Characterization of Chondrogenic Gene Expression and Cartilage Phenotype Differentiation in Human Breast Adipose-Derived Stem Cells Promoted by Ginsenoside Rg1 In Vitro

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Key Words
Human breast adipose-derived stem cells • Chondrogenesis • Cell-based therapy • Induced differentiation • Cell proliferation • Ginsenoside Rg1

Abstract

Background/Aims: Investigating and understanding chondrogenic gene expression during the differentiation of human breast adipose-derived stem cells (HBASCs) into chondrogenic cells is a prerequisite for the application of this approach for cartilage repair and regeneration. In this study, we aim to characterize HBASCs and to examine chondrogenic gene expression in chondrogenic inductive culture medium containing ginsenoside Rg1. Methods: Human breast adipose-derived stem cells at passage 3 were evaluated based on specific cell markers and their multilineage differentiation capacity. Cultured HBASCs were treated either with basic chondrogenic inductive conditioned medium alone (group A, control) or with basic chondrogenic inductive medium plus 10 µg/ml (group B), 50 µg/ml (group C), or 100 µg/ml ginsenoside Rg1 (group D). Cell proliferation was assessed using the CCK-8 assay for a period of 9 days. Two weeks after induction, the expression of chondrogenic genes (collagen type II, collagen type XI, ACP, COMP and ELASTIN) was determined using real-time PCR in all groups. Results: The different concentrations of ginsenoside Rg1 that were added to the basic chondrogenic inductive culture medium promoted the proliferation of HBASCs at earlier stages (groups B, C, and D) but resulted in chondrogenic phenotype differentiation and higher mRNA expression of collagen type II (CO-II), collagen type XI (CO-XI), acid phosphatase (ACP), cartilage oligomeric matrix protein (COMP) and ELASTIN compared with the control (group A) at later stages. The results reveal an obvious positive dose-effect relationship between...
ginsenoside Rg1 and the proliferation and chondrogenic phenotype differentiation of HBASCs in vitro. **Conclusions:** Human breast adipose-derived stem cells retain stem cell characteristics after expansion in passage 3 and serve as a feasible source of cells for cartilage regeneration in vitro. Chondrogenesis in HBASCs was found to be prominent after chondrogenic induction in conditions containing ginsenoside Rg1.

**Introduction**

The loss of cartilage tissue due to trauma, tumor or age-related degeneration is generally associated with a poor prognosis that requires long-term follow-up treatment, which brings about an ongoing clinical challenge in orthopedic reparative & reconstructive surgery. Due to the limited vascularization of cartilage tissue, chondrocytes exhibit poor proliferative activity and regenerative capacity in vivo. This limitation often leads to the accelerated development of osteoarthritis or the remodeling of cartilage defects with fibrous or fibrocartilaginous tissue, which shows a decreased mechanical potential compared with hyaline cartilage.

Autologous chondrocyte transplantation (ACT) was the first chondrocyte tissue engineering technique to be applied in daily clinical practice and was first successfully performed by Brittberg in 1994 [1]. This technique consists of three main steps, including the isolation of chondrocytes from healthy cartilage tissue, chondrocyte cultivation or expansion in vitro over 2-3 weeks, and re-injection of chondrocytes into the injured cartilage, covered with a periosteal flap [1]. This method gained international acceptance within the orthopedic surgery field [2, 3] and was further refined by adding biomaterials such as coated scaffolds, membranes, and different matrices [4, 5]. Despite this wide acceptance, several studies have revealed certain problems and limitations of ACT, including cell leakage, the requirement for high cell concentrations, and apoptosis of the re-injected chondrocytes. In addition, the major shortcomings of this procedure remain: only smaller cartilage defects can be addressed, and an adjunct-qualified laboratory unit within the surgical department is necessary [6].

In the last several decades, an increasing number of studies have focused on mesenchymal stem cells isolated from bone marrow in vitro and in vivo. However, bone marrow procurement causes considerable discomfort to the patient and yields a relatively small number of harvested cells. More recent animal and clinical studies have shown that adipose-derived stem cells (ASCs) are capable of repairing damaged soft tissue, bone or cartilage defects [7–13]. ASCs represent a readily available abundant supply of mesenchymal stem cells that can be obtained from lipo-aspirates [11, 12]. It is simple to expand ASCs to large numbers in vitro compared with bone marrow-derived mesenchymal stromal cells (BMSCs) [14], and there is less cell heterogeneity in ASCs than BMSCs due to the mixture of hematopoietic and mesenchymal stem cells [15–17]. Similar to BMSCs, various studies have described the plasticity of ASCs to differentiate towards chondrocytes, osteoblasts, adipocytes, myocytes (cardiomyocytes, smooth muscle, and skeletal muscle cells) and neural phenotype cells in different inductive culture systems [18–22]. Although BMSCs are considered to be a valuable source for bone tissue regeneration in human diseases, the capacity of autologous BMSCs to differentiate toward functional bone-forming osteoblasts remains relatively limited for bone regeneration in vivo [23]. Osteogenesis or chondrogenesis is defined by a series of events starting with commitment to an osteogenic or chondrogenic lineage by mesenchymal cells. However, the differentiation potency of ASCs toward osteogenic or chondrogenic cell lines is not as strong as BMSCs under the same conditions [24, 25]. To promote their ability to differentiate into chondrogenic cell lines and increase their proliferative capacity, on the basis of our previous work demonstrating the effect of ginsenoside Rg1 on the proliferation and neural phenotype differentiation of human adipose-derived stem cells in vitro [26], in this study, we introduced ginsenoside Rg1 as a supplement in cell cultures to observe its ability to promote the proliferation and chondrogenic differentiation of HBASCs in vitro.
Materials and Methods

Preparation of ginsenoside Rg1

Ginsenoside Rg1 (protopanaxatriol extract monomer; Sigma, St. Louis, MO, USA), which is a colorless semi-crystalline material, was easily dissolved in pyridine and acetone (both at a 100 μg/mL final concentration). The chemical name of ginsenoside Rg1 is (3β, 6α, 12β)-20-(β-D-glucopyranosyloxy)-3, 12-dihydroxydammar-24-en-6-yiβ-D-glucopyranoside. Its molecular formula is C42H72O14, and its molecular weight is 801.01. Its chemical structure formula is presented in Fig. 1.

Patient consent and ethical approval

The present study was approved by the institutional ethical review board of Zhongshan Hospital of Sun Yat-Sen University (Zhongshan, Guangdong, China). Written informed consent was provided by the donor patient.

Isolation and expansion of HBASCs

HBASCs were isolated from spare fat tissue from 6 female patients who underwent reduction mammoplasty or breast prolapse repair (Table 1). For each sample, 150 g of fat tissue was cut into small sections and was washed with phosphate-buffered saline (PBS) to eliminate red blood cells. Then, the adipose tissue was finely minced and digested with 0.1% collagenase for 60 min at 37°C with vigorous agitation. Following centrifugation at 260 ×g for 5 min, the cell pellet, mainly consisting of HBASCs, was resuspended in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS). The suspended cells were seeded onto dishes to expand the HBASCs. The cultures were incubated at 37°C with 5% carbon dioxide. The first medium change was conducted 24 h after seeding, and nonadherent cells were discarded. Thereafter, the medium was replaced every 3 days. Specific differentiation medium and culture conditions are described in the following relevant results sections. Cultured cells were observed using a microscope to assess expansion and cell morphology. After reaching 80%-90% confluence, the primary culture (P0) was trypsinized using 0.05% trypsin-EDTA (Invitrogen) and passaged at a culture expansion ratio of 1:3 until passage 3 (P3).

Flow cytometry analysis

Flow cytometry was performed on the HBASCs at P3. The HBASCs were trypsinized using Accutase (Innovative Cell Technologies, San Diego, CA, USA). The cell suspension was subsequently centrifuged at 260 ×g for 10 minutes and washed with DPBS containing 0.5% bovine serum albumin (Sigma). HBASCs were filtered through a 70-mm nylon membrane, and the number of cells was determined with a hemocytometer. A total of 1×10⁶ cells were incubated with either fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for 30 minutes. The following CD surface markers were tested: CD29/PE, CD31/PE, CD34/FITC, CD44/FITC, CD45/FITC, CD49d/FITC, CD73/FITC, CD90/FITC, CD105/PE, HLA-ABC/FITC and HLA-DR-DP-DQ/FITC (BD Biosciences, NJ, USA). Ten thousand events were acquired for each CD surface marker in a Becton Dickinson FACSCalibur flow cytometer. The data analysis was performed using CellQuestPro acquisition software (BD Bioscience, Franklin Lakes, NJ, USA).

Table 1. Information of patients

<table>
<thead>
<tr>
<th>SN of patients</th>
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<th>Age (year)</th>
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later; the cells were switched to media containing different concentrations of ginsenoside Rg1, including 3 treatment groups (10, 50, or 100 µg/ml ginsenoside Rg1) and a control (without ginsenoside Rg1) for up to 9 days. The proliferation of HBASCs was determined using the CCK-8 assay (Kumamoto, Japan) and measured via microplate reader scanning (ELx800, BioTek) at 450 nm as previously described [27].

Adipogenic induction of HBASCs
Isolated HBASCs were expanded in culture until passage 3 (P3) in an equal volume mixture of Ham’s F-12 medium and Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS (control medium). The HBASCs were induced in adipogenic induction medium that contained 200 µM indomethacin, 10 µM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 µM dexamethasone (Sigma) at a seeding density of 300 cells/mm². The media were replaced every three days, and the cells were maintained in culture for up to two weeks. Adipogenic differentiation was determined by Oil Red O staining. The Oil Red O staining procedure was as follows (1). Air dry the cultured cells for 30 minutes, and then fix in 1% formalin for 5 minutes (2). Rinse in 3 changes of distilled water. Place in absolute propylene glycol for 5 minutes to avoid transferring water into the Oil Red O. (3) Stain in Oil Red O solution for 8 minutes in 60°C oven (4). Rinse in 85% propylene glycol solution for 5 minutes (5). Rinse in distilled water and stain in Gill’s hematoxylin solution for 30 seconds (6). Wash thoroughly in running water for 3 minutes and rinse in 2 changes of distilled water (7). Mount with glycerin jelly or aqueous mounting medium (8). As a result, lipids will appear red, and the nuclei will appear pale blue.

Osteogenic induction of HBASCs
HBASCs at passage 3 were induced in osteogenic induction medium containing 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 µg/ml ascorbate-2-phosphate (Sigma) at a seeding density of 300 cells/mm². The cultures were fed with the osteogenic medium every three days for up to three weeks. After three weeks, osteogenic differentiation was determined via Alizarin Red staining. The alizarin Red staining procedure for calcium precipitation (mineralization) was as follows (1). Fix cells in 2.5% glutaraldehyde (freshly prepared in 1x PBS buffer) for 10-15 minutes at room temperature (2). Remove the fixation solution, and wash cells with 1x PBS (pH adjusted to 4.2) 1-2 times (3). Add a filtered 2% Alizarin Red solution to the fixed cells and incubate them at 37°C for 10-20 minutes (Note: monitor staining under a microscope every 2-5 min) (4). Remove the Alizarin Red solution, and rinse cells with PBS 1-2 times (5). Record the staining results through microphotography (positively stained nodules will appear orange-red).

Neurogenic induction of HBASCs
HBASCs at passage 3 were induced in neurogenic pre-induction medium containing 1 mM mercaptoethanol (BME, Sigma) for 24 hours, which was then replaced by neurogenic inductive medium containing 5 mM BME and 20 ng/ml basic fibroblast growth factor (bFGF, Sigma) at a seeding density of 300 cells/mm² for 7 days. After 7 days, the cultures were fixed in 10% formalin, and neurogenic differentiation was determined via immunohistochemical staining for neuron-specific enolase (NSE); the procedure was as follows (1). Fix cells in 2.5% glutaraldehyde (freshly prepared in 1x PBS buffer) for 10-15 minutes at room temperature (2). Remove the fixation solution, and wash cells with 1x PBS (pH adjusted to 4.2) 1-2 times (3). Add a filtered 2% Alizarin Red solution to the fixed cells and incubate them at 37°C for 10-20 minutes (Note: monitor staining under a microscope every 2-5 min) (4). Remove the Alizarin Red solution, and rinse cells with PBS 1-2 times (5). Record the staining results through microphotography (positively stained nodules will appear orange-red).

Chondrogenic induction of HBASCs
Four groups of HBASCs at P3 were cultured in T25 flasks at a cell density of 1×10^5 cells/cm² in basic chondrogenic induction medium (BCIM, control, group A) or in BCIM plus 10 µg/ml (group B), 50 µg/ml (group C), or 100 µg/ml ginsenoside Rg1 (group D). The basic chondrogenic induction medium contained F12:DMEM (1:1) supplemented with 1% FBS, 10 ng/ml transforming growth factor-β1 (TGF-β1), 50 ng/ml insulin-like growth factor 1 (IGF-1), 6.25 µg/ml transferrin, 100 nM dexamethasone, and 1% antibiotics and biochemistry.
antimycotics (Sigma). The morphological features of the cultured cells were monitored every day using an inverted light microscope (Olympus, Shinjuku-ku, Tokyo, Japan). HBASCs were harvested after two weeks of culture for Alcian blue staining and quantification of chondrogenic gene expression in all groups. The procedure was as follows (1). Deparaffinize slides and hydrate with distilled water (2). Stain in Alcian blue solution for 30 minutes (3). Wash in running tap water for 2 minutes, and rinse in distilled water (4). Counterstain in nuclear Fast Red solution for 5 minutes, and wash in running tap water for 1 minute (5). Dehydrate through 95% alcohol and 2 changes of absolute alcohol, applied for 3 minutes each (6). Clear in xylene or a xylene substitute (7). Mount with resinous mounting medium. As a result, agminated cells will secrete strongly acidic sulfated mucosubstances and appear blue.

**Total RNA isolation and quantitative PCR analysis**

Total RNA was extracted from cultured HBASCs after the second week of culture in either the control medium or the chondrogenic induction medium using the TRI Reagent (Molecular Research Center; Cincinnati, OH) according to the manufacturer's protocol. The HBASCs were homogenized in the TRI reagent and centrifuged at 12,000 rpm for 15 minutes at 4°C to separate the cell debris. Total RNA was precipitated with 10 µL of Polyacryl Carrier (Molecular Research Center). The total RNA pellet was then washed with 75% ethanol, dissolved in RNase- and DNase-free water (Invitrogen) and stored at -80°C until use. Complementary DNA (cDNA) was synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen). The reaction mixture and protocol followed the manufacturer’s recommendations. The reaction cycle was 10 min at 23°C, 60 min at 42°C, and 10 min at 94°C. To quantify the expression levels of chondrogenic genes after the second week of culture in the chondrogenic induction medium or control medium, quantitative polymerase chain reaction (qPCR) was performed using cDNA as the template. The genes of interest were CO-II, CO-XI, ACP, COMP and ELASTIN. The specific primer sequences were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) based on the published GenBank database sequences (Table 2). qPCR was performed in a Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA), and the data were analyzed using the Bio-Rad iCycler software. Each qPCR mixture consisted of iQ SYBR Supermix (Bio-Rad), forward and reverse primers (5 µM of each) and 1 µl of cDNA template. The following PCR conditions were applied: cycle 1 - 95°C for 3 minutes (1×); and cycle 2 - step 1, 95°C for 10 seconds, and step 2, 61°C for 30 seconds (40×). The PCR cycles were followed by melting curve analysis to determine the specificity of the PCR products. Next, the data were normalized to the expression of the housekeeping gene GAPDH. The formulas for calculating the relative mRNA expression from the data are as follows:

Relative mRNA expression: $2^{\Delta\Delta C_T}$

$\Delta\Delta C_T = C_T$ for the housekeeping gene – $C_T$ for the gene of interest

$C_T$ = threshold cycle (relative measure of the concentration of the target in the amplification reaction).

**Statistical analysis**

The data are presented as the mean±SD. The gene expression data for collagen type II, collagen type XI, the aggrecan core protein, COMP and ELASTIN in the four groups after induction for two weeks were

<table>
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<th>Chondrogenic Gene</th>
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<td>Human Collagen Type II, Col II</td>
<td>F: 5'-CTATCTGGGACGAGCAGCTGGCA-3'</td>
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<tr>
<td></td>
<td>R: 5'-ATGGGTTGCAAATGTAATGAGG-3'</td>
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<td>F: 5'-CAGCTTTACCGCCACCTCC-3'</td>
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<tr>
<td></td>
<td>R: 5'-ACCGCAGGAAATCCCTTCG-3'</td>
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<tr>
<td>Cartilage Oligomeric Protein, COMP</td>
<td>F: 5'-AATCTGGGAGAGGAGGT-3'</td>
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<td></td>
<td>R: 5'-TGTCCTTTGGTCTGTTCC-3'</td>
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<tr>
<td>ELASTIN</td>
<td>F: 5'-GGCCTGGAGGAAACCTCTTT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCACCAACTCTGGGACACC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-TCCCCTAGCTGAAGGGGAA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGAGGAGTGGGTCCTGT-3'</td>
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subjected to one-way analysis of variance with Tukey’s post-hoc test and were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL). P < 0.05 were considered statistically significant.

Results

Characterization and immunophenotype of HBASCs

Following initial isolation and expansion, homogeneous HBASCs growing in a monolayer with a spindle-shaped morphology were observed after culture for 1~2 weeks (Fig. 2A). These HBASCs presented a strong proliferation capacity. The HBASCs reached 80%-90% confluence 7 days after initial seeding for the first passage. During subsequent culture, these cells reached the same confluence within 3-4 days, with a 1:3 split ratio in the HBASCs of P3 (Fig. 2B). These observations demonstrated that HBASCs resemble other ASCs in terms of their morphology and proliferation capacity [26, 28]. The HBASCs displayed positive staining for the mesenchymal surface markers CD29, CD44, CD49d, CD73, CD90, CD105 and HLA-ABC/FITC, as determined through flow cytometry analysis (Table 3). The HBASCs in passage 3 exhibited high levels of expression of CD29, CD44, CD73, CD90 and CD105, with more than 90% of the total cell population being stained, while more than 80% of the total cell population showed positive staining for CD49d. More than 99% of the HBASCs expressed the histocompatibility antigen molecule HLA-ABC. In contrast, only a small proportion (less than 6%) of the HBASCs expressed the hematopoietic markers CD31, CD34 and CD45 and the histocompatibility antigen class II HLA-DR-DP-DQ. It has been reported that MSCs derived from different tissue sources express similar, but not identical, patterns of cell surface markers, possibly due to differences in tissue sources and donor age [29, 30]. CD105, CD90 and CD44 are the three main positive markers used for MSCs, while CD34 is the most frequently reported negative marker. CD49d and CD31 have also been described as a positive marker and a negative marker, respectively [28].

Adipogenic, osteogenic and neurogenic differentiation of HBASCs

After 14 days of culture in the adipogenic induction medium, lipid droplets had formed in the induced HBASCs, and positive Oil Red O staining further confirmed the formation of lipid droplets in the culture (Fig. 2C). Under osteogenic induction conditions, after 21 days, dark extracellular matrix material was detected following the induction period. The
deposition of calcified matrix by the cells was confirmed by positive staining with Alizarin Red (Fig. 2D). Under neurogenic induction conditions, after 7 days, the majority of cells had become bipolar or multipolar neuron-like cells, displaying long neurites similar to those of axons or dendrites. These cells were positive for NSE and nestin (Fig. 2E). The corresponding negatively stained controls are shown in Fig. 2F, G, H.

Influence of ginsenoside Rg1 on the cell proliferation of HBASCs

During the chondrogenic induction process, CCK-8 tests were performed on the 4 experimental groups of HBASCs (i.e., the groups exposed to basic chondrogenic inductive medium plus 10, 50, or 100 μg/mL of ginsenoside Rg1) and the basic chondrogenic inductive medium control group. The results, expressed as growth curves, clearly demonstrated that ginsenoside Rg1 promoted the cell proliferation of HBASCs during the chondrogenic differentiation process. Beginning 3 d after treatment, the proliferation rate of the ginsenoside Rg1 groups (B, C and D) was significantly higher than that of the control group (group A) (P < 0.001, Fig. 3). The results show that ginsenoside Rg1 has obvious positive dose-dependent effects on cell proliferation.

Morphological features of HBASCs during chondrogenic induction

After the HBASCs of the 4 groups had been incubated in the chondrogenic medium for 48 hours, the cells began to move closer to one another and form cell aggregates (Fig. 4A-D). This aggregation demonstrated that cellular condensation activity started shortly after chondrogenic induction. After 14 days, the cell aggregates became larger in the BCIM culture (Fig. 5A). In the BCIM plus 10 μg/ml ginsenoside Rg1 group, most of the cells in the culture were involved in aggregate formation, and empty areas were observed in the culture flasks (Fig. 5B). However, on day 14 in the BCIM plus 50 and 100 μg/ml ginsenoside Rg1 groups, the cell aggregates became darker in color, which indicated the formation of dense cell aggregates with increased extracellular matrix production (Fig. 5C and D). All four groups showed positive staining for Alcian blue, and the blue staining, indicating the density of cell aggregates, increased gradually from group A to group D.

Sequential expression of chondrogenic genes in HBASCs

After 14 days of induction, the HBASCs cultured in BCIM plus 10, 50 and 100 μg/ml ginsenoside Rg1 (groups B, C and D) displayed significantly higher expression of chondrogenic genes (mRNA of CO-II, CO-XI, ACP, COMP and ELASTIN) than the control group.
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Fig. 4. Morphological characterization of HBASCs following chondrogenic induction for 48 hours. (A) basic chondrogenic induction without Rg1; (B) chondrogenic induction with 10 μg/ml Rg1; (C) chondrogenic induction with 50 μg/ml Rg1; (D) chondrogenic induction with 100 μg/ml Rg1; (E) normal HBASCs without induction. Bar=100 μm.

Fig. 5. Characterization of HBASCs prior to and following chondrogenic induction, showing Alcian blue staining following chondrogenic induction for 2 weeks. (A) Basic chondrogenic induction without Rg1; (B) chondrogenic induction with 10 μg/ml Rg1; (C) chondrogenic induction with 50 μg/ml Rg1; (D) chondrogenic induction with 100 μg/ml Rg1; (E) normal HBASCs without induction. Bar=100 μm.

(only BCIM, group A). The specific data were as follows: the mRNA level of CO-II was 2.36- to 5.72-fold higher compared with the control group (P < 0.05); the mRNA level of CO-XI was 2.87- to 4.24-fold higher compared with the control group (P < 0.05); the mRNA level of ACP was 2.93- to 5.25-fold higher compared with the control group (P < 0.05); the mRNA level of COMP was 1.98- to 5.50-fold higher compared with the control group (P < 0.05); and the mRNA level of ELASTIN was 2.25- to 4.64-fold higher compared with the control group (P < 0.05). The results show that ginsenoside Rg1 has obvious positive dose-dependent effects on chondrogenic differentiation (Fig. 6).

Discussion

Mesenchymal stem cells (MSCs) are multipotent mesoderm-derived progenitor cells that can be isolated from various human tissues, including bone marrow (BMSCs), umbilical
cord blood (CBSCs), muscular tissue (MDSCs), and adipose tissue (ASCs). MSCs derived from human adipose tissue have been successfully differentiated into functional adult white or brown fat cells and into neural, muscle, tendon, bone, or cartilage cells [31–33]. However, different tissue sources and conditions as well as isolation techniques have led to variability in MSC-initiating populations. To minimize MSC population variability, the initial population of cells and the differentiated progeny are defined by examining the expression of specific cell surface markers. In addition to different surface antigen profiles, the individual therapeutic capacities of MSCs populations can also differ greatly. For instance, in the treatment of myocardial infarction, MSCs from discrete populations exhibit differences in healing performance for cardiac regeneration [34] but possess nearly equal chondrogenic differentiation capacities \textit{in vitro} and \textit{in vivo} [35, 36]. Additional factors, such as age and sex, have marked effects on the proliferation and differentiation capacities of ASCs. For example, ASCs from elderly donors (>60 years of age) display lower proliferation rates and impaired osteogenic and chondrogenic differentiation, whereas adipogenic differentiation is independent of donor age [37]. The donor’s gender must also be taken into consideration because muscle-derived stem cells from female donors show a higher potential for cartilage regeneration and repair [38]. The differentiation potential and mechanical properties of ASCs also decline with extended cell passaging [39]. Therefore, many protocols and tissue engineering strategies utilize cells between the second and fifth passages. Compared with other types of MSCs, a shift toward utilizing ASCs has recently taken place because of their

Fig. 6. Relative mRNA expression analysis of the chondrogenic marker genes collagen type II, collagen type XI, ACP, COMP and ELASTIN in the 0 μg/ml Rg1 control (A), 10 μg/ml Rg1 (B), 50 μg/ml Rg1 (C) and 100 μg/ml Rg1 (D) groups. The relative expression of collagen type II in Rg1-supplemented groups (B: 10 μg/ml Rg1, C: 50 μg/ml Rg1, and D: 100 μg/ml Rg1) were significantly higher compared with the control group (A: without Rg1), and the significant difference of relative expression including collagen type XI, ACP, COMP and ELASTIN also existed in Rg1-supplemented groups compared with the control. The results are shown as the mean±SD (n =3, *P<0.05).
better bioavailability and greater ease of harvesting via liposuction or from breast operations. In this study, we successfully isolated ASCs from human breast adipose tissues and identified their multilineage differentiation capacity.

Recent progress in the field of cartilage tissue engineering has led to new and exciting research related to regenerative medicine. This interdisciplinary field is focused on the development of biological substitutes that restore, maintain or improve tissue function by applying the principles of engineering and the life sciences [40, 41]. Strategies that stimulate cartilage regeneration to reduce or treat complications are becoming more important for treating articular cartilage injury and disease due to the increase in life expectancy and ageing of the world population. Cell-based approaches for chondrogenesis and cartilage regeneration are widely considered to be the most effective, as they are able to efficiently sustain the physiological osteogenic process in vivo. Indeed, the most promising field for the application of ASCs is cartilage reconstruction/regeneration [42, 43].

Ginsenoside Rg1, a steroidal saponin that is abundant in ginseng, is one of the most active components of ginseng and contributes to many of its effects. Previous studies have shown that ginsenoside Rg1 can induce differentiation of mouse embryonic stem cells into neurons in vitro via the GR-MEK-ERK1/2-PI3K-Akt signaling pathway in addition to inducing the proliferation and angiogenesis of endothelial progenitor cells and inhibiting their senescence [44, 45]. Furthermore, our previous work demonstrated that ginsenoside Rg1 can promote the cell proliferation and neural phenotype differentiation of human adipose-derived stem cells in vitro [24]. However, whether ginsenoside Rg1 could promote the chondrogenic differentiation of HBASCs was unknown prior to this study. In this work, we showed for the first time that different concentrations of ginsenoside Rg1 promote cell proliferation when HBASCs at passage 3 are not undergoing inductive differentiation in Dulbecco’s minimum essential medium (DMEM), according to the obtained cell growth curves. Comparison of cell growth under supplementation with 10 µg/ml (group B), 50 µg/ml (group C), or 100 µg/ml ginsenoside Rg1 in DMEM revealed a slightly higher growth rate than in medium without ginsenoside Rg1. Moreover, it demonstrated a dose-dependent effect of ginsenoside Rg1 on the cell proliferation of HBASCs at earlier stages in vitro. It is feasible that the HBASCs could be used as a cartilage source in regenerative medicine. Subsequently, we further investigated the influence of ginsenoside Rg1 on the chondrogenic differentiation of these cells. Accordingly, we aimed to optimize the chondrogenic culture conditions for the in vitro induction of HBASCs by testing different concentrations of ginsenoside Rg1 in basic chondrogenic inductive medium. Our results regarding cell growth in basic chondrogenic inductive medium (BCIM) plus 10 µg/ml, 50 µg/ml and 100 µg/ml ginsenoside Rg1 revealed a markedly higher growth rate compared with the BCIM-only treatment. The different concentrations of ginsenoside Rg1 that were added to the BCIM markedly promoted the proliferation of HBASCs at earlier stages. In the current study, we added different concentrations of ginsenoside Rg1 to the BCIM during the culture of HBASCs and found that this supplementation significantly enhanced the mRNA expression levels of chondrogenic genes, such as collagen type II (relative density from 2.36- to 5.72-fold), collagen type XI (relative density from 2.87- to 4.24-fold), ACP (relative density from 2.93- to 5.25-fold), COMP (relative density from 1.98- to 5.50-fold) and ELASTIN (relative density from 2.25- to 4.64-fold), when HBASCs were maintained in BCIM for 14 days compared with the control group. These findings not only demonstrate the potential use of ginsenoside Rg1 for cartilage regeneration but also support the theory that ginsenoside Rg1 has a broad range of effects on cell differentiation and proliferation. Thus, within a certain concentration range of ginsenoside Rg1, the results revealed a dose-dependent effect of ginsenoside Rg1 on the proliferation at earlier stages and chondrogenic differentiation at later stages of HBASCs in vitro. However, chondrogenesis is hypothesized to be regulated by sequential exposure to chondrogenic induction factors in a specific timeframe. Therefore, further studies should evaluate the sequential combinations of various growth factors to achieve more complete chondrogenesis in HBASCs. Furthermore, the use of HBASCs for cartilage regeneration in vivo will be studied in the future.

KARGER
Conclusions

Our data show that the different concentration conditions of ginsenoside Rg1 have a significant effect on the behavior of HBASCs. The results demonstrated that ginsenoside Rg1 exerts an obvious effect on stimulating the cell proliferation of HBASCs at earlier stages in vitro. It also shows a significant potential for promoting the chondrogenic differentiation of HBASCs at later stages in vitro. Moreover, the results revealed a dose-dependent effect of ginsenoside Rg1 on the proliferation and chondrogenic differentiation of HBASCs in vitro. These effects of ginsenoside Rg1 may facilitate the potential use of HBASCs for cartilage repair and regeneration.

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