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CD105 promotes chondrogenesis of synovium-derived mesenchymal stem cells through Smad2 signaling

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ABSTRACT

Mesenchymal stem cells (MSCs) are considered to be suitable for cell-based tissue regeneration. Expressions of different cell surface markers confer distinct differentiation potential to different subpopulations of MSCs. Understanding the effect of cell surface markers on MSC differentiation is essential to their targeted application in different tissues. Although CD105 positive MSCs possess strong chondrogenic capacity, the underlying mechanisms are not clear. In this study, we observed a considerable heterogeneity with respect to CD105 expression among MSCs isolated from synovium. The $CD105^+$ and $CD105^-$ synovium-derived MSCs (SMSCs) were sorted to compare their differentiation capacities and relative gene expressions. $CD105^+$ subpopulation had higher gene expressions of *AGG*, *COL II* and *Sox9*, and showed a stronger affinity for Alcian blue and immunofluorescent staining for aggrecan and collagenase II, as compared to those in $CD105^-$ cells. However, no significant difference was observed with respect to gene expressions of *ALP*, *Runx2*, *LPL* and *PPARγ*. $CD105^+$ SMSCs showed increased levels of Smad2 phosphorylation, while total Smad2 levels were similar between the two groups. There was no difference in activation of Smad1/5. These results were further confirmed by CD105-knockdown in SMSCs. Our findings suggest a stronger chondrogenic potential of $CD105^+$ SMSCs in comparison to that of $CD105^-$ SMSCs and that CD105 enhances chondrogenesis of SMSCs by regulating TGF-β/Smad2 signaling pathway, but not Smad1/5. Our study provides a better understanding of CD105 with respect to chondrogenic differentiation.

1. Introduction

Articular cartilage is an avascular tissue that has a very limited healing capacity after injury caused by trauma or degenerative disease. Although several surgical treatments, such as microfracture or mosaicplasty, have been used with some success, the results have largely been unsatisfactory [1,2]. Cell-based tissue engineering is a promising therapeutic modality for cartilage repair [3]. However, selection of appropriate seed cells is a key issue in cartilage tissue engineering.

Mesenchymal stem cells (MSCs) are considered to be well-suited to potential application since they are convenient to harvest and have pluripotent capability [4,5]. However, MSCs are a heterogeneous group of cell subpopulations with no universal surface markers, although several markers, such as CD29, CD44, CD90 and CD105, have been used to identify MSCs [6]. Different subtypes with different cell surface markers have distinct proliferation and differentiation features [7]. For example, CD49f regulates the differentiation potential of MSCs and maintains pluripotency [8]. CD90 positive selection is known to enhance osteogenic capacity of human adipose-derived stem cells, and may prove to be an effective marker of a highly osteogenic subtype of MSCs for bone tissue engineering [9]. Since surface markers play an important role in control of MSC differentiation, it is essential to study the effects of surface markers on the differentiation potentials of MSCs.

CD105, also known as Endoglin, is a transmembrane

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glycoprotein that serves as a cell surface marker of MSCs [10]. CD105 is involved in a variety of biological processes, such as angiogenesis, through regulation of cell cycle, migration and differentiation on endothelial cells [11]. Further, expression of CD105 has been shown to correlate with extracellular matrix production by chondrocytes, and whose expression was shown to increase both during *in vitro* dedifferentiation of chondrocytes as well as in osteoarthritis cartilage *in vivo* [12]. Recent studies have demonstrated a stronger chondrogenic potential in CD105 positive ($CD105^+$) MSCs which implies their greater intrinsic capability to repair the cartilage defect [13]. However, the mechanism by which CD105 regulates chondrogenesis in MSCs is yet to be elucidated.

CD105 is an auxiliary receptor for the transforming growth factor-beta (TGF-β) which is involved in regulation of MSC differentiation [14]. TGF-β regulates cell functions through TGF-β receptor II and two other distinct receptors, activin-receptor like kinase-1 (ALK1) and ALK5, which mediate the phosphorylation of the downstream proteins Smad1/5 or Smad2/3 respectively, by balancing signals from these two pathways [12,15]. Aside from the canonical Smad signaling pathway for TGF-β, a host of other non-Smad signaling pathways that operate via TGF-β receptors have been identified. These include signal transduction via mitogen-activated protein kinase (MAPK) pathways [16].

In the present study, we hypothesized that CD105 enhances chondrogenesis of MSCs via regulation of TGF-β/Smad signaling. We isolated MSCs from synovium and sorted $CD105^+$ and $CD105^-$ synovium-derived MSCs (SMSCs). The effect of CD105 on chondrogenesis of SMSCs was observed and the underlying mechanisms investigated.

2. Materials and methods

2.1. Harvest of synovial tissues and isolation of cells

Synovial tissue was harvested from primary knee osteoarthritis patients who underwent total knee arthroplasty. Written informed consent was obtained from all patients prior to the use of their tissues. The study protocol was approved by the Ethics Committee at the Zhongshan Hospital, Fudan University.

All procedures of cell separation are described in our previous study [17]. Briefly, synovial tissues were digested with 0.25% trypsin and 0.1% collagenase II (Sigma–Aldrich, St. Louis, MO, USA) for 0.5 h and 2 h at 37 °C, respectively. Digested cells were filtered through a 70-μm nylon filter (Becton Dickinson, Franklin Lakes, NJ, USA) and resuspended in growth medium which consisted of DMEM with 10% FBS (Gibco, Invitrogen, Carlsbad, CA, USA). The medium was changed after 1 day to remove non-adherent cells and then replaced twice per week.

2.2. Flow cytometric analysis and cell sorting

SMSCs were harvested by enzymatic digestion, centrifuged and washed twice in phosphate-buffered saline. Aliquots of 2×10^5 cells were resuspended and directly stained with 5 μL fluorescein isothiocyanate (FITC)-labeled antibodies against CD29, CD45 or CD105 and phycoerythrin (PE) - labeled anti-CD44 (eBioscience, San Diego, CA, USA) and kept in dark at 4 °C for 30 min. Cytometric analysis and cell sorting were performed using a BD FACSAria™ II sorter. Data were processed using FlowJo software (FlowJo, Inc., Ashland, OR).

2.3. SMSC differentiation

FACS-sorted $CD105^+$ and $CD105^-$ SMSCs were plated onto 12-well culture plate at a density of 2×10^4 cells for osteogenesis and adipogenesis, or 5×10^5 cell pellets for chondrogenesis. SMSC differentiation was induced using hMSC adipogenic, osteogenic or chondrogenic culture medium (Cyagen Biosciences, Santa Clara, CA, USA), respectively. After 7 days of induction, relative gene expressions were measured by Reverse transcription polymerase chain reaction (RT-PCR). Cells were also stained with alkaline phosphatase for assessment of osteogenesis on the 14th day; cell pellets were stained with Alcian blue for aggrecan and immunofluorescence stain for collagenase II after 21 days.

2.4. Quantitative real-time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA). cDNA was obtained by reverse-transcription using PrimeScript RT Master Mix (Takara, Japan). Quantitative real-time PCR was performed using 2× SYBR Green PCR Master Mix using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA), under the following conditions: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 64 °C for 34 s, and a melt cure step. The target gene expression levels for each experiment were normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table 1. All experiments were performed in triplicates.

2.5. Western blot analysis

Cell lysates were extracted using RIPA lysis buffer (Beyotime, China) containing PMSF and phosphatase inhibitor (Roche, Switzerland) at 4 °C. The extracts were prepared using Cytoplasmic Extraction Reagents (Pierce Biotechnology, USA). Protein samples were separated by 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% bovine serum albumin (BSA; Beyotime, China) for 2 h, washed, and then incubated with primary antibodies for p-Smad2, Smad2, p-Smad1/5, Smad1/5, GAPDH, and CD105 (Cell Signaling Technology, USA) overnight at 4 °C. After washing, the membrane was incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected using enhanced chemiluminescence method (Pierce, IL). The protein expressions were quantified using densitometry with ImageJ software (National Institutes of Health, USA) and normalized to relative GAPDH expressions. All experiments were performed in triplicates.

2.6. SMSC transfection

$CD105^+$ SMSCs were transfected with either control lentiviral vectors or CD105 shRNA lentiviral vectors established by Genechem Biotechnology (Shanghai, China), as described in the instructions. Three CD105 specific target sequences were chosen and the efficiency of CD105 knockdown was assessed by Western blot and RT-PCR (Supplementary data). The control sequence and most effective sequence for CD105 used were *5′-TTCTCCGAACGTGTCACGT-3′* and *5′-CTTCTACACAGTACCCATA-3′*

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA). All data are expressed as mean ± standard deviation (SD). Student's *t*-test was used to assess between-group differences. P values < 0.05 were considered indicative of a statistically significant between-group difference.