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Potent *in vitro* chondrogenesis of CD105 enriched human adipose-derived stem cells

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ABSTRACT

Adipose-derived stem cells (ASCs) are considered as a promising cell source for cartilage regeneration. However, the heterogeneity of this cell source may affect their ability in cartilage formation. It is therefore necessary to establish an efficient method for isolating the cells that have chondrogenic potential. To date, no specific markers have been reported to be able to isolate such a cell population from human adipose tissue. In recent studies, endoglin (CD105) has been known as a relatively specific marker for identifying mesenchymal stem cells, but no studies show it is related to chondrogenic potential of human ASCs. In this study, human cells from adipose tissue were isolated, cultured, and sorted according to CD105 expression. The sorted cells were then subjected to adipogenic, osteogenic, and chondrogenic induction to confirm their multi-potentiality. In adipogenic conditions, CD105− cells showed stronger Oil Red staining and higher expression of adipose-specific genes compared to CD105+ cells. By contrast, CD105+ cells exhibited better osteogenic potential with stronger Alizarin Red staining and higher expression of osteogenic specific genes than CD105− cells. Noticeably, CD105+ cells also exhibited a much stronger chondrogenic potential than CD105− cells, with stronger collagen II staining and higher gene expression of collagen II and aggrecan. Most importantly, CD105+ cells could form a homogeneous cartilage-like tissue when seeded into a biodegradable scaffold and cultured in chondrogenic media for 8 weeks. These results indicate that sorting of ASC subpopulation with CD105 as a marker may allow better *in vitro* chondrogenesis and thus provide an important implications for cartilage regeneration and reconstruction using autologous cells from adipose tissue.

1. Introduction

The cell source for cartilage tissue engineering is always a difficult yet important issue [1]. Adipose-derived stem cells (ASCs) have been widely reported as mesenchymal stem cells (MSCs) with high proliferative and multi-lineage potential, including osteogenic, chondrogenic, and adipogenic lineages [2–4]. ASCs can also be obtained in abundance from autologous fat tissue with minimal injury to the donor site, making them a desirable cell source for cartilage regeneration [5,6]. Our previous studies demonstrated that autologous ASCs could repair porcine osteochondral defects [7] when seeded on polyglycolic acid (PGA) unwoven fibers. However, the ASC-scaffold constructs failed to form homogeneous cartilage-like tissue *in vitro* during chondrogenic induction, forming sporadic islands of cartilage instead (unpublished data). Those results differed obviously from the promising results using bone marrow stem cells (BMSCs) [8]. In fact, several studies have shown that ASCs may have limited chondrogenic potential [9–11], and some researchers even suggest that ASCs might not be suitable at all for cartilage regeneration [11].

The reason for the conflict between two types of MSCs may lie in the heterogeneity of ASCs [2,12]. The pool population of ASCs obtained from adipose tissue using current methods of isolation is quite heterogeneous, containing many kinds of non-MSCs, including adipose precursor cells, fibroblasts, endothelial cells, and smooth muscle cells etc. Most of these non-MSCs actually have no chondrogenic potential and thus might interfere with

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chondrogenesis of ASCs. To address this concern, it is necessary to establish an efficient method for separating MSCs with chondrogenic potential from the rest of the cell population.

Although the surface makers related to human ASCs have been widely studied [3,13,14], less attention has been paid to define the relationship between a surface marker and the chondrogenic potential, leading to the lack of efficient methods for isolating chondrogenic cells from adipose-derived cells. In recent studies, endoglin (CD105) has been reported as a relatively specific marker for identifying MSCs. Aslan et al. reported that CD105+ cells in a pool population of BMSCs exhibited stronger stemness than CD105− cells [15]. Other studies found that CD105+ cells from human synovial fluid or bone marrow exhibited strong chondrogenic potential [16,17]. Ishimura et al. also found that CD105+ cells from mouse adipose-derived vascular fraction showed strong chondrogenic potential [18]. Nonetheless, no studies have shown the correlation of CD105 with the chondrogenic potential of human ASCs. Especially, no studies have reported whether human ASCs can form homogeneous cartilage-like tissue *in vitro* when seeded into a biodegradable scaffold.

In this study, cells were isolated from human adipose tissue and sorted using CD105 as the surface marker. The cell morphology, colony-forming ability, multi-lineage potential, as well as their chondrogenesis in 3D biodegradable scaffolds were compared between CD105+ and CD105− cells to explore the feasibility of constructing homogeneous cartilage-like tissue *in vitro* utilizing the cells from human adipose tissue.

2. Materials and methods

2.1. Cell culture

The human adipose tissue was harvested from the patients who underwent liposuction procedures in Shanghai 9th People's Hospital. Similar to previously described methods [2], cells were isolated from adipose tissue by 0.075% collagenase (NB4, Serva, France) treatment and cultured in tissue culture plates with MesenPRO RS™ Medium (GIBCO) at 37 °C in a 5% CO_2 humidified incubator. All experimental protocols involving human tissue and cells were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

2.2. Flow cytometry and sorting

The expression of CD105 in adherent cells were examined by fluorescence-activated cell sorting (FACS, Beckman Coulter, USA) after different times in culture (freshly isolated, 16 h, 4 days, 7 days, and 10 days) [19]. Monoclonal anti-human CD105/Endoglin–Phycoerythrin (anti-CD105–PE, R&D Systems) was used.

According to the pattern of CD105 expression, after 16 h of culture, the adherent cells were sorted by FACS with anti-CD105–PE antibody [20]. The CD105+ and CD105− cells were then collected and cultured in the same conditions for the subsequent experiments.

2.3. Proliferation assay

Cell proliferation was assayed as described previously [21]. Briefly, the CD105+ or CD105− cells were seeded into 24-well plates at a density of 1×10^4 cells per well. Cells were removed from the wells ($n = 3$) every day for 8 days and counted using a hemacytometer in order to construct growth curves.

2.4. Colony forming unit-fibroblast (CFU-F) assay

CFU-F assay was performed using modified techniques described previously [22]. The CD105+ or CD105− cells were cultured in MesenPRO RS™ Medium at a density of 1000 cells per 100 mm dish. The media were changed every 3 days. After 14 days of culture, the cells were fixed with 4% paraformaldehyde and stained with Giemsa solution. The number of colonies (diameter ≥2 mm) was counted.

2.5. In vitro differentiation

According to previously established methods [19], CD105+ or CD105− cells were cultured in adipogenic, osteogenic, and chondrogenic media to evaluate the multi-lineage potential of sorted cells. For the chondrogenic conditions, an optimized growth factor combination (10 ng/mL TGF-β3, 10 ng/mL BMP-6, 50 ng/mL IGF-1, 0.1 μM dexamethasone, R&D Systems) was used according to a newly published literature [23]. The adipogenic, osteogenic, and chondrogenic potentials were confirmed through gene expression and staining of their corresponding specific markers. The cells were cultured in regular culture medium (DMEM plus 10% FBS) as a control.

2.6. Real-time PCR

Total RNA was extracted from the cells after multi-lineage induction and reverse polymerase transcription (RT) was performed to obtain cDNA according to previously described methods [24]. Real-time PCR was performed with a continuous fluorescence detector (Stratagene MX3000P) by monitoring SYBR Green fluorescent dye (SYBR Green PCR master mix, Applied Biosystems) bound to double-strand DNA. The GAPDH mRNA level was quantified as an internal control. The forward and reverse primer sequences are listed in Table 1.

2.7. Magnetic-activated cell sorting (MACS)

To obtain abundant, functional CD105+ or CD105− cells in a short time for 3D cartilage construction, MACS was used for cell sorting. Consistent with FACS, we also chose 16 h of cell culture as the time point for cell sorting. According to previously described methods [15], the adherent cells were harvested, incubated with microbeads that were directly conjugated with mouse anti-human CD105 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), and separated using AutoMACS separation device and column (Miltenyi Biotec GmbH). The CD105+ and CD105− cells were then collected and cultured in the same conditions for 3D cartilage construction.

2.8. In vitro 3D cartilage construction

Polylactic acid (PLA) coated polyglycolic acid (PGA) scaffold was prepared similar to previously established methods [25]. Briefly, 6 mg of PGA unwoven fibers were compressed into a cylinder shape with 5 mm diameter and 2 mm thickness and 0.2 mL of 0.1% PLA in dichloromethane (Sigma) was added to solidify the scaffold shape. MACS-sorted CD105+ or CD105− cells (5×10^6 in 0.1 mL) were then evenly added dropwise into the scaffolds. The cell-scaffold constructs were incubated *in vitro* for 4–5 h at 37 °C with 95% humidity and 5% CO_2 to allow for complete adhesion of the cells to the scaffold. Then, chondrogenic media were added to cover the constructs, and the culture media were changed every 3 days. All the constructs were kept in culture for 8 weeks to allow for 3D cartilage formation *in vitro*.

2.9. Histological analysis

As previously described [8], the engineered tissue was fixed, embedded in paraffin, and sectioned into 5 μm slices. The sections were stained with hematoxylin and eosin (HE) to evaluate histological structure and with Safranin-O and anti-collagen II to evaluate cartilage matrix deposition in the engineered tissue.

2.10. Statistical analysis

The differences between CD105+ and CD105− populations in CFU-F, positive area of Oil Red and Alizarin Red staining, as well as gene expression by real-time PCR, were analyzed using a Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

Table 1
Reverse transcriptase-polymerase chain reaction (RT-PCR) primers.

Gene	Primer size (bp)	Primer sequences
PPARγ	313	Sense: 5-AGATTCTCCTATTGACCCAG-3 Antisense: 5 AGGCTCCACTTTGATTGC 3
LPL	273	Sense: 5-ATGAGCCTGTAATCCT-3 Antisense: 5-AAACGGTCCTTGTCTA-3
RUNX2	317	Sense: 5-ACTGGGCCCTTTTTCAGA-3 Antisense: 5-GCGGAAGCATTCTGGAA-3
ON	369	Sense: 5-GATGAGGACAACAACCTTCTGAC-3 Antisense: 5-TTAGATCACAAGATCCTTGTCGAT-3
COL II	293	Sense: 5-ATTGGAGCCCTGGATGAGC-3 Antisense: 5-GCGAGACTTGCGTCTACCC-3
Agreecan	353	Sense: 5-CACCACCTACAAACGC-3 Antisense: 5-GGCACGATGGGAAAT-3
GAPDH	213	Sense: 5-CGGATTTGGTCGTATTGGG-3 Antisense: 5-CGCTCCTGGAAGATGGTGAT-3