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Induction of chondrogenesis and expression of superficial zone protein (SZP)/lubricin by mesenchymal progenitors in the infrapatellar fat pad of the knee joint treated with TGF-β1 and BMP-7

Sang Yang Lee *, Toshiyuki Nakagawa, A. Hari Reddi

Center for Tissue Regeneration and Repair, Department of Orthopaedic Surgery, University of California—4635 Second Avenue, Room 2000, Sacramento, CA 95817, USA

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ABSTRACT

Superficial zone protein (SZP) is a key mediator of boundary lubrication of articular cartilage in joints. In this investigation, we made the unexpected discovery that SZP was expressed in infrapatellar fat pad (IFP) from bovine knee. Quantitative analysis of secreted proteins in the medium of the IFP stromal cells demonstrated a significant stimulation by TGF-β1 and BMP-7. Real-time PCR analysis revealed the SZP expression was up-regulated by TGF-β1 and BMP-7. Chondrogenically differentiated IFP progenitor cells were stimulated by TGF-β1 and BMP-7 to synthesize and secrete SZP. SZP mRNA was significantly up-regulated by chondrogenic induction for 21 days. These findings indicate that the stimulation of SZP expression by TGF-β and BMP-7 may lead to functional improvement of damaged intraarticular tissues and that IFP progenitor cells may be a potential useful source for inducing superficial zone of articular cartilage by tissue engineering for regeneration of damaged articular cartilage due to osteoarthritis

Superficial zone protein (SZP) is a homologous to lubricin [1], and is a large proteoglycan that is synthesized and secreted into synovial fluid by chondrocytes in the superficial zone of articular cartilage and synovial cells in the joint [2–5]. SZP is known to function as a boundary lubricant in articular joints and reduces the coefficient of friction of the articular cartilage surface [6,7]. Loss of SZP has been associated with the pathogenesis of osteoarthritis (OA) [8,9]. Therefore, SZP is considered as a critical factor for maintenance of joint function and homeostasis in health and disease. SZP has also been identified in meniscus and ligament/tendon [10], where it may function as a lubricant. Previously, we demonstrated the regulation of SZP expression by transforming growth factor-β1 (TGF-β1) in the different tissue compartments of the knee; articular cartilage, synovium, meniscus, and anterior/posterior cruciate ligament [11] and by bone morphogenetic protein-7 (BMP-7) in synovial cells [5,12]. However, the expression of SZP and the factors regulating SZP secretion have not been clarified in another tissue compartment of the knee such as the infrapatellar fat pad (IFP). The IFP lies intra-articularly but extrasynovially to the joint and fills the total anterior knee compartment in all the joints [13]. MacConaill [14] suggested that IFPs occupied dead space in a joint, maintaining the joint cavity and promoting efficient lubrication. In view of the findings, we hypothesized that the IFP might be another source of SZP found in knee synovial fluids. Thus, our first aim was to clarify whether SZP is expressed in IFP and to determine the effects of TGF-β1 and BMP-7 on the SZP expression in primary cultured stromal cells from IFP.

The optimal function of articular cartilage is critical for locomotion in humans and animals. OA is the most common form of arthritis, affecting 24.3 million US adults [15]. Treatment of OA is a critical unmet need in medicine for the regeneration of damaged articular cartilage in the elderly. Articular cartilage tissue engineering using stem/progenitor cells has been pursued by many investigators, as it is promising for biological repair of articular cartilage damage due to injuries or arthritis [16]. In recent years, chondrogenic progenitor cells have been identified in various adult tissues, including bone marrow [17], muscle [18], and synovium [19]. More recently, progenitor cells with chondrogenic potential have been identified within IFP of the knee [20–23]. Because of practical access and minimal donor-site morbidity, mesenchymal progenitors from the IFP are of interest as a potential source for the repair of cartilage defect of the knee. However, little is known about the ability of these cells to generate SZP after chondrogenic differentiation. As SZP is a key mediator in boundary lubrication which is a primary function of articular cartilage, the localization of SZP-secreting cells at the surface of tissue-engineered cartilage may be critical for proper lubricating function. The potential of secreted SZP in chondroprogenitor cells, which were derived from bone marrow [24] and synovium [12], have been investigated. Our

* Corresponding author. Fax: +1 916 734 5750.
E-mail address: sangyang@beige.plala.or.jp (S.Y. Lee).

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hypothesis is that mesenchymal progenitor cells from IFP have SZP-producing ability after chondrogenesis and could be used as an optimal source for the superficial zone cartilage tissue engineering. Thus, the second objective of this study was to investigate the potential of SZP expression in IFP-mesenchymal progenitor cells after in vitro chondrogenic differentiation induced by TGF-β1 and BMP-7 using an aggregate pellet culture system.

Material and methods

Cell isolation and culture. Stifle (knee) joints from 3-month-old calves were obtained from a local abattoir. The IFP tissue was harvested from the interior areas of the capsule excluding synovial regions. Dissected tissue was cut into small pieces and digested with 0.1% Collagenase P (Roche, Indianapolis, IN) for 1.5 h at 37 °C. The floating mature adipocytes were separated from stromal fraction by centrifugation at 800*g* for 5 min at 4 °C. The sedimented stromal cells were resuspended in Dulbecco's modified Eagle medium/F12 (DMEM/F12 Gibco, Grand Island, NY) and the cells were enumerated. For primary culture, isolated stromal cells were plated as monolayers at a density of 1×10^5 cells/well in 12-well culture plates in DMEM/F-12 containing 50 μg/ml ascorbate-2-phosphate (Sigma, St. Louis, MO), 0.1% bovine serum albumin (BSA, Sigma), and penicillin–streptomycin (PS, Gibco) (Medium-A), and 10% fetal bovine serum (FBS, Gibco), and incubated. Following 24 h equilibration in the culture medium, cells were switched to serum-free chemically defined fresh Medium-A with ITS™+ Premix (BD Bioscience, Bedford, MA) in the presence or absence of 10 ng/ml of TGF-β1 (R&D systems, Minneapolis, MN) and/or 300 ng/ml BMP-7 (a generous gift from Dr. D. Rueger, Stryker Biotech, Hopkinton, MA) and incubated for 3 days. These concentrations were based on our previous experiments [4,5,12,13]. For chondrogenic differentiation, isolated cells were plated at 10^3 cells/cm^2 in 75 cm^2 flasks in the growth medium, DMEM-high glucose (Gibco) supplemented with PS and 10% FBS. Then cells were expanded for 14 days (passage 0) and were harvested with 0.05% trypsin–0.02% EDTA (Gibco), and passaged at 10^3 cells/cm^2 into new flasks for further expansion. Mesenchymal progenitor cells from passage 2 were used in the following chondrogenic differentiation assay.

Chondrogenic differentiation in pellet culture. For chondrogenic differentiation, pellet cultures were performed as three-dimensional (3D) cultures [25,26]. Cells (2.5×10^5) in the 15 ml polypropylene tube were centrifuged at 2000 rpm for 5 min to form a pellet. The pellets were cultured for 21 days in chondrogenic medium (CM) consisted of DMEM-high glucose with 100 nM dexamethasone (Sigma), 50 μg/ml ascorbate-2-phosphate, 0.1% BSA, 1 mM sodium pyruvate (Gibco), 0.4 mM proline (Sigma), 1% ITS™+ Premix, 10 ng/ml TGF-β1, and 300 ng/ml BMP-7 [27]. Pellets without TGF-β1 and BMP-7 treatment served as a control. Medium was changed twice per week. The pellets were embedded in paraffin, cut into 5 μm sections, and stained with toluidine blue (Fisher Scientific, Fair Lawn, NJ).

Immunohisochemistry. For immunohistochemical localization studies, freshly isolated IFP tissues and cultured cell pellets were fixed with 4% paraformaldehyde (Fisher Scientific) and embedded in paraffin, and sectioned at 5 μm intervals. The sections were probed for SZP using monoclonal antibody (mAb) S6.79 (a generous gift from Dr. T. Schmid, Rush Medical College, Chicago, IL) with a 1:5000 dilution [6,11,28]. Standard immunohistochemical staining was performed using VECATATIN® ABC reagent and ImmPACT™ DAB peroxidase substrate (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol, without counterstaining.

Enzyme-linked immunosorbent assay (ELISA) analysis of SZP protein. The spent medium from primary culture and pellet culture was harvested for subsequent analysis. At the end of the treatment interval, culture medium was frozen. SZP accumulation was quantitatively analyzed in duplicate by a sandwich ELISA using S6.79 mAb and purified SZP as standard, as previously described [5,6,11,12].

Real-time polymerase chain reaction (PCR) analysis. For the analysis of SZP mRNA expression, total RNA was extracted from the primary cultured cells after 0, 24, 48, and 72 h of stimulation using RNeasy mini kit (Qiagen, Valencia, CA). Total RNA were also extracted from undifferentiated IFP stromal cells at the end of passage 2 (time-zero control) and induced pellets at day 21 for the analysis of chondrocyte-related markers. Total RNA was reverse transcribed into single-strand cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was on the cDNA with an ABI PRISM® 7700 Sequence Detection Systems and SYBR® Green regents (both from Applied Biosystems) following the recommended protocols. The primers for bovine GAPDH (forward; 5′-GGCGCCAAGAGGGTCAT-3′; reverse: 5′-GTGGTTCACGCCCATCACA-3′), SZP (forward: 5′-AG AAAACCCGATGGCTATGA-30; reverse: 50-TCGCCCATCAGTCTAAGGA C-3′), type II collagen (Col II, forward: 5′-GCATTGCCTACCTGG ACGA A-3′; reverse: 5′-CGTTGGAGCCCTGGATGA-3′), type I collagen (Col I, forward: 5′-ACATGCCGAGACTTGA GACTCA-3′; reverse: 5′-GCATCCATAGTACATCCTTGGTTAGG-3′), Sox9 (forward; 5′-ACGC CGAGCTCAGCAAGA-3′; reverse: 5′-CACGAACGGCCGCTTCT-3′ and aggrecan (forward: 5′-GCGGGTGCGGGTCAA-3′; reverse: 5′-TAGA ATCCCGGAGTCATTGGA-3′) were designed with Primer Express software (Applied Biosystems) to span more than 1intron on genomic DNA to avoid nonspecific fluorescent emission derived from contaminating genomic DNA [11]. The level of each target gene was normalized to GAPDH levels and expressed relative to the time-zero control culture levels ($\Delta\Delta C_T$ methods; Applied Biosytems) [29].

Statistical analysis. All the quantitative data were presented as means ± standard deviation. Paired *t*-test was performed using StatView statistics (SAS Institute Inc., Cary, NC) to determine the deference between the control and treated cells.

Results

The distribution of SZP in freshly isolated IFP

Sections of the IFP tissues from four different calves stained intensely positive for SZP (Fig. 1A). The reaction product was found diffusely distributed in the IFP tissues. The IFP was a heterogeneous structure containing adipose tissue, connective tissue, and blood vessels. No positive staining was observed in vessels. Sections treated with the non-specific mouse IgG showed no positive signals (data not shown).

TGF-β1 and BMP-7 modulate the SZP expression in primary IFP stromal cells

We next investigated whether stromal cells from IFP expressed SZP in primary monolayer culture. Since the accumulation of SZP in the culture medium increased linearly in a time-dependent manner in our previous studies [4,5], the culture media were analyzed on day 3. ELISA analysis revealed that IFP stromal cells synthesized and secreted SZP protein into the culture medium (Fig. 1B). TGF-β1 exhibited a robust capacity to stimulate SZP accumulation. BMP-7 also significantly up-regulated the accumaltion of SZP. Compared to the control (SZP concentration; 2.7 μg/ml), there was a 4.8-fold increase (12.6 μg/ml) by treatment of 10 ng/ml TGF-β1, and 1.6-

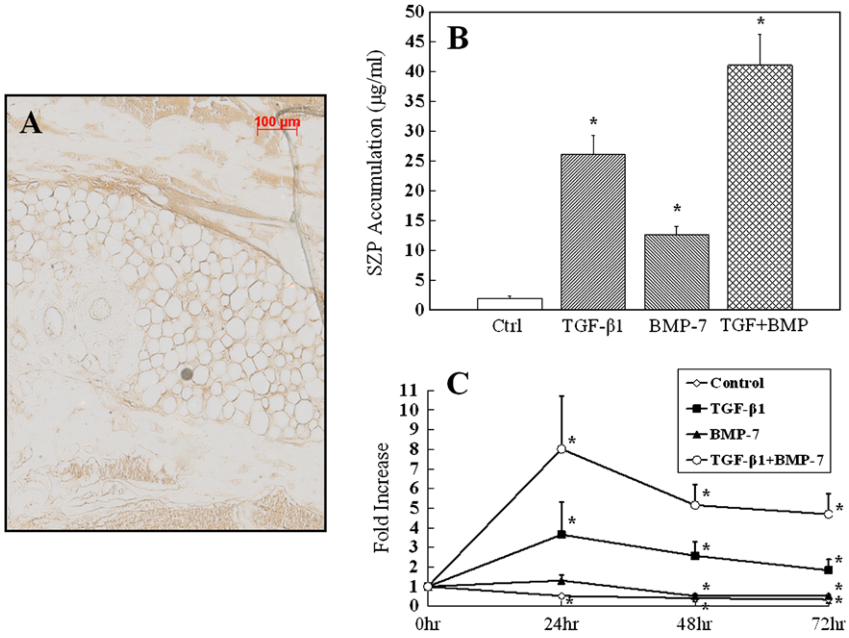

Fig. 1. (A) Immunolocalization of the SZP in the bovine infrapatellar fat pad (IFP). Scale Bar = 100 μm. (B) Effects of TGF-β1 and BMP-7 on SZP accumulation in the medium of primary IFP stromal cells. SZP accumulation in spent medium after the 3-day treatment was quantified by ELISA (i = 4–6: $^{*}p < 0.01$, compared with the values of the control cultures (Ctrl, open bars)). (C) Effects of TGF-β1 and BMP-7 on the expression SZP mRNA in primary IFP stromal cells. Time-course response (0–72 h after treatment) for SZP mRNA induction was assessed by quantitative real-time PCR. The fold increase in treated and untreated cultures is expressed relative to the levels in the time-zero controls ($n = 4$: $^{*}p < 0.01$, compared with the values of the time-zero control cultures).

fold (4.3 μg/ml) by 300 ng/ml BMP-7, respectively. There was an additive action of TGF-β1 and BMP-7 for accumulation of SZP (15.7 μg/ml).

The expression of SZP mRNA in IFP stromal cells was dramatically enhanced by concurrent treatment of TGF-β1 and BMP-7 during the entire experiment period (24–72 h, Fig. 1C). Stimulation of SZP expression reached the maximal level at 24 h with 8-fold increase over control. Treatment with TGF-β1 alone enhanced the expression of SZP mRNA during the entire experiment period. However, the expression of SZP mRNA was unaffected (at 24 h) or decreased at 48 and 72 h by BMP-7.

Induction of chondrogenesis of IFP-mesenchymal progenitor cells by TGF-β1 and BMP-7

Pellets cultured in chondrogenic medium (CM) containing TGF-β1 and BMP-7 for 21 days were larger in gross than those treated without growth factors (control) (Fig. 2A and B). The chondrogenic potential of IFP-mesenchymal progenitor cells was evaluated histologically for the presence of proteoglycan with toluidine blue staining. Treatment with CM resulted in a greater extent of proteoglycan deposition (Fig. 2D). In contrast, control pellets exhibited a fibrous structure, and deposition of proteoglycan were not detected (Fig. 2C).

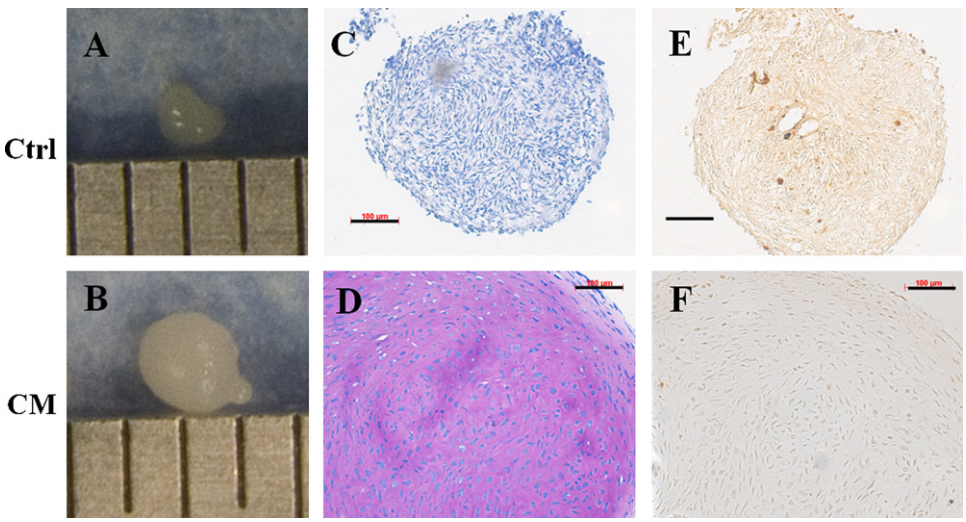

Fig. 2. (A,B) Macroscopic appearance of the pellets cultured for 21 days. IFP mesenchymal progenitor cells (passage 2) were treated with chondrogenic medium (CM) including TGF-β1 and BMP-7 or without them (ctrl). Scale Bar = 1 mm. (C–F) Toluidine blue staining (C,D) and Immunohistochemistry for SZP (E,F) of cells cultured in pellets of IFP stromal cells. Scale Bar = 100 μm.

Chondrogenesis of pellets after 21 days induction were further confirmed by real-time PCR (Fig. 3). Expression of Col II (Fig. 3A), Sox 9 (Fig. 3C), and aggrecan (Fig. 3D) dramatically increased in pellets cultured with CM compared to the control pellets and the time-zero control. Treatment with CM induced a major increase in the expression of Col II, 25-fold in Sox 9, 190-fold in aggrecan compared to the time-zero controls, respectively. There was a slight decrease in Col I expression compared to the time-zero control (Fig. 3B). These results were in accordance with the histological findings.

SZP expression in chondrogenically differentiated IFP-mesenchymal progenitor cells

We then evaluated whether IFP-mesenchymal progenitor cells expressed SZP protein and mRNA after chondrogenic differentiation. Both control pellets and pellets treated with CM for 21 days showed positive staining for SZP (Fig. 2E and F). The SZP content was lower in the treated pellets than in control pellets. The results of ELISA analysis demonstrated that the accumulation of SZP was significantly up-regulated with CM treatment compared to the controls (Fig. 4A). Real-time PCR revealed that pellets treated with CM for 21 days exhibited significant higher expression (10-fold increase) of SZP mRNA than that of time-zero control (Fig. 4B).

Discussion

The present study demonstrated that SZP is present within IFP of the knee and is synthesized and secreted by IFP stromal cells. We investigated the modulation of SZP expression in primary IFP stromal cells by using TGF-β1 and BMP-7 and a monolayer culture system. Treatment with TGF-β1 significantly increased SZP mRNA expression in IFP stromal cells. A corresponding robust increase in SZP protein accumulation by TGF-β1 was also observed. BMP-7 stimulated SZP accumulation, although the effect was modest compared to TGF-β1. Concurrent treatment with TGF-β1 and BMP-7 resulted in significantly higher expression of SZP compared with that observed upon control group. The finding of an additive action of TGF-β1 and BMP-7 revealed the importance of the TGFβ/BMP superfamily members in the regulation of SZP expression in IFP stromal cells. These trends in regulation of SZP expression were similar to those previously observed in bovine synovial cells [5,11]. Taken together, our findings indicated that IFP could play an important role for maintaining proper lubricating function of artic-

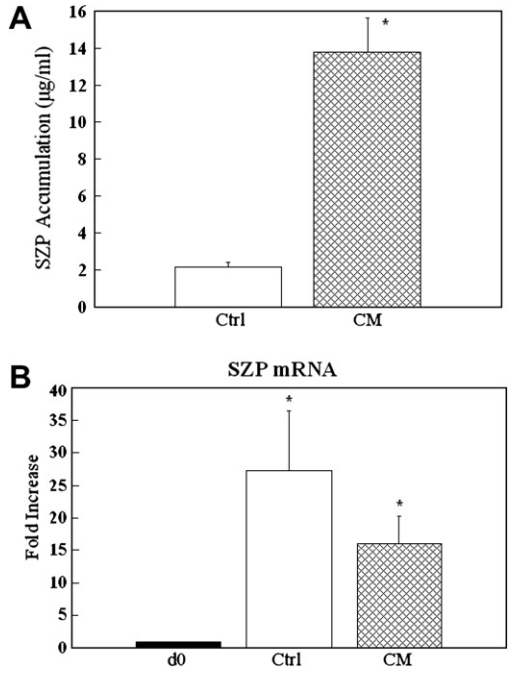

Fig. 4. (A) SZP accumulation in the medium after the 21-day chondrogenic differentiation (CM, cross hatched bars) was quantified by ELISA ($n = 4$–6: $^{*}p < 0.01$, compared with the values of the control cultures (ctrl, open bars)). (B) SZP mRNA expression levels after the 21-day chondrogenic differentiation was analyzed by quantitative real-time PCR. The fold in chondrogenic medium (CM) and control cultures (ctrl) is expressed relative to the levels in the time-zero controls (d0, black bars) ($n = 4$: $^{*}p < 0.01$, compared with the values of the time-zero controls).

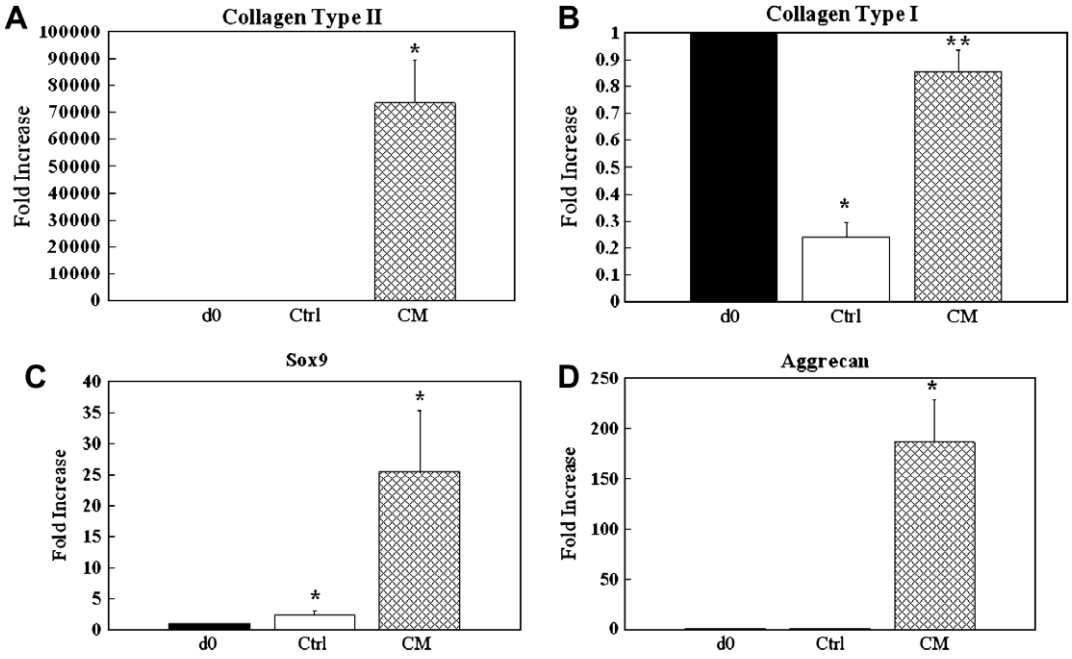

Fig. 3. Quantitative real-time PCR analysis to determined gene expression of Col II (A), Col I (B), Sox 9 (C), and Aggrecan (D) after 21 days of culture. The increase in chondrogenic medium (CM, cross hatched bars) and control cultures (ctrl, open bars) is expressed relative to the levels in the time-zero controls (d0, black bars) ($n = 4$–6: $^{*}p < 0.01$; $^{**}p < 0.05$, compared with the values of the time-zero controls).

ular joint by producing SZP into synovial fluid, and therefore contribute to maintain healthy joint function and homeostasis. The stimulation of SZP secretion in IFP stromal cells by TGF-β1 and/or BMP-7, as demonstrated here, may lead to functional improvement of damaged intraarticular tissues and ameliorate the pathology of joint function in arthritis, providing an efficient means to improve joint lubrication.

Another notable finding of this study is that expanded IFP-mesenchymal progenitor cells had the ability to synthesize and secrete SZP after chondrogenic differentiation. Initially, we investigated the in vitro chondrogenic potential of IFP-mesenchymal progenitor cells. We demonstrated that IFP-mesenchymal progenitor ells cultured for 21 days under 3D conditions and the treatment with TGF-β1 and BMP-7 [27] exhibited chondrogenic differentiation activities by expressing chondrocyte specific genes such as Col II, Sox 9, and aggrecan, and positive staining of proteoglycan with toluidine blue. These results were consistent with previous published reports [20–23]. We then demonstrated that chondrogenically differentiated IFP-mesenchymal progenitor cells produced SZP in 3D pellet culture. Immunohistochemical staining revealed that SZP was localized within chondrogenically differentiated pellets as well as control pellets. The ELISA analysis showed the presence and up-regulation of the SZP accumulation in media after 21-day chondrogenic induction. Finally, real-time PCR analysis showed significant up-regulation of SZP mRNA in pellets after chondrogenic differentiation for 21 days. Since sequentially passaged chondrocytes exhibited decreases in SZP expression [30], it is noteworthy that IFP-mesenchymal progenitor cells could maintain SZP-secreting phenotype even after passaging.

The present results have important clinical implications. Superficial zone chondrocytes selectively synthesize and secrete SZP, whereas the middle zone and deep zone chondrocytes has little or no capacity for SZP accumulation [2,5–7,11]. Thus, SZP has been considered as a zonal molecular marker for superficial zone of articular cartilage. Since the general paradigm of tissue engineering strategies is to mimic the structure of a tissue and native environment as closely as possible to encourage the restoration of its structure and function [31], SZP produced from a superficial layer of tissue-engineered cartilage was considered as a critical factor [32]. Gleghorn et al. suggested that controlling localization of SZP in engineered cartilage may be critical for proper lubricating function [24]. Fabrication of a functional tissue-engineered cartilage could be dependent on localizing SZP secreting cells at the surface. Our results indicated that IFP-mesenchymal progenitor cells may be possible and useful source of chondroprogenitor cells with SZP-producing ability. Giving its location, accessibility, and great expandability [20], IFP could be promising cell source for creating a superficial zone of articular cartilage by tissue engineering.

Acknowledgments

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