Image taken 48 h after infection. WNT3A expression was validated by western blotting using anti-WNT3A antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was western blotted using the anti-GAPDH antibody as a loading control. (B) Promotion of cell growth was evaluated by colorimetric tetrazolium salt (MTT) assay. (C, D) WNT3A-induced down-regulation of the differentiation markers *COL2A1*, *aggrecan*, and *SOX9*, evaluated by quantitative PCR (C; $n = 6$). At 48 h after infection, MSCs were seeded into aggregates and cultured in chondrogenic medium for 21 days. The accumulation of matrix proteoglycan in aggregates was detected by toluidine blue staining (D). Statistical analysis was performed using unpaired *t* tests. Error bars indicate mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ vs mock group.

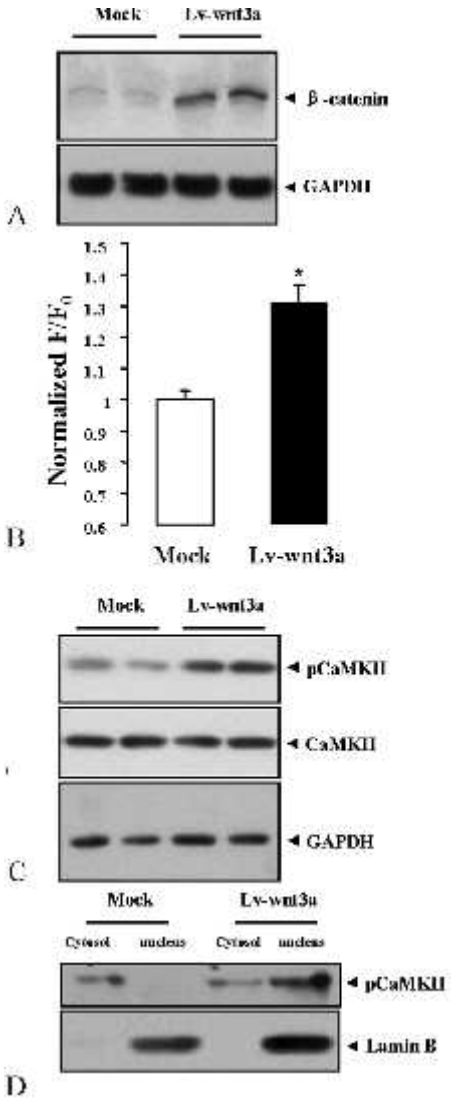

Figure 2. WNT3A activates both the canonical and the non-canonical Wnt pathways in MSCs. (A) Western blotting for β-catenin in the cytoplasmic fraction of MSCs infected with WNT3A lentivirus or vehicle control for 24 h. (B) WNT3A induced Ca2+ mobilization in MSCs. MSCs were infected with WNT3A lentivirus or vehicle control for 48 h and then subjected to fluorimetric determination of Ca2+ mobilization (n = 9). Statistical analysis was performed using unpaired t tests. Error bars indicate mean ± SEM. *, $P < 0.05$, vs. mock group. (C, D) WNT3A promotes intracellular Ca2+-mediated phosphorylation of CaMKII and its nuclear translocation at 48h post WNT3A lentivirus infection. (C) WNT3A promotes phosphorylation of CaMKII. hosphorylated CaMKII and total CaMKII were examined by western blotting using specific antibodies. GAPDH was western blotted using an anti-GAPDH antibody as a loading control. (D) WNT3A promotes the nuclear translocation of phosphorylated CaMKII. At 48 h after infection, MSCs were seeded into aggregates and cultured in chondrogenic medium for 21 days. Western blot analysis of nuclear and cytoplasmic fractions was performed using control and WNT3A-overexpressing MSC aggregates. Lamin B was measured as a loading control for the nuclear fraction.

MSCs infected with the control lentivirus, WNT3A-overexpressing MCSs displayed an approximately 2-fold increase in proliferation. *COL2A1*, *aggrecan*, and *SOX9* mRNAs were dramatically down-regulated as a result of WNT3A upregulation. The primary function of chondrocytes is the production of a specialized ECM that is rich in sulfated glycosaminoglycans (GAGs), and we therefore examined the effects of WNT3A on chondrocyte function, as reflected by ECM production. Elevated expression of WNT3A in MSCs decreased the accumulation of matrix proteoglycans, as shown by toluidine blue staining. Collectively, these results suggest that WNT3A is involved in the proliferation and differentiation of MSCs.

4.2. WNT3A activates both the canonical and the non-canonical Wnt pathways in MSCs

WNT3A activates both the canonical and non-canonical Wnt pathways in a variety of cells (38-41). We tested specific markers of both pathways to determine which of them was activated by WNT3A in MSCs. As shown in Figure 2A, forced expression of WNT3A caused upregulation of β-catenin, indicating that WNT3A activated the canonical Wnt pathway in MSCs. The non-canonical Wnt/Ca^{2+}/CaMKII pathway has been shown to play a role in chicken chondrocyte maturation (42). Ca^{2+} mobilization occurred in WNT3A lentivirus-infected MSCs. We also demonstrated that forced expression of WNT3A led to an increase in the phosphorylated form of CaMKII, but had no effect on the expression of endogenous CaMKII. Furthermore, western blotting showed that WNT3A expression increased nuclear localization of the phosphorylated form of CaMKII. These results demonstrate that the non-canonical Wnt pathway was also activated by forced expression of WNT3A in MSCs.

4.3. Inhibition of the Wnt canonical pathway by Dickkopf-related protein 1 (DKK1) abrogates WNT3A-induced proliferation but does not rescue the loss of the chondrocyte phenotype

To confirm the mechanism indicated by upregulation of β-catenin and accumulation of the phosphorylated form of CaMKII, we used specific inhibitors of both the canonical and the non-canonical Wnt pathways in WNT3A lentivirus-infected MSCs. DKK1 competes with Frizzled receptors and their co-receptors, low-density lipoprotein receptor-related (LRPs) co-receptors and inhibits the β-catenin-mediated canonical Wnt pathway (43). DKK1 exerted an inhibitory effect on WNT3A-enhanced proliferation (Figure 3A), but had no significant effect on the proliferation of MSCs infected with control lentivirus. These results suggest that WNT3A promotes MSC proliferation via the canonical Wnt pathway. We also tested if activation of the canonical Wnt pathway might be responsible for the inhibition of chondrocyte differentiation. The results demonstrated that blocking the canonical Wnt pathway with DKK1 did not rescue WNT3A-induced downregulation of *COL2A1*, *aggrecan*, and *SOX9* mRNAs; indeed, treatment of MSCs with DKK1 alone actually promoted the downregulation of *COL2A1*, *aggrecan*, and *SOX9*. DKK1 therefore exerted a synergistic effect on the downregulation of these genes,

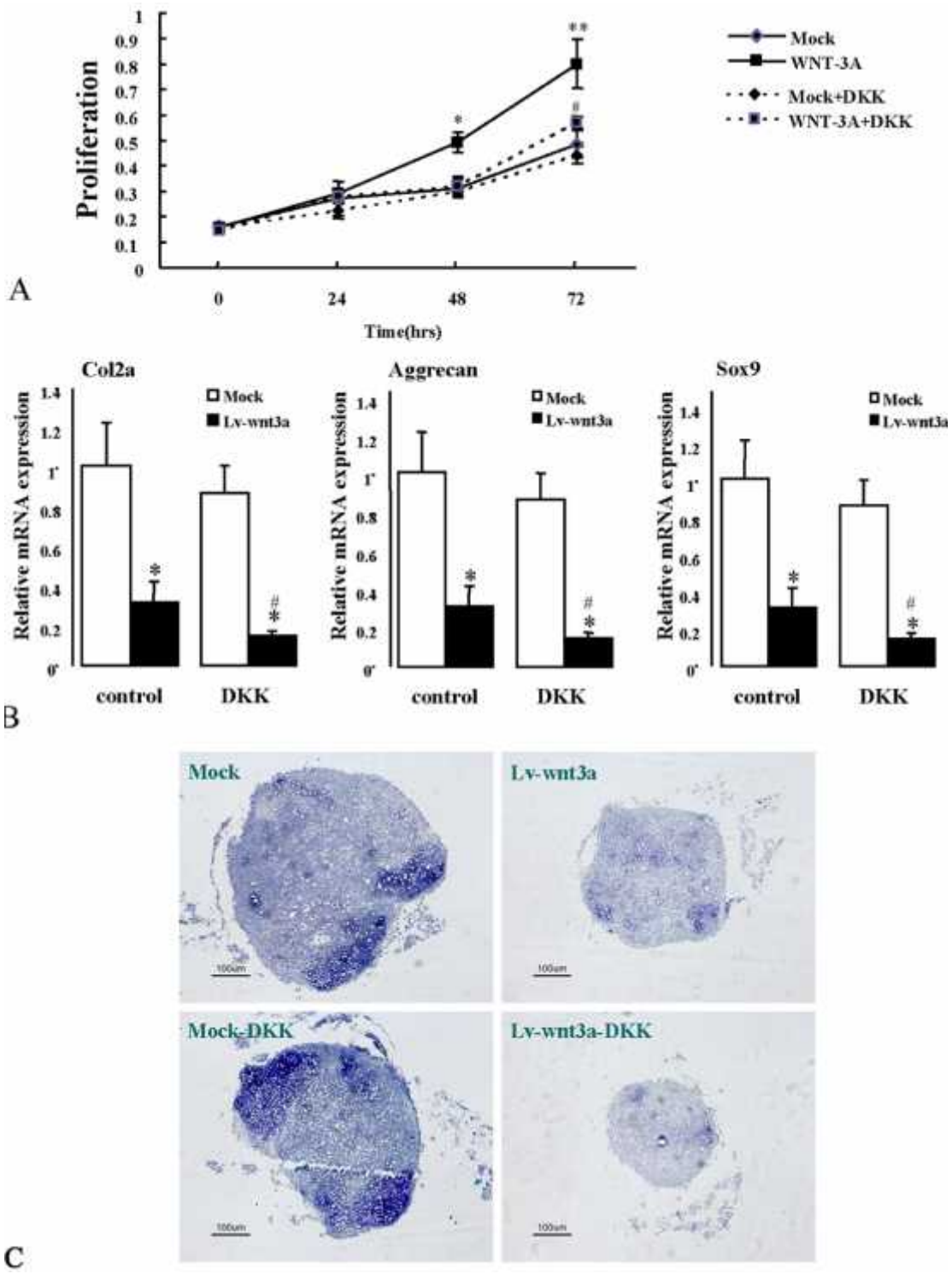

Figure 3. Inhibition of the Wnt canonical pathway by DKK1 abrogates WNT3A-induced proliferation but does not rescue the loss of the chondrocyte phenotype. MSCs were infected with WNT3A lentivirus or vehicle control for 48 h. Cells were then treated for 24 h with 100 ng/mL of DKK1. (A) Promotion of cell growth was evaluated by colorimetric tetrazolium salt (MTT) assay. (B) MSCs were harvested and subjected to gene expression analysis by quantitative PCR (n = 6). Data were analyzed using unpaired t tests. Error bars indicate mean ± SEM. *P < 0.05; **P < 0.005 vs. mock group; $^{\#}P$ < 0.05 vs. control group. (C) MSCs were seeded into aggregates and cultured in chondrogenic medium for 21 days. The accumulation of matrix proteoglycan in the aggregates was detected by toluidine blue staining.

together with upregulation of WNT3A. DKK1 and WNT3A also acted synergistically to decrease the accumulation of highly-sulfated GAGs. These results demonstrate that the canonical Wnt pathway had a positive effect on WNT3A-induced MSC proliferation but an inhibitory effect on chondrogenesis.

4.4. Inhibition of the Ca^{2+}/CaMKII pathway rescues loss of the chondrocyte phenotype but has little effect on WNT3A-induced proliferation of MSCs.

Because of the positive effect of the canonical Wnt pathway, we speculated that a second pathway, such as the non-canonical Wnt pathway, must account for the decrease in chondrogenesis induced by WNT3A. We used the specific non-canonical pathway inhibitor KN93 to test this hypothesis. KN93 rescued the expression of *COL2A1*, *aggrecan*, and *SOX9* (Figure 4B), resulting in moderate elevations of *COL2A1*, *aggrecan*, and *SOX9* mRNAs in MSCs infected with the control lentivirus, but dramatic increases in MSCs infected with WNT3A lentivirus. However, the expression levels of these genes in cells treated with KN93 were still lower than those in control cells. In accordance with the expression of chondrocyte-lineage markers, toluidine-blue staining revealed that KN93 itself had a moderate effect on chondrogenesis, but also partially rescued the decrease of chondrogenesis induced by WNT3A. These results thus support our hypothesis. We also tested the role of the non-canonical Wnt pathway in WNT3A-induced proliferation of MSCs. KN93 slightly enhanced WNT3A-induced MSC proliferation, but had no effect on the proliferation of MSCs infected with the control lentivirus.

5. DISCUSSION

The results of the present study demonstrate that lentiviral-mediated expression of WNT3A can serve as an effective means of protein delivery, inhibiting chondrogenesis by MSCs in aggregate cultures. Forced expression of the Wnt family member WNT3A resulted in an increase of MSC proliferation via the canonical Wnt/β-catenin pathway, and a simultaneous inhibition of chondrogenesis of MSCs via the non-canonical Wnt/ Ca^{2+}/CaMKII pathway.

These results, indicating that WNT3A activates both the canonical and the non-canonical Wnt pathways, are broadly in agreement with the study of Nalesso at al. (38), but there were important differences between the studies. The current study utilized MSCs instead of chondrocytes to study the effects of WNT3A on chondrogenesis, and demonstrated that overexpression of WNT3A in MSCs resulted in downregulation of *COL2A1*, *aggrecan*, and *SOX9* mRNAs. DKK1 had a synergistic effect on the downregulation of these genes by WNT3A. KN93 is a selective CaMKII inhibitor, which partially rescued the chondrogenesis inhibition induced by WNT3A. Purified WNT3A protein augments the intracellular Ca^{2+} concentration in neurons, leading to the activation of several Ca^{2+}-sensitive proteins, including CaMKII and protein kinase C (PKC). The WNT5A and Wnt11 ligands induce Ca^{2+} release and activate PKC and CaMKII (23), and WNT3A activates phosphatidylinositol signaling and PKC in murine ST2 cells to trigger osteoblastogenesis (41). We speculate that WNT3A-activated PKC may also be responsible for MSC chondrogenesis inhibition, but to a lesser degree. In addition to the Ca^{2+}/CaMKII pathway, the PCP pathway, which plays a role in the regulation of cytoskeletal structure, is also classified as a non-canonical Wnt signaling pathway. However, the involvement of PKC or other pathways like PCP in WNT3A-induced chondrogenesis inhibition requires further investigation. Nalesso *et al.* used recombinant WNT3A and found that extracellular signaling may only affect SOX9. The present study used lentivirus to increase the expression levels of WNT3A, and although Wnts are secreted proteins, the possible role of cytoplasmic WNT3A in our study cannot be ruled out, as another Wnt, WNT5A, has been shown to be cytoplasmic in SW1353 cells (44,45). Forced expression of WNT3A may thus result in cytoplasmic accumulation and secretion of WNT3A. Our results suggest that *COL2A1* and *aggrecan* may respond to cytoplasmic WNT3A, and elucidation of the signaling mediated by cytoplasmic WNT3A requires further investigation.

MSCs are present in a number of tissues, and their multipotency enables them to contribute to postnatal tissue repair and regeneration (2). The availability of MSCs makes them a potent candidate for tissue engineering. MSCs can differentiate into multiple cell lineages, suggesting the existence of specific mechanisms to control the switch and regulate the differentiation programs of these cells (46). It is therefore important to understand the specific signaling networks that regulate the differentiation and maintenance of MSCs. In addition to chondrogenesis, WNT3A also plays a role in osteogenesis, and it has been reported that exposure of adult human MSCs to WNT3A during the process of differentiation suppresses osteogenesis (41,46). We speculate that WNT3A acts as a key switch, controlling proliferation and differentiation in MSC lineage commitment. The inhibition of MSC osteogenesis induced by WNT3A was accompanied by increased cell proliferation, resulting from both increased proliferation during expansion of undifferentiated MSCs, and a decreased proportion of differentiated MSCs in the whole cell mass. Inhibition of canonical Wnt signaling using a dominant negative TCF1 enhanced MSC osteogenesis (46). Thus the increased number of undifferentiated MSCs may reduce the extracellular signals acting on individual cells. Boland *et al.* suggest that canonical Wnt signaling plays a role in the maintenance of the undifferentiated, proliferating progenitor MSC population, whereas non-canonical Wnt signaling facilitates osteogenic differentiation. The current study showed that inhibition of the canonical Wnt pathway failed to rescue the expression of the chondrogenesis marker genes *COL2A1*, *aggrecan*, and *SOX9*; on the contrary, inhibition of the canonical Wnt pathway had a synergistic effect on the inhibition of chondrogenesis. Wnt 3A, as a representative canonical Wnt member, thus also plays a role in the non-canonical Wnt pathway. WNT5A activates the non-canonical Wnt pathway to promote osteogenesis (47), but our data suggest that WNT3A inhibits chondrogenesis via the same pathway. Thus, different contexts of MSC

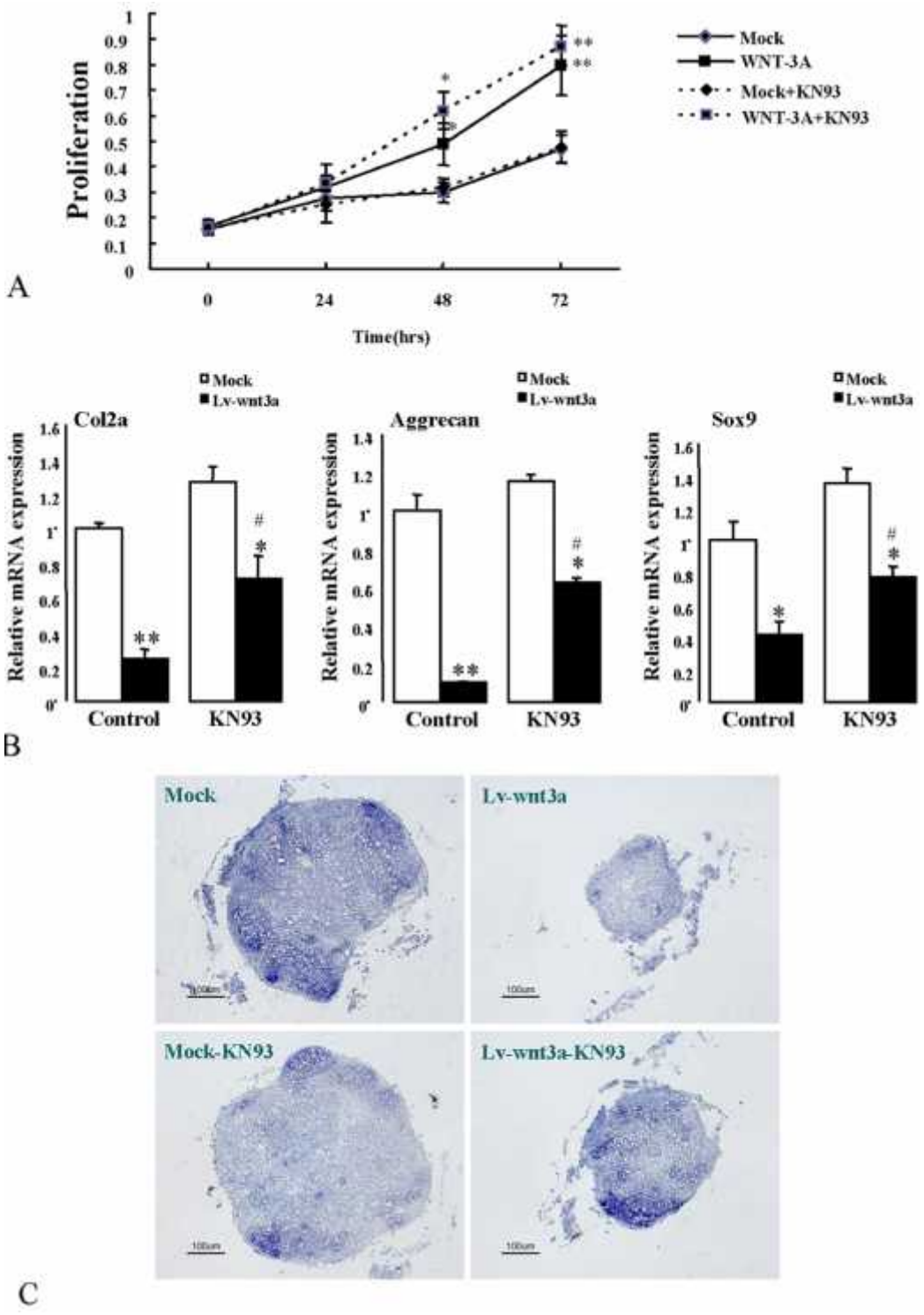

Figure 4. Inhibition of the Ca^{2+}/CaMKII pathway rescues loss of the chondrocyte phenotype but has little effect on WNT3A-induced proliferation in MSCs. MSCs were infected with WNT3A lentivirus or vehicle control for 48 h. Cells were then treated for 24 h with the CaMKII inhibitor KN93 or its inactive analogue KN92 (both 10 μM). (A) Promotion of cell growth was evaluated by colorimetric tetrazolium salt (MTT) assay. (B) MSCs were harvested and subjected to gene expression analysis by quantitative PCR ($n = 6$). Data were analyzed using unpaired t tests. Error bars indicate mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.005$, vs. mock group; $^{\#}P < 0.05$, vs. control group. (C) MSCs were seeded into aggregates and cultured in chondrogenic medium for 21 days. The accumulation of matrix proteoglycans in aggregates was detected by toluidine blue staining.

chondrogenesis and osteogenesis may lead to distinct outcomes via common signaling pathways.

Other members of the Wnt family, including the non-canonical Wnt, WNT5A, also function as a switch in the course of osteogenesis (47). Overexpression of Wnt 5a results in enhanced osteogenic differentiation. Proliferation is the opposite of osteogenesis, and it is therefore possible that the canonical Wnt pathway regulates proliferation, while the non-canonical Wnt pathway controls differentiation. Canonical Wnt signaling results in a reversible outcome of MSC differentiation, in contrast to non-canonical Wnt signaling. It has been reported that in WNT3A/WNT5A co-treatment, WNT5A was able to suppress WNT3A-mediated canonical Wnt signaling, with concomitant reductions in both canonical Wnt transcriptional activity and cell proliferation (47). We hypothesize that WNT3A overexpression, followed by treatments that are able to inhibit both the canonical and non-canonical Wnt pathways, might increase the number of chondrocytes and enhance chondrogenesis. At the first stage, overexpression of WNT3A might increase the pool of MSCs via the canonical Wnt pathway, thus providing an abundant cell source for chondrogenesis. During the second stage, apart from the introduction of growth factors required to initiate differentiation, blockade of either the canonical or non-canonical Wnt pathways, or blocking the expression of WNT3A would enhance chondrogenesis. Further studies are needed to investigate this hypothesis. Abrogation of the canonical Wnt pathway is a prerequisite for MSC differentiation, and loss-of-function mutations of co-receptors such as LRP5 would thus perturb the induction of proliferation via canonical Wnt signaling, and transfer the signals to promote differentiation via non-canonical Wnt signaling.

Delivery of MSCs that are genetically modified to secrete certain biological factors has been reported to stimulate the formation of cartilage in animal models (48,49). Alterations in Wnt signaling may act to both maintain a pool of progenitor cells and to modulate the lineage commitment in differentiation. Thus, selective changes in Wnt signaling may represent a useful target for cell-based therapy. Combined regulation of the expression of some genes should be targeted to identify ways to initiate chondrocyte differentiation and maintain the articular cartilage phenotype long-term. Once these problems have been solved, the coordinated expression of multiple genes in MSCs using complex regulatory systems shows promise for fulfilling the potential of gene therapy for stem-cell-based cartilage repair.

In conclusion, the results of this study demonstrate that WNT3A activates both the canonical and the non-canonical Wnt pathways in MSCs. These pathways play different roles under different experimental conditions. WNT3A promotes the proliferation of undifferentiated MSCs via the β-catenin-mediated canonical Wnt pathway in the setting of proliferation, and in contrast, WNT3A inhibits MSCs chondrogenesis during differentiation through the CaMKII-mediated non-canonical Wnt pathway. WNT3A-activated canonical and non-canonical pathways both function in a reversible manner. These results provide new insights into the delicate regulation of the Wnt signaling cascade involved in the chondrogenesis of MSCs.

6. ACKNOWLEDGMENTS

This project was financially supported by the National Key Technology R&D Program (No: 2007BA104B09)

7. REFERENCES

1. Palmer GD, Steinert A, Pascher A, Gouze E, Gouze J,Betz O, Johnstone B,Evans CH, and Ghivizzani SC. Gene-induced chondrogenesis of primary mesenchymal stem cells *in vitro*. *Mol Ther* 12, 219-228. (2005)

2. Caplan A I, and Bruder SP: Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* 7, 259-264. (2001)

3. Richter W: Cell-based cartilage repair: illusion or solution for osteoarthritis. *Curr Opin Rheumatol* 19, 451-456. (2007)

4. Vinatier C, Gauthier O, Masson M, Malard O, Moreau A, Fellah BH, Bilban M, Spaethe R, Daculsi G, and Guicheux J: Nasal chondrocytes and fibrin sealant for cartilage tissue engineering. *J Biomed Mater Res A* 89, 176-185. (2009)

5 .Takahashi K, Yamanaka S: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676. (2006)

6. Selvaraj V, Plane JM, Williams AJ, Deng W: Switching cell fate: the remarkable rise of induced pluripotent stem cells and lineage reprogramming technologies. Trends in Biotechnology 28, 214-223 (2010)

7. Zhao XY, Li Wei, Lv Zhuo, Liu Lei, Tong Man, Hai Tang, Hao Jie, Guo Chang-Long: iPS cells produce viable mice through tetraploid complementation. Nature 461, 86-90. (2009)

8. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: Blastocysts Embryonic Stem Cell Lines Derived from Human. Science 282, 1145-1147. (1998)

9. Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC: Review: ex vivo engineering of living tissues with adult stem cells. Tissue Eng 12, 3007-3019 (2006)

10. Gimble JM, Katz AJ, Bunnell BA: Adipose-derived stem cells for regenerative medicine. *Circ Res* 100, 1249-1260. (2007)

11. Liao YH, Verchere CB, Warnock GL: Adult stem or progenitor cells in treatment for type 1 diabetes: current progress. *Can J Surg* 50,137-142. (2007)

12. Mimeault M, Hauke R, Batra SK: Stem Cells: A Revolution in Therapeutics-Recent Advances in Stem Cell Biology and Their Therapeutic Applications in Regenerative Medicine and Cancer Therapies. *Clin Pharmacol Ther* 82, 252-264. (2007)

13. Christoforou N, Gearhart JD: Stem Cells and Their Potential in Cell-Based Cardiac Therapies. *Prog Cardiovasc Dis* 49, 396-413. (2007)

14. Le Blanc K, Ringdén O: Mesenchymal stem cells: properties and role in clinical bone marrow transplantation. *Curr Opin Immunol* 18, 586-591. (2006)

15. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 213, 341-347 (2007)

16. Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, and Owen M: Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*. *Clin Orthop* 151, 294-307 (1980)

17. Haynesworth SE, Goshima J, Goldberg VM, and Caplan AI: Characterization of cells with osteogenic potential from human marrow. *Bone* 13: 81-88. (1992)

18. Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 276: 71-74. (1997)

19. Phinney DG, Prockop DJ: Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem Cells. 25: 2896-2902 (2007)

20. Chanda D, Kumar S, Ponnazhagan S: Therapeutic potential of adult bone marrow-derived mesenchymal stem cells in diseases of the skeleton. *J Cell Biochem* 111:249-257. (2010)

21. Du SJ, Purcell SM, Christian JL, McGrew LL, Moon RT: Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in Xenopus embryos. *Mol Cell Biol* 15:2625-2634. (1995)

22. Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT: The Wnt/Ca2t pathway: A new vertebrate Wnt signaling pathway takes shape. *Trends Genet* 16,279-283 (2000)

23. Komiya Y, Habas R: Wnt signal transduction pathways. *Organogenesis* 42:68-75. (2008)

24. Miller JR, Hocking AM, Brown JD, Moon RT: Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. *Oncogene* 1855, 7860-7872. (1999)

25. Logan CY, and Nusse R: The Wnt signaling pathway in development and disease: *Annu Rev Cell Dev Biol* 20, 781-810. (2004)

26. Moon RT, Kohn AD, De Ferrari GV, Kaykas A: WNT and beta-catenin signalling: Diseases and therapies. *Nat Rev Genet* 5:691-701. (2004)

27. Murdoch B, Chadwick K, Martin M, Shojaei F, Shah KV, Gallacher L, Moon RT, Bhatia M: Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells *in vivo*. *Proc Natl Acad Sci USA*100:3422-3427. (2003)

28. Kawano Y, Kypta R: Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 116, 2627-2634. (2003)

29. Nelson WJ, Nusse R: Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 303, 1483-1487. (2004)

30. Macdonald BT, Semenov MV, and He X: SnapShot: Wnt/beta-catenin signaling. *Cell* 131, 1204.e1-1204.e2. (2007)

31. Nalesso G, Sherwood J, Bertrand J, Pap T, Ramachandran M, De Bari C, Pitzalis C, and Dell'accio F: WNT-3A modulates articular chondrocyte phenotype by activating both canonical and noncanonical pathways. *J Cell Biol* 193, 551-564. (2011)

32. Semenov MV, Habas R, Macdonald BT, and He X: SnapShot: noncanonical Wnt signaling pathways: *Cell* 131, 1378.e1-1378.e2. (2007)

33. Church V, Nohno T, Linker C, Marcelle C, and Francis-West P: Wnt regulation of chondrocyte differentiation. *J Cell Sci* 115, 4809-4818. (2002)

34. Akiyama, H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR: Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev* 18, 1072-1087. (2004)

35. Lories RJ, Peeters J, Bakker A, Tylzanowski P, Derese I, Schrooten J, Thomas JT, and Luyten FP: Articular cartilage and biomechanical properties of the long bones in Frzb-knockout mice. *Arthritis Rheum* 56, 4095-4103. (2007)

36. Chen M, Zhu M, Awad H, Li TF, Sheu TJ, Boyce BF, Chen D, and O'Keefe RJ: Inhibition of beta-catenin signaling causes defects in postnatal cartilage development. *J Cell Sci* 121, 1455-1465. (2008)

37. De Boer J, Siddappa R, Gaspar C, van Apeldoorn A, Fodde R, Van Blitterswijk C: Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells. *Bone* 34, 818-826. (2004)

38. Etheridge SL, Spencer GJ, Heath DJ, Genever PG: Expression profiling and functional analysis of wnt signaling mechanisms in mesenchymal stem cells. *Stem Cells* 22, 849-860. (2004)

39. Samarzija I, Sini P, Schlange T, Macdonald G, Hynes NE: WNT3A regulates proliferation and migration of HUVEC via canonical and non-canonical Wnt signaling pathways. *Biochem Biophys Res Commun* 386, 449-454. (2009)

40. Endo Y, Wolf V, Muraiso K, Kamijo K, Soon L, Uren A, Barshishat-Küpper M, Rubin JS: Wnt-3a-dependent cell motility involves RhoA activation and is specifically regulated by dishevelled-2. *J Biol Chem* 280, 777-786. (2005)

41. Tu X, Joeng KS, Nakayama KI, Nakayama K, Rajagopal J, Carroll TJ, McMahon AP, Long F: Noncanonical Wnt signaling through G protein-linked PKCdelta activation promotes bone formation. *Dev Cell* 12, 113-127. (2007)

42.Taschner MJ, Rafigh M, Lampert F, Schnaiter S, Hartmann C: Ca^{2+}/Calmodulin-dependent kinase II signaling causes skeletal overgrowth and premature chondrocyte maturation. *Dev Biol* 317, 132-146. (2008)

43. Endo Y, Beauchamp E, Woods D, Taylor WG, Toretsky JA, Uren A, Rubin JS: Wnt-3a and Dickkopf-1 stimulate neurite outgrowth in Ewing tumor cells via a Frizzled3- and c-Jun N-terminal kinase-dependent mechanism. *Mol Cell Biol* 28, 2368-2379. (2008)

44. Da Forno PD, Pringle JH, Hutchinson P, Osborn J, Huang Q, Potter L: WNT5A expression increases during melanoma progression and correlates with outcome. *Clin Cancer Res* 14, 5825-5832. (2008)

45. Yamamoto H, Oue N, Sato A, Hasegawa Y, Yamamoto H, Matsubara A: WNT5A signaling is involved in the aggressiveness of prostate cancer and expression of metalloproteinase. *Oncogene* 29, 2036-2046. (2010)

46. Boland GM, Perkins G, Hall DJ, Tuan RS: Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. *J Cell Biochem* 93, 1210-1230. (2004)

47. Baksh D, Boland GM, and Tuan RS: Cross-talk between Wnt signaling pathways in human mesenchymal stem cells leads to functional antagonism during osteogenic differentiation. *J Cell Biochem* 101, 1109-1124. (2007)

48. Mason JM, Grande DA, Barcia M, Grant R, Pergolizzi RG, and Breitbart AS: Expression of human bone morphogenic protein 7 in primary rabbit periosteal cells: potential utility in gene therapy for osteochondral repair. *Gene Ther* 5, 1098-1104. (1998)

49. Gelse K, von der Mark K, Aigner T, Park J, and Schneider H: Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. *Arthritis Rheum* 48, 430-441. (2003)

Abbreviations: MSCs: Mesenchymal stem cells; CaMKII: calcium/calmodulin-dependent kinase II; ECM: extracellular matrix; iPSCs: induced pluripotent stem cells; ESCs: embryonic stem cells; FZD: Frizzled; DSH: Dishevelled; TCF: T-cell factor; CaMKII: calcium/calmodulin-dependent kinase II; PCP: planar cell polarity; AHACs: adult human articular chondrocytes; (TGF)-β: transforming growth factor; DMEM: Dulbecco's modified Eagle's medium; PBS: phosphate buffered saline; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GFP: green fluorescent protein; GAGs: glycosaminoglycans; DKK1: Dickkopf-related protein 1

Key Words: Wnt3A; Mesenchymal, Stem Cells,Induced, Pluripotent, Adult, Human, Articular, Chondrocytes

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