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Original Article

Inhibition of RUNX2 expression promotes differentiation of MSCs correlated with SDF-1 up-regulation in rats

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Abstract: Hypertrophic differentiation of differentiated MSCs has been a main barrier in application of mesenchymal stem cells (MSCs) for cartilage repair in the treatment of osteoarthritis (OA). Runt-related transcription factor 2 (RUNX2) is a well-known Runt domain containing transcription factor promoting OA progression through inducing chondrocyte hypertrophy. However, the molecular mechanisms of RUNX2 involved in differentiated MSCs hypertrophy remains not be fully understood. Thus, we firstly isolated MSCs from male Sprague-Dawley rats and induced them to be differentiated MSCs. Then real time-polymerase chain reaction (RT-PCR) assay was used to determine the expression levels of cytokines, differentiated MSCs markers and hypertrophic markers. The results showed the expression levels of cytokines (IL-1β, IL-6, IL-10, HIF-1α, TNF-α, TGF-β and SDF-1) and differentiated MSCs markers (ACAN, COL2A1 and SOX9) were significantly up regulated in differentiated MSCs, while the expression levels of hypertrophic markers (ALP, COLXA1, RUNX2 and MMP13) were obviously down regulated. Then the expression of RUNX2 was silenced in MSCs. RUNX2-silencing suppressed the expression levels of hypertrophic markers and elevated differentiated MSCs markers. Furthermore, with the elevated concentration of SDF-1, the expression levels of RUNX2 along with hypertrophic markers were significantly down regulated. In conclusion, RUNX2-silencing could inhibit hypertrophic differentiation of differentiated MSCs through up-regulating SDF-1, which suggest RUNX2 might be as a potential molecular target against OA and deserves further investigation.

Keywords: Osteoarthritis, MSCs differentiation, RUNX2, SDF-1

Introduction

Osteoarthritis (OA) is one of the most common and disabling diseases characterized by progressive degradation of joint cartilage [1]. Despite some achievement has been made in pharmacologic therapy, it is largely ineffective due to poorly understanding the molecular mechanisms underlying OA progression.

Currently, cell-based cartilage regeneration therapies have been demonstrated to be promising technologies. The mesenchymal stem cells (MSCs) has been widely applied to repair cartilage and can be differentiated marked by Aggrecan (ACAN), Collagen type II (Col2A1) and Sex determining region Y box 9 (SOX9) [2, 3]. Differentiated MSCs are identified as predominant cell type in mature articular cartilage, which are responsible for the maintenance and repair of normal extracellular matrix. Unfortunately, the phenotype of MSCs in cartilage repair is unstable [4]. Differentiated MSCs has been found to switch toward hypertrophic differentiation [5, 6], which may drive the onset and progression of OA [7]. Thus, if we could control hypertrophic differentiation, we will find an effective strategy for cartilage repair in the treatment for OA. However, the exact molecular mechanism underlying hypertrophic differentiation has not been fully understood.

Studies have indicated runt-related transcription factor 2 (RUNX2) is a well-known Runt domain containing transcription factor regulating chondrocyte maturation [8]. RUNX2 was previously shown to be a candidate gene promotes OA progression through chondrocyte hypertrophy [9, 10]. Enhancer-binding protein-β is a potent regulator of skeletal growth and OA development, which could cooperate with RUNX2 to control cartilage degradation with matrix metalloproteinase-13 (MMP-13) as the target and HIF-2α as the inducer [11]. Over-

expression of RUNX2 could alter bone remodeling in vivo directed by the MMP-13 promoter [12]. Interestingly, RUNX2, Collagen X, MMP-13 and ALP have been identified as hypertrophic markers and presented overexpressed in hypertrophic differentiation. Some signal pathways of chondrocyte hypertrophy, including WNT, BMP and IHH were deregulated by other signal factors, which consequently results in activating the transcription factor RUNX2. Activated RUNX regulated the transcription of hypertrophic markers like Collagen X, MMP-13 and VEGF [13]. Based on these evidences, we could infer RUNX2 might play a crucial role in hypertrophy occurrence. In addition, research has focused largely on the inflammatory cytokines, in particular interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [14]. Chemokines, as a family of small, soluble cytokines, have been shown to influence cell morphology and differentiation [15]. Of particular interest in cartilage biology is stromal cell-derived factor-1 (SDF-1), which is an 8 kDa Chemokine originally isolated from bone marrow stromal cells [16]. Related study has indicated that SDF-1 knockdown mice exhibit significant developmental abnormalities that lead to embryo death [17]. Similar studies suggest that SDF-1 play an important role in the development of OA, which responses to differentiated MSCs expressed functional chemokines receptors and released MMP-3 and MMP-13 [18]. In addition, TGF-β, as a growth factor, has shown to improve osteoblastic proliferation and bone tissue engineering [19]. These findings strongly support that RUNX2 and SDF-1 are involved in hypertrophy of MSCs and differentiated MSCs.

This study was performed to explore further the role of RUNX2 and SDF-1 in OA pathogenesis. Our results revealed that RUNX2 could promote hypertrophy of differentiated MSCs, which is closely related with up-regulation of hypertrophic markers and cytokines and down-regulation of SDF-1. What's more, elevated SDF-1 could suppress RUNX2 and hypertrophic markers. Our findings will provide evidences for the role of RUNX2 and SDF-1 in the occurrence of OA and fundamental information for future new therapy for OA.

Materials and methods

Ethics statement

All human/animal studies have been approved by The Institute Research Medical Ethics Committee of Fifth Affiliated Hospital of Sun Yat-Sen University. All human studies have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

Isolation and culture of MSCs

Bone mesenchymal stem cells (MSCs) were generated from male Sprague-Dawley rats (aged 6 weeks; 200-250 g) provided by Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). Briefly, femurs were isolated from the anesthetized rats and washed by Dulbecco's modified Eagle's medium (low glucose) containing 10% fetal bovine serum (Hyclone, Logan, Utah), 100 mg/ml streptomycin (Hyclone) and 100 units/ml penicillin (Invitrogen). The flushing medium was applied to nylon mesh and cells were harvested from filtered fluid by centrifugation for 5 min at 50 g. MSCs were re-suspending in medium and cultured in 37°C 5% CO_2 at a density of 5×10^6 cells/ml. After 3 days incubation, adherent cells were passaged. The fourth generation cells were stained with phycoerythrin-conjugated anti-CD45, CD44 (BD Biosciences Pharmingen), CD34, CD14 (Invitrogen), CD105 (R&D Systems), and CD90 (eBioscience) antibodies and analyzed by flow cytometry to determine the MSC phenotype of the cells.

Differentiated MSCs differentiation and analysis

To induce MSCs differentiation into differentiated MSCs, MSCs were cultured in medium that contained high glucose DMEM supplemented with 10 ng/ml recombinant TGF-β1 (PeproTech), 100 mM dexamethasone (Sigma-Aldrich), 1 mM sodium pyruvate, 0.2 Mm ascorbic acid 2-phosphate (Sigma-Aldrich) and ITS plus Premix (BD Biosciences). The medium was changed every 3 d. Differentiation was assessed at the molecular level after 21 days for chondrogenic differentiation

Plasmid construction, packaging and infection

To study the effect of RUNX2 in MSCs differentiation into differentiated MSCs, short hairpin RNA (shRNAs) targeted RUNX2 gene were designed and synthesized, inserted into pFH-L vector to generate constructed plasmids. The

Table 1. Primers used in this study

Gene	Primer	Primer sequence
BMP2	Forward	5'-AGGAGGAGGAGGCGAAGAAA-3'
BMP2	Reverse	5'-CCTGGGGAAGCAGCAACA-3'
IL-1β	Forward	5'-TGGAGAGTGTGGATCCCAAG-3'
IL-1β	Reverse	5'-GGTGCTGATGTACCAGTTGG-3'
IL-6	Forward	5'-ATAGTCCTTCCTACCCCAATTTCC-3'
IL-6	Reverse	5'-GATGAATTGGATGGTCTTGGTCC-3'
IL-10	Forward	5'-GAGAAGCATGGCCCAGAAATC-3'
IL-10	Reverse	5'-GAGAAATCGATGACAGCGCC-3'
HIF-1α	Forward	5'-GGACGATGAACATCAAGTCAGCA-3'
HIF-1α	Reverse	5'-GGAATGGGTTCACAAATCAGCAC-3'
TGF-β	Forward	5'-CAGTACAGCAAGGTCCTTGC-3'
TGF-β	Reverse	5'-ACGTAGTAGACGATGGGCAG-3'
TNF-α	Forward	5'-CTGAACTTCGGGGTGATCGG-3'
TNF-α	Reverse	5'-GGCTTGTCACTCGAATTTTGAGA-3'
SDF-1	Forward	5'-TGCATCAGTGACGGTAAACCA-3'
SDF-1	Reverse	5'-TTCTTCAGCCGTGCAACAATC-3'
ACAN	Forward	5'-CATGAGAGAGGCGAATGGAA-3'
ACAN	Reverse	5'-TGATCTCGTAGCGATCTTTCTTCT-3'
COL2A1	Forward	5'-TGGGTGTTCTATTTATTTATTGTCTTCCT-3'
COL2A1	Reverse	5'-GCGTTGGACTCACACCAGTTAGT-3'
SOX9	Forward	5'-AGTACCCGCACCTGCACAAC-3'
SOX9	Reverse	5'-TACTTGTAGTCCGGGTGGTCTTTC-3'
ALP	Forward	5'-CCGATGGCACACCTGCTT-3'
ALP	Reverse	5'-GGAGGCATACGCCATCACAT-3'
COLXA1	Forward	5'-CATGCCTGATGGCTTCATAAA-3'
COLXA1	Reverse	5'-AAGCAGACACGGGCATACCT-3'
MMP13	Forward	5'-CGATGAAGACCCCAACCCTAA-3'
MMP13	Reverse	5'-ACTGGTAATGGCATCAAGGGATA-3'
RUNX2	Forward	5'-GACGAGGCAAGAGTTTCACC-3'
RUNX2	Reverse	5'-GGACCGTCCACTGTCACTTT-3'

recombined pFH-L vector was transfected into 80% confluent cells with packing plasmids pVSVG-I and pCMVΔR8.92 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For lentivirus infection, MSCs were seeded into six-well plates and transfected with shRNA targeted to RUNX2 and control, respectively.

Real time-polymerase chain reaction (RT-PCR)

Total RNA was isolated from MSCs and differentiated MSCs RNeasy isolation kit (Qiagen, USA), as previously described [20]. The 1 μg of total RNA was transcribed into cDNA by SuperScript II Kit (Invitrogen). The mRNA expression for genes was evaluated on the BioRad Connet Real-Time PCR platform with SYBR Green PCR core reagents using 20 μl reaction mixtures (2 × SYBR premix ex taq 10 μl, forward and reverse primers (2.5 μM) 0.5 μl, cDNA 5 μl, ddH_2O 4.5 μl). The β-actin was used as an internal control. The reaction procedure was initiated with initial denaturation at 95°C for 1 min followed by 40 cycles including 5 s denaturation at 95°C and 20 s annealing extension at 60°C. The primers were designed by using Primers Express software (Bio-Tools Incorporated, Edmonton, AB, Canada), which presented in **Table 1**. The $2^{-\Delta\Delta CT}$ value method [21] was used to calculate the mean of triplicate CT value for genes.

Western blot analysis

Total protein was extracted from MSCs cells using 2 × SDS Sample Buffer (100 mM Tris-Hcl (pH 6.8), 4% SDS, 10% glycine and 10 mM EDTA). The proteins were separated by 10% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane under 300 mA for 2.5 h. The membrane was blocked using 5% skim milk at room temperature for at least 1 h and probed with primary antibodies against ACAN, COL2A1, SOX9, ALP, COLXA1, MMP13, RUNX2 and GAPDH. Then the membrane was washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:5000; NO.SC-2054; Santa Cruz) for 2 h at room temperature, followed by detection using enhanced chemiluminescent detection reagents. GAPDH was used as an internal control.

Statistics analysis

SPSS 13.0 software was used to analyze experimental data. All the data were expressed as mean ± standard deviation (SD) from three independent experiments. The difference was evaluated by nonparametric test. The *p* value less than 0.05 was considered to be statistically significant.

Results

Characterization of MSCs

It has been reported that mice MSC cells expressed the surface antigens including CD44

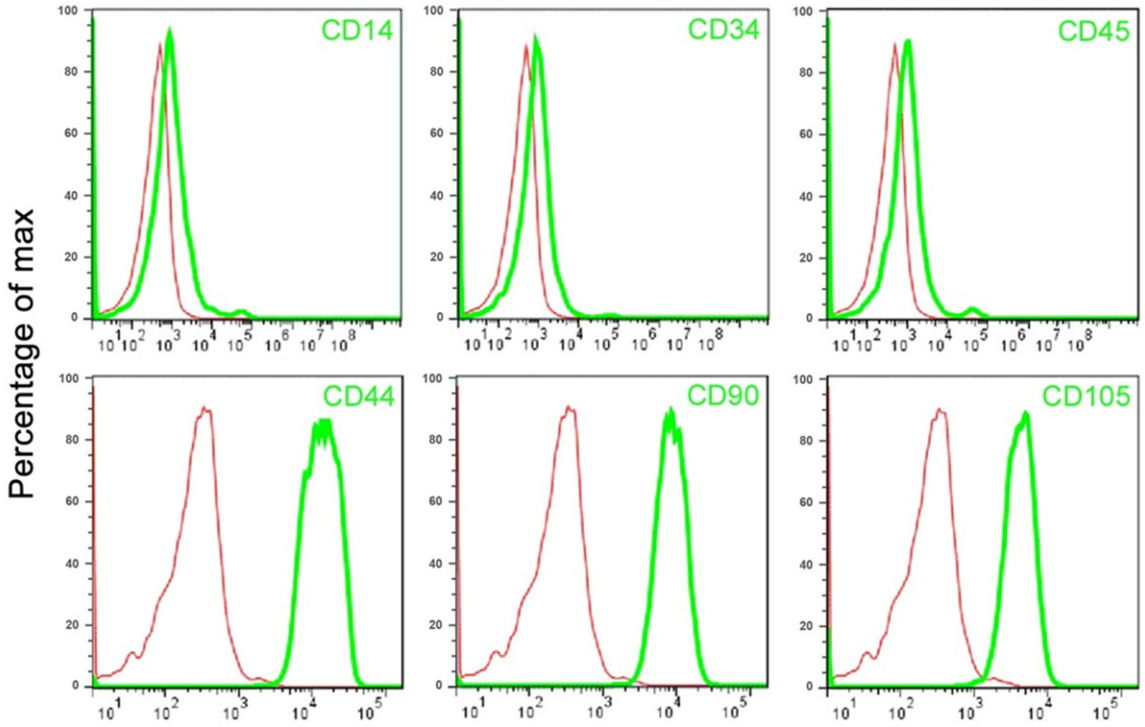

Figure 1. Positive expression of mesenchymal stem cell makers (CD44, CD90 and CD105) and negative expression of hematopoietic markers (CD14, CD34 and CD45) from mice bone marrow (BM) derived cells by flow cytometry analysis.

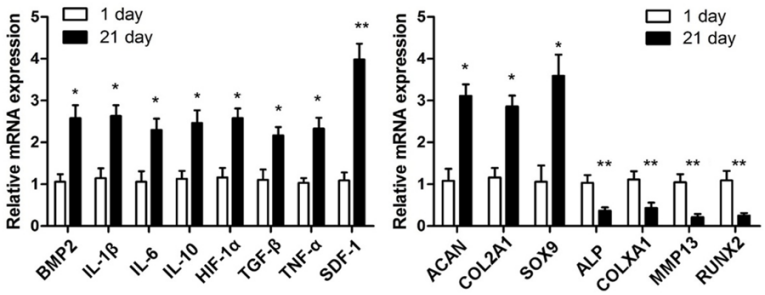

Figure 2. Chondrogenesis of MSCs, as confirmed by differentiated MSCs markers: SOX9, ACAN and COL2A1 using RT-PCR analysis.

and CD90 and CD105. Based on the flow cytometry analysis, we found MSCs expressed CD44, CD90 and CD105, but not CD14, CD34 and CD45. The positive cells were over 90%, demonstrating a high purity of MSCs. However expression of hematopoietic markers CD14, CD34 and CD45 was low, indicating a low contamination of hematopoietic cells in the isolated MSC (**Figure 1**).

MSCs differentiation

The mRNA expression of cytokines, differentiated MSCs and hypertrophic markers was tested to evaluate the differentiated status of MSCs after 21 days of induction. Statistical analysis showed a significant difference in the expression of all genes between MSCs and differentiated MSCs. As shown in **Figure 2**, the mRNA expression of cytokines, including IL-1β, IL-6, IL-10, HIF-1α, TNF-α, TGF-β and SDF-1 were obviously up regulated in differentiated MSCs compared those in MSCs. The mRNA expression of differentiated MSCs markers [22], including ACAN, COL2A1 and SOX9 were significantly increased in differentiated MSCs. Whereas, the levels of hypertrophic markers such as ALP, COLXA1, MMP13 and RUNX2 [13, 23, 24] were remarkably reduced in differentiated MSCs compared in those in MSCs ($P < 0.05$, $P < 0.01$, $P < 0.001$). These results showed MSCs cells were successfully induced into differentiated MSCs.

Knockdown of RUNX2 promotes MSCs differentiation

RUNX2 has been reported to be a key transcription factor that regulates osteogenesis [25] and

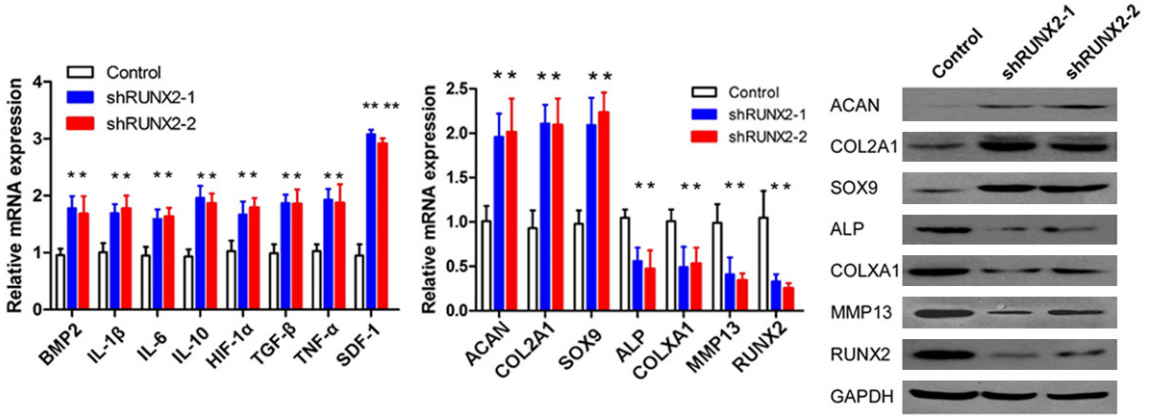

Figure 3. Knockdown of RUNX2 altered the expression levels of cytokines, differentiated MSCs and hypertrophic markers, as determined by RT-PCR and Western blot analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

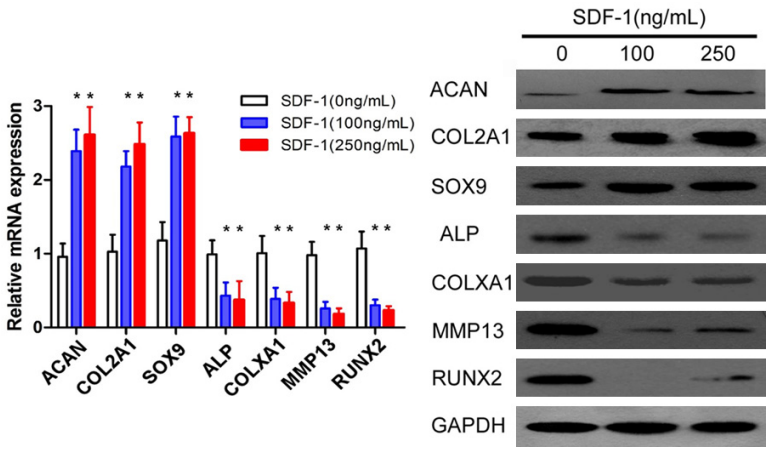

Figure 4. Elevated level of SDF-1 altered the expression levels of differentiated MSCs and hypertrophic markers, as determined by RT-PCR and Western blot analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

involved in activation of hypertrophic differentiation of articular differentiated MSCs [26]. To investigate the role of RUNX2 in chondrogenic differentiation, we successfully constructed RUNX2-silencing MSCs cell model and determined the levels of RUNX2 in MSCs after RUNX2 knockdown. As shown in **Figure 3**, knockdown of RUNX2 significantly up regulated several cytokines including IL-1β, IL-6, IL-10, HIF-1α, TNF-α, TGF-β. Interestingly, the mRNA expression of SDF-1 was remarkably increased in MSCs after RUNX2 knockdown. What's more, knockdown of RUNX2 obviously activated differentiated MSCs markers (ACAN, COL2A1 and SOX9), while suppressed hypertrophic markers (ALP, COLXA1 and MMP13). Collectively, RUNX2 might play an important role in hypertrophic differentiation of differentiated MSCs via up down regulating SDF-1 level.

Elevated SDF-1 level promotes MSCs differentiation

SDF-1 has been identified as a chemokines originally isolated from bone marrow stromal cells [16] and demonstrated to play an important role in the development of OA [27]. Our results showed SDF-1 was significantly up regulated in the process of MSCs differentiation into differentiated MSCs. To further confirm the SDF-1 whether affected MSCs differentiation, the effect of a series concentration of SDF-1 on the differentiated MSCs and hypertrophic markers was determined. As shown in **Figure 4**, up regulation of SDF-1 significantly increased the mRNA and protein levels of differentiated MSCs markers (ACAN, COL2A1 and SOX9) and decreased the mRNA and protein levels of hypertrophic markers (ALP, COLXA1, RUNX2 and MMP13). These finding indicated that both knockdown of RUNX2 and elevated SDF-1 levels could promoted the differentiation process from MSCs to differentiated MSCs.

Discussion

OA is a disabling disease characterized by progressive degradation of joint cartilage. Study has shown the development of OA may be caused by activation of hypertrophic differentiation of articular differentiated MSCs [26]. During hypertrophic differentiation of differentiated MSCs in OA, several hypertrophic mark-

ers, such as RUNX2, MMP-13 have been demonstrated to be overexpressed [13]. To further investigated the role of these hypertrophic markers in the differentiation process of differentiated MSCs, we firstly determined the levels of some cytokines, hypertrophic markers and differentiated MSCs markers between MSCs and differentiated MSCs and found differentiated MSCs markers (ACAN, COL2A1 and SOX9) were significantly increased in differentiated MSCs, which suggest we have successfully induced MSCs differentiation.

RUNX2, as a well-known Runt domain containing transcription factor, promotes OA progression through chondrocyte hypertrophy [13]. To further illustrate its role in differentiated MSCs hypertrophy, the expression of RUNX2 was specific knocked down in MSCs. Both qPCR and Western blot analysis indicated that knockdown of RUNX2 significantly suppressed the expression levels of ALP, COLXA1 and MMP-13, but promoted the expression levels of cytokines, chemokine SDF-1 and differentiated MSCs markers (ACAN, COL2A1 and SOX9). These evidences strongly suggest RUNX2 is a crucial factor for promoting chondrocyte hypertrophy. Interestingly, knockdown of RUNX2 remarkably activated the level of SDF-1. As our best knowledge, SDF-1 is a small cytokine that belong to the chemokine, which is expressed in the area of inflammatory bone destruction and has suppressive effect on osteoclastogenesis [28].

In organ culture, the high concentration of SDF-1 (250 ng/ml) was observed comparable to levels in the synovial fluid of osteoarchritic knees and can readily penetrate the articular cartilage [29, 30]. This suggests SDF-1 can stably accumulate around differentiated MSCs. In our investigation, up regulation of SDF-1 could significantly suppress the expression level of RUNX2 to promote MSCs differentiate into differentiated MSCs through activating differentiated MSCs markers (ACAN, COL2A1 and SOX9).

Based on these findings, we propose that the expression of RUNX2 was inversely related with the expression of SDF-1 in regulating MSCs differentiation into differentiated MSCs.

In summary, this study reveals the crucial role of RUNX2 in promoting chondrocyte hypertrophy might via activating the hypertrophic markers (ALP, COLXA1 and MMP-13) and negatively regulating the expression of SDF-1. These findings will provide an experiment basis for RUNX2 as a potential molecular target against OA. Additionally, further investigations will be needed to illuminate the exact molecular mechanism underlying RUNX2 and SDF-1 regulating hypertrophic differentiated MSCs.

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Disclosure of conflict of interest

None.

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