pISSN 1738-2696 · eISSN 2212-5469
http://dx.doi.org/10.1007/s13770-015-0113-4
ORIGINAL ARTICLE

TERM

Supplementation of Growth Differentiation Factor-5 Increases Proliferation and Size of Chondrogenic Pellets of Human Umbilical Cord-Derived Perivascular Stem Cells

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In recent years, perivascular stem cells (PVCs) have gained increasing attention as a promising source for regenerative medicine due to their greater differentiation potential compared to multipotent mesenchymal stem cells (MSCs). It has been reported that growth differentiation factor-5 (GDF-5) is involved in regulating proliferation and chondrogenic differentiation of MSCs. In this study, we investigated the effect of GDF-5 on the proliferation and chondrogenic differentiation of PVCs isolated from human umbilical cords. The supplementation of PVC cultures with GDF-5 (100 ng/mL) significantly enhanced their proliferative rate and augmented the size of pellets in micromass pellet cultures for chondrogenic induction. Although similar expression levels of chondrogenic-related genes were observed in chondrogenic pellets treated with GDF-5 compared to the pellets without GDF-5 treatment, these results indicate that supplementation of GDF-5 is able to acquire more chondrocytes when starting with equal amount of PVCs. Our study suggests that GDF-5 is an effective agent for the enhancement of PVC proliferation, thereby achieving a higher number of chondrocytes that are applicable in therapeutic doses for cartilage regeneration.
Tissue Eng Regen Med 2015;12(3):181-187

Key Words: Perivascular stem cells; Growth differentiation factor-5; Proliferation; Chondrocytes

INTRODUCTION

Articular cartilage possesses a weak capacity for self-repair due to its lack of repair system like cytokines, vessels and chondrocytes [1,2]. Therefore, the repair of cartilage injuries is considered one of the most important clinical and scientific challenges. Surgical techniques including multiple drilling, microfracture, and autologous chondrocyte transfer have been developed and clinically applied for the repair of damaged cartilage [3,4]. However, these techniques are faced with problems including limited proliferative and de-differentiation potential of chondrocytes, invasive procedures, and the sacrifice of normal cartilage [5,6]. In order to overcome the major drawbacks of previous techniques, stem cell-based therapy has been evaluated as an alternative approach to replace autologous chondrocyte transfer for cartilage repair [7-9]. In recent years, perivascular stem cells (PVCs), which represent ubiquitous ancestors of mesenchymal stem cells (MSCs), have gained increasing attention as a promising candidate for cartilage regeneration due to their greater proliferation rate and capacity for osteogenic, adipogenic and chondrogenic differentiation when compared to bone marrow-derived MSCs (BM-MSCs) [10-12]. PVCs can be isolated from various fetal and adult tissues including human adipose tissue, umbilical, placenta and skeletal muscle and

Received: December 16, 2014
Revised: January 24, 2015
Accepted: February 2, 2015
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are being extensively tested in a number of preclinical studies for various diseases [10].

Generation of sufficient number of functional chondrocytes from multipotent stem cells remains a major issue for fulfilling the increasing demands of cartilage repair. Basic fibroblast growth factor and transforming-growth factor (TGF)-β1 have been reported to enhance proliferation and chondrogenic differentiation of MSCs derived from human synovium and bone marrow, respectively [13,14]. Lee et al. [15] demonstrated that the combination of TGF-β1 and bone morphogenic protein-2 provided a favorable condition for chondrogenic differentiation of human synovium-derived MSCs. Notably, it has been reported that growth differentiation factor-5 (GDF-5) supports the differentiation of MSCs into chondrocytes and cartilage development [16-24]. Although some exogenous factors have been described to promote the *in vitro* generation of chondrocytes from MSCs, no experiments have been performed to identify exogenous factors for *in vitro* generation of chondrocytes from PVCs.

Based on previous studies on MSCs, we hypothesized that supplementation of GDF-5 in PVC cultures or in micromass pellet cultures will positively affect their proliferation and chondrogenic differentiation. In this study, we investigated the effect of GDF-5 on proliferation, morphology, generation of micromass pellets, and chondrogenesis of PVC isolated from human umbilical cord (HUC). Our study suggests that GDF-5 is an effective agent for the enhancement of PVC proliferation, thereby obtaining a higher number of chondrocytes that are applicable in therapeutic doses for cartilage regeneration.

MATERIALS AND METHODS

Isolation and expansion of HUCPVCs

HUCs were obtained from full-term deliveries by caesarian section with IRB approved donors' written consent at Kangwon National University hospital. HUCPVCs were isolated and cultured as described previously [25]. Briefly, adherent cells migrated from HUC vessels were cultured for 13−16 days in α-MEM (Gibco, CA USA) supplemented with 10% FBS (Hyclone, UT, USA), 1% penicillin/streptomycin and 1 mM L-glutamine. On reaching 70−80% confluency, HUCPVCs were detached by treatment with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma, MO, USA) and subcultured at about 6500 cells/cm^2. After 2nd passage, cells were frozen and kept in liquid nitrogen until use.

Flow cytometry

The immunophenotypic characterization was performed by flow cytometry using the following fluorochrome-conjugated mouse anti-human antibodies: CD45-APC, CD31-PE, CD34-FITC, CD146-FITC, CD44-APC, and CD90-APC (all BD Biosciences). Single cell suspension were detached with 0.05% trypsin-EDTA and resuspended in 1% FBS-PBS at passage 2. The cells were filtered through a 70 μm filter and incubated for 1 hr at 4°C. Negative control is nonspecific IgG of the corresponding cells. Live cells were analyzed for cell surface markers using an Accuri C6 (BD Biosciences) and Accuri C6 analysis software (BD Biosciences). For DNA content analysis, single cell suspension from chondrogenic pellets was stained with 7-aminoactinomycin-D and analyzed using Accuri 6.

In vitro multilineage differentiation of HUCPVCs

Adipogenic differentiation

The HUCPVCs (4.5×10^3 cells per cm^2) were plated onto 12-well tissue culture plates and cultured as a monolayer in the presence of adipogenic supplements (Gibco) for 21 days. Cells were harvested after 21 days, then subjected to stain with Oil Red O (Lifeline Cell Technology, MD, USA) to visualize the intracellular accumulation of lipid vacuoles and quantified by spectrophotometry at A_{540}.

Osteogenic differentiation

The HUCPVCs were plated onto 12-well tissue culture plate at a density of 4.5×10^3 cells per cm^2 and cultured as a monolayer in the presence of osteogenic supplements (Gibco) for 21 days. On day 21, cultures were fixed in 4% PFA for 10 min and stained with Alizarin Red S (Lifeline Cell Technology) to visualize mineralization in osteogenic cell cultures and quantified by spectrophotometry at A_{405}.

Chondrogenic differentiation

The HUCPVCs (passage 3) were trypsinized using 0.05% trypsin-EDTA and washed with the expansion medium. HUCPVCs (2.5×10^5 cells per tube) were pelleted by centrifugation at 1500 rpm for 10 min and resuspended in StemPro™ chondrogenic medium (Gibco no.A10071-01) supplemented with 100 ng/mL of GDF-5 (R&D, MN, USA). The pellets were maintained with chondrogenic culture medium in the presence or absence of GDF-5 in 5% CO_2 at 37°C for 21 days and medium changed every 3 days. The dose and duration of GDF-5 for chondrogenic differentiation was determined based on previous studies [20,21]. On day 21, the chondrogenic differentiation potential of HUCPVCs was evaluated by hematoxylin and eosin staining and Alcian blue stain. Briefly, the chondrogenic pellets were fixed in 4% PFA, dehydrated, and embedded in paraffin for histology to detect sulfated proteoglycans by Alcian blue staining.

Proliferation assay and morphometric analysis

To evaluate effects of GDF-5 on the proliferation and morphological changes of PVCs, HUCPVCs (passage 2) were plated onto 6-well tissue culture plates at a density of 2.4×10^4 cells per well and cultured in the presence or absence of GDF-5 (100 ng/mL) for 5 days. Viable cells were counted using Moxi Z automated cell counter (ORFLO Technologies, ID, USA). Image J software was used to measure the cell area.

Table 1. Primer sequences for qRT-PCR

Genes		Sequences
Collagen IIA1 (COL2A1)	F	GCCAC CTTCTCACTGCTCA
	R	TAGGACAGGCAGCACCTCTT
Aggrecan (ACAN)	F	CGAAGGAAGAGTGTCGGAAG
	R	CTTGGACCTCTTTGGAGCTG
SOX-9	F	GGAGGAGAAGAGAAGGGGTG
	R	CTTAGGAAGGCGCGGGGTGG
GAPDH	F	TGCACCACCAACTGCTTAGC
	R	GGCATGGACTGTGGTCATGAG

RT-PCR: quantitative real-time polymer chain reaction

Quantitative RT-PCR

Total ribonucleic acid (RNA) was extracted from chondrogenic pellets and PVC cultures using RNeasy mini kit (Qiagen, Limburg, the Netherlands) according to the manufacturer's manual. cDNA was made with 1 μg of RNA using TOPscript™ RT Dry mix (Enzynomics, Daejeon, Korea) and subsequent quantitative real-time polymer chain reaction (RT-PCR) were carried out in triplicate using GoTaq Green Masterer Mix (Promega, WI, USA) on StepOne™ Real-Time PCR system (Applied Biosystems, CA, USA). Amplifications were performed using the following conditions: 5 min at 95°C followed by 40 cycles of denaturation (15 sec at 95°C), annealing and extension (1 min at 60°C) with FAM signal reading after extension. All data were normalized to GAPDH. The primer sequences used are shown in Table 1.

Data analysis

Values for all measurements are presented as mean±standard deviation. Comparisons for all experiments were performed with Student's t-test. Significance levels were set at $p<0.05$.

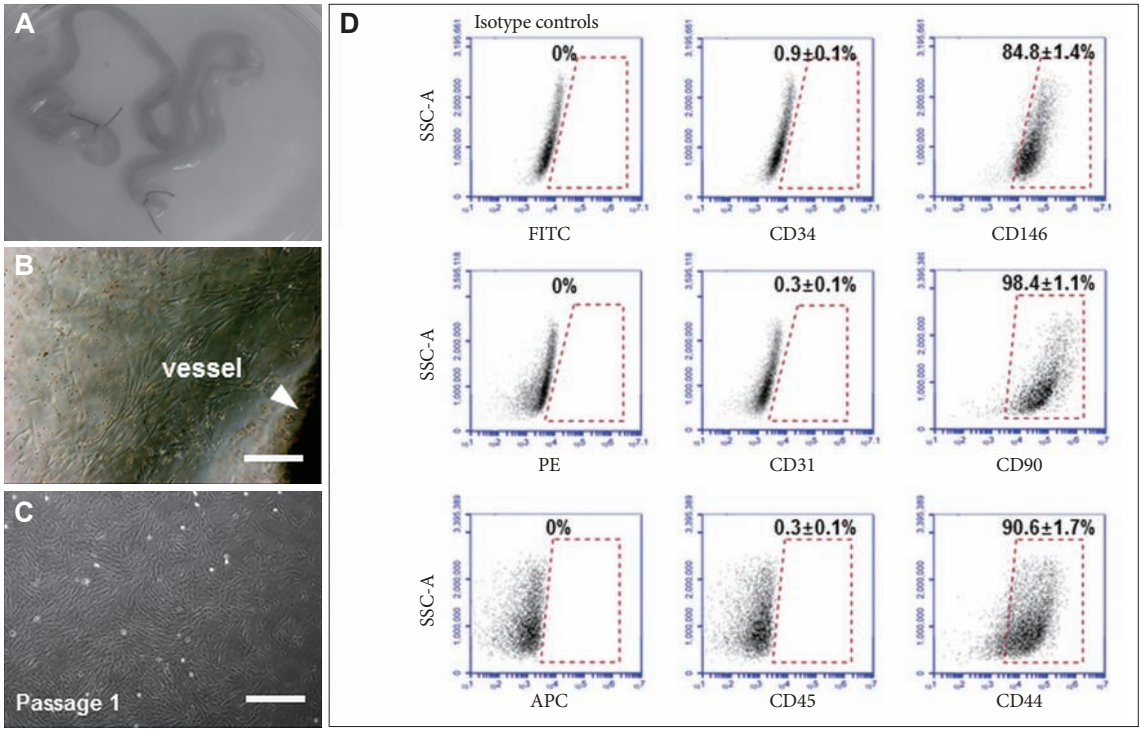

Figure 1. Isolation and immunophenotypic analysis of PVCs from HUCs. (A) A dissected vessel removed from HUCs was ligated at both ends and seeded into a 100-mm. (B) On day 7–10 post-plating, the fibroblast-like cells migrated from the vessels appeared as a colony (the white arrow indicates the vessel). (C) PVC colonies were collected and subcultured when the colonies are too dense to spread out. Scale bars, 100 μm. (D) Representative FACS dot plots for immunophenotypic analysis of isolated HUCPVCs. Frequencies shown in each dot plot indicate that average and standard deviation (n=3). PVCs: perivascular stem cells, HUCs: human umbilical cords, FACS: fluorescence-activated cell sorting, FITC: fluorescein isothiocyanate, PE: phycoerythrin, APC: allophycocyanin, SSC-A: side scatter-area.

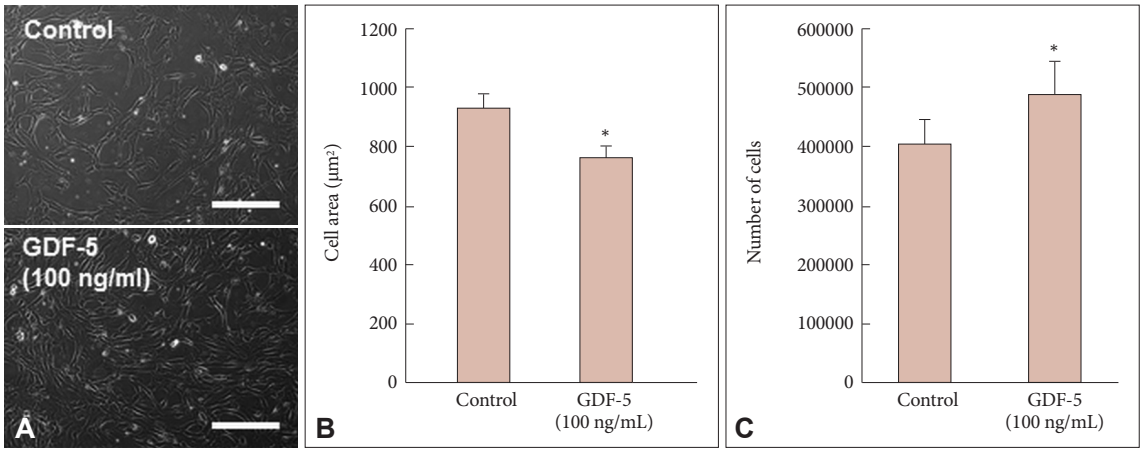

Figure 2. Effects of GDF-5 supplementation on proliferation of PVCs. (A) HUCPVCs (passage 3) were plated at a density of 6×10^4 cells per well and treated with 100 ng/mL of GDF-5 for 5 days. Scale bar, 100 μm. (B) Measurement of cell area (μm²) in PVC cultures treated or non-treated with GDF-5. (C) GDF-5 promotes proliferation of HUCPVCs. Bars indicate mean±SD. GDF *p<0.05. GDF-5: growth differentiation factor-5, PVCs: perivascular stem cells, HUCs: human umbilical cords.

RESULTS

Supplementation of GDF-5 promotes the proliferative rate of HUCPVCs

HUCPVCs are normally located in the perivascular regions of human umbilical vessels including arteries and veins, which were isolated according to our previous non-enzymatic isolation method [25]. Dissected HUC vessels were first ligated at both ends and then plated into a dish containing the expansion medium (Fig. 1A). On day 7–10 post plating, fibroblast-like cells that migrated from the vessels appeared as a colony-forming unit and were successfully subcultured (Fig. 1B and C). To characterize the cell surface antigens of the isolated cells, flow cytometry was employed to analyze these cells at passage 2. These cells displayed a high expression level of CD44 (90.6±1.7%), CD90 (98.4±1.1%), and CD146 (84.8±1.4%), which are markers for MSCs and PVCs, and a negative expression of CD31 (0.3±0.1%), CD34 (0.9±0.1%), and CD45 (0.3±0.1%) (Fig. 1D). We first asked if supplementation of GDF-5 influences on morphology and proliferation of HUCPVCs *in vitro*. HUCPVCs were cultured in the presence or absence of GDF-5 (100 ng/mL) for 5 days. During *in vitro* expansion of HUCPVCs without GDF-5, the cells are relatively bigger and flattened compared to the cells that were expanded in medium supplemented with GDF-5 (Fig. 2A). Measurement of cell area supports this morphological change by supplementation of GDF-5 (Fig. 2B). In addition, the number of HUCPVCs was significantly increased in the presence of GDF-5 (p<0.05) (Fig. 2C). These results suggest that GDF-5 could be an effective agent for the enhancement of PVC proliferation and induces a change in morphology.

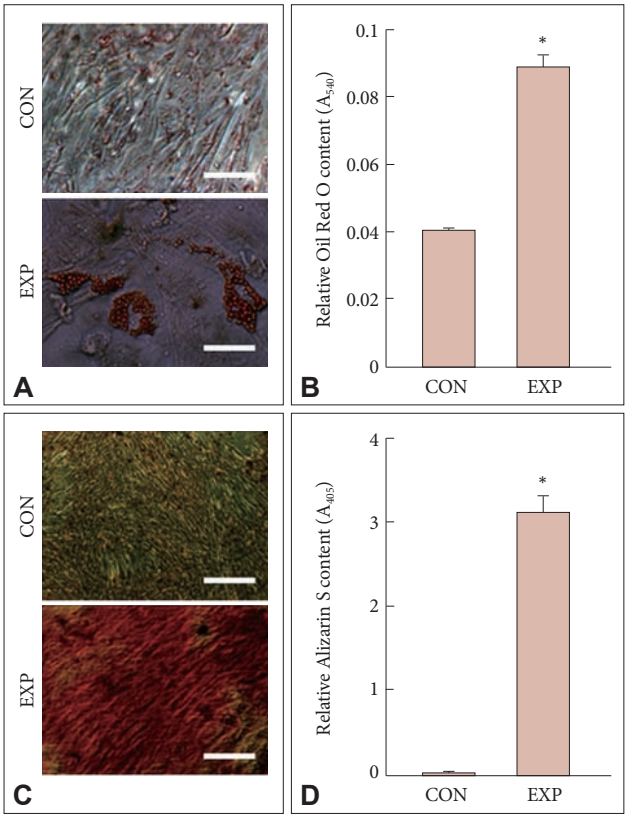

Figure 3. Adiopgenic and costeogenic differentiation potential of HUCPVCs. (A) Representative images of Oil Red O stained HUCPVCs cultured under adipogenic conditions. Scale bars, 100 μm. (B) Measurements of Oil Red O contents using spectrophotometry. Bars indicate mean±SD. (C) Representative images of Alizarin Red S stained HUCPVCs cultured under osteogenic condition. Scale bars, 100 μm. (D) Measurements of Alizarin Red S contents using spectrophotometry. Bars indicate mean±SD. *p<0.01. CON: control cultures, EXP: experimental cultures with differentiation conditions, PVCs: perivascular stem cells, HUCs: human umbilical cords, SD: standard deviation.

Supplementation of GDF-5 increases size of chondrogenic pellets

We then asked if supplementation of GDF-5 influences the chondrogenic differentiation of HUCPVCs. We first evaluated the multilineage differentiation potential of HUCPVCs. Under adipogenic conditions, HUCPVCs showed the ability to generate adipocytes positively stained for Oil Red O (Fig. 3A). Spectrophotometric quantification further revealed efficient adipogenic induction of HUCPVCs (Fig. 3B). In addition, osteoblasts positively stained with Alizarin Red S were seen in HUCPVCs cultured under osteogenic conditions (Fig. 3C and D). We next generated micromass pellets by centrifugation of HUCPVCs cultured in the presence or absence of GDF-5 (100 ng/mL) supplementation to determine the effects of GDF-5 on size of pellet and the ability to differentiate into chondrogenic lineage. Alcian blue staining and qPCR analysis revealed that HUCPVCs have the ability to differentiation into chondrocytes. Alcian blue staining for sectioned chondrogenic pellets showed glycosaminoglycan expression upon chondrogenic media treatment (Fig. 4A). COL2A1 and ACAN (markers for chondrocytes) transcripts were strongly expressed in chondrogenic pellets cultured under chondrogenic condition compared to the control (p<0.01) (Fig. 4B). Interestingly, size of pellets generated from HUCPVCs treated with GDF-5 were significantly increased compared to non-treated cells (p<0.01) (Fig. 4C). In addition, flow cytometric analysis showed higher percentage of DNA contents in GDF-5-treated pellets in comparison with the control (p<0.05) (Fig. 4D). However, there were no significant differences in the expression levels of COL2A1, ACAN, and SOX-9 mRNAs between those cells, irrespective of

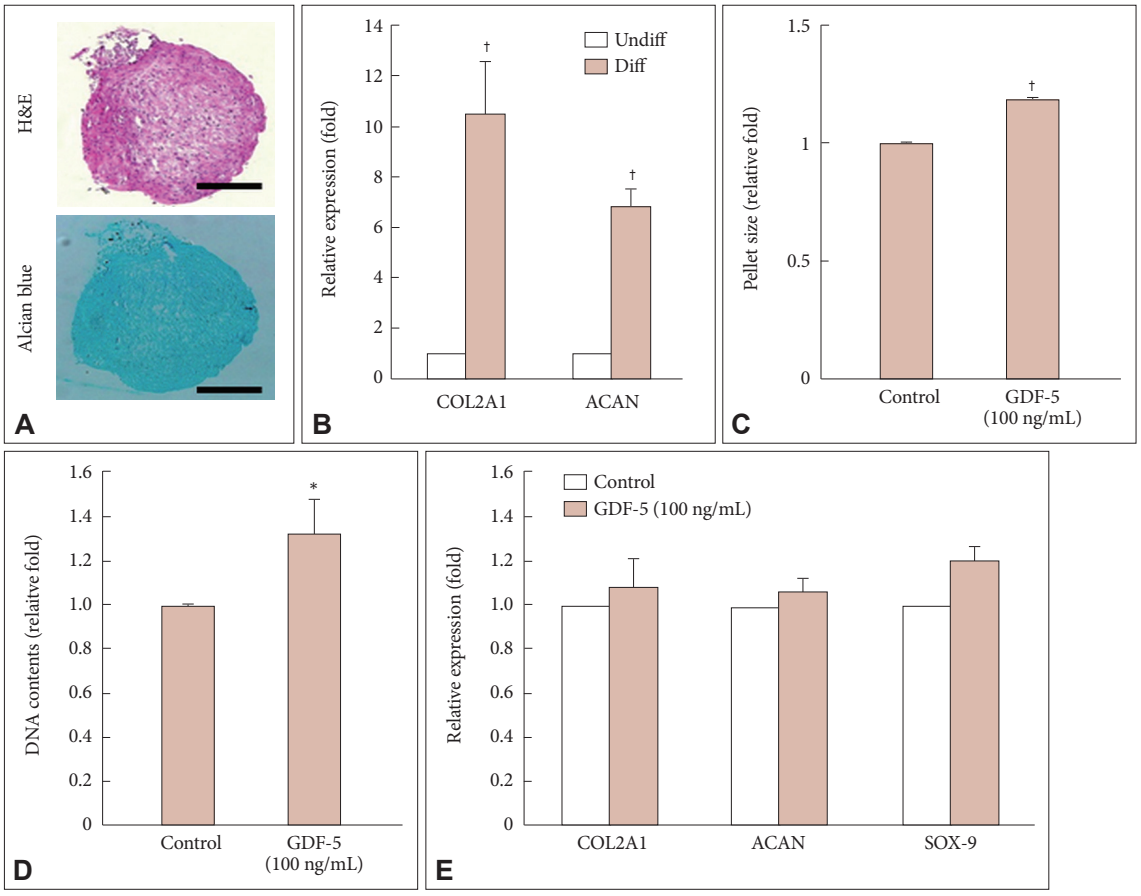

Figure 4. Effects of GDF-5 supplementation on chondrogenic differentiation of HUCPVCs. (A) H&E and Alcian blue staining of developing chondrogenic pellets. Immunohistochemistry analysis on sectioned pellets showing glycosaminoglycan expression upon chondrogenic media treatment. Scale bars, 500 μm. (B) qRT-PCR anaylsis showing increased expression of cartilage markers, collagen IIA1 (COL2A1) and aggrecan (ACAN), in chondrogenic differentiation as compared to undifferentiation. (C) Histomorphometric cell size analysis of chondrogenic pellets of HUCPVCs on day 21. The pellet size of aggregates treated with GDF-5 (100 ng/mL) is significantly increased compared to without GDF-5. (D) Flow cytometic analysis for the measurement of DNA content in chondrogenic pellets. (E) No significant differences in expression levels of COL2A1, ACAN, and SOX-9 mRNAs were observed in both groups. Bars indicate mean±SD (n=3, triplicate). *p<0.05, †p<0.01. H&E: hematoxylin and eosin staining, COL2A1: collagen type IIA1, GDF-5: growth differentiation factor-5, PVCs: perivascular stem cells, HUCs: human umbilical cords, qRT-PCR: quantitative real-time polymer chain reaction, SD: standard deviation.

the GDF supplementation to the pellets during chondrogenic induction (Fig. 4E). These results suggest that supplementation of GDF-5 produced more chondrocytes when starting with equal amount of HUCPVCs by augmenting the size of chondrogenic pellets.

DISCUSSION

PVCs are currently being considered and tested in preclinical trials as potential therapeutic cells for patients with various diseases including cartilage injury due to their greater differentiation potential compared to the "gold standard" BM-MSCs [12,26]. We have previously established a non-enzymatic (NE) method to isolate PVCs from HUCs [25]. This NE method provides PVCs with greater clonogenic capacity and osteogenic differentiation potential compared to PVCs obtained by the enzymatic treatment. In this study, we used NE-HUCPVCs to determine if they have the ability to differentiate into chondrocytes and if they are influenced by supplementation of GDF-5 in terms of proliferation and chondrogenic differentiation. We demonstrated that the exposure of HUCPVC cultures to GDF-5 (100 ng/mL) was shown to increase cell yield. This result is consistent with other studies in which GDF-5 promotes cellular proliferation of murine and human MSCs. For example, it has been reported that treatment of GDF-5 led to vigorous proliferation of rabbit and rat adipose-derived MSCs [16,24]. Similarly, Al-Sharabi et al. [23] showed that supplementation of GDF-5 enhanced cellular proliferation of human dental pulp-derived MSC cultures. On the other hand, supplementation of GDF-5 did not alter the proliferation rate of human BM-MSCs [27]. Hatakeyama et al. [28] found that the presence of 500 ng/mL of GDF-5 had no effect on the proliferation of mouse dental pulp cells after 1 to 7 days of cultures. This discrepancy may be due to origin of the cells or the tissue source and the presence of absence of GDF-5 receptor in those tissues. However, the molecular mechanisms are still unknown. We also found that GDF-5 augmented the size of pellets in micromass cultures for chondrogenic induction of HUCPVCs. This result was supported by two independent research groups, which demonstrated that GDF-5 improved self-assembly of MSCs derived from chick embryonic limb and human bone marrow [18,19]. Furthermore, GDF-5 induced condensation was inhibited upon interference of gap junction mediated communication, suggesting that gap junction is in the action of GDF-5 in PVC condensation and chondrogenesis.

Several studies showed that supplementation of chondrogenic MSC pellet cultured with GDF-5 enhanced the chondrognic differentiation of MSCs by increasing the expression levels of chondrogenic-related genes [16-24]. However, in our study, this phenomenon was not observed in HUCPVCs. This discrepancy may be due to distinct phenotypic and functional differences between PVCs and MSCs. It has been reported that PVCs have greater clonogenic capacity and differentiation potentials compared to MSCs and can be purified by pericyte markers such as CD146 and NG2, indicates that PVCs are a relatively more homogenous population than MSCs [10-12,25]. In addition, our NE isolation method offers a way to isolate more homogenous PVC population compared to the conventional enzymatic treatment method [25]. These functional and methodological differences may result in no response of PVCs to GDF-5 treatment in chondrogenic differentiation. Therefore, further studies will be needed to investigate the global gene expression profiles of PVCs and MSCs, which will help provide a better understanding of the therapeutic potential of PVCs.

In conclusion, to the best of our knowledge, our study is the first to describe the role of GDF-5 on the proliferation and chondrogenic differentiation of PVCs. Although similar expression levels of chondrogenic-related genes were observed in chondrogenic pellets treated with GDF-5 compared to the pellets without GDF-5 treatment, the present study indicates that supplementation of GDF-5 acquires more chondrocytes when starting with equal amount of PVCs. Together, our study suggests that GDF-5 is an effective agent for the enhancement of PVC proliferation, thereby obtaining a higher number of chondrocytes that are applicable in therapeutic doses for cartilage regeneration.

Acknowledgements

This study was supported by a grant of the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A120262). This research was also supported by The Health Fellowship Foundation.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no human or animal experiments carried out for this article.

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