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A predominantly articular cartilage-associated gene, *SCRG1*, is induced by glucocorticoid and stimulates chondrogenesis *in vitro*

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Summary

Objective: Cartilage tissue engineering using multipotential human mesenchymal stem cells (hMSCs) is a promising approach to develop treatment for degenerative joint diseases. A key requirement is that the engineered tissues maintain their hyaline articular cartilage phenotype and not proceed towards hypertrophy. It is noteworthy that osteoarthritic articular cartilage frequently contains limited regions of reparative cartilage, suggesting the presence of bioactive factors with regenerative activity. Based on this idea, we recently performed cDNA microarray analysis to identify genes that are strongly expressed only in articular cartilage and encode secreted gene products. One of the genes that met our criteria was *SCRG1*. This study aims to analyze *SCRG1* function in cartilage development using an *in vitro* mesenchymal chondrogenesis system.

Methods: Full-length *SCRG1* cDNA was subcloned into pcDNA5 vector, and transfected into hMSCs and murine C3H10T1/2 mesenchymal cells, placed in pellet cultures and micromass cultures, respectively. The cultures were analyzed by reverse transcription-polymerase chain reaction for the expression of *SCRG1* and cartilage marker genes, and by histological staining for cartilage phenotype.

Results: Induction of *SCRG1* expression was seen during *in vitro* chondrogenesis, and was dependent on dexamethasone (DEX) known to promote chondrogenesis. Immunohistochemistry revealed that SCRG1 protein was localized to the extracellular matrix. Forced expression of *SCRG1* in hMSCs suppressed their proliferation, and stimulated chondrogenesis in C3H10T1/2 cells, confirmed by reduced *collagen type I* and elevated *collagen type IIB* expression.

Conclusion: These results suggest that *SCRG1* is involved in cell growth suppression and differentiation during DEX-dependent chondrogenesis. *SCRG1* may be of utility in gene-mediated cartilage tissue engineering.
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Key words: SCRG1, Articular cartilage, Chondrogenesis, Dexamethasone, Cell proliferation, Tissue engineering.

Introduction

Normal synovial joint function is dependent on the maintenance of its articular cartilage layer. Degeneration of articular cartilage results from osteoarthritis (OA) and many other forms of severe arthritis, leading to ossification and dysfunction of the joint[1,2]. OA is the most common form of arthritis and the second most common cause of long-term disability among adults in the United States[1]. Current treatments for cartilage repair are less than satisfactory, and rarely restore full function or return the tissue to its native normal state. There is thus a need for improved treatment modalities[3,4].

Tissue engineering of articular cartilage is considered to be a promising approach to develop methods for the treatment for OA and severe arthritis in patients[3,4]. Specifically, multipotential human adult mesenchymal stem cells (hMSCs) represent a candidate cell source[4–7]. Human MSCs are found in various tissues, in particular the bone marrow, and have been shown to have the ability to differentiate into multiple mesenchymal lineage, including bone, muscle, ligament, tendon, adipose and marrow stroma[5,8–16]. Specifically, human MSCs have also been shown to form cartilage *in vivo* and *in vitro*[9–11,14,15,17]. However, it is unknown whether this nascent cartilage tissue could remain hyaline and not proceed into a hypertrophy pathway seen in endochondral skeletal tissues, and successfully acquire the phenotype of articular cartilage[3,18].

A number of recent studies suggest that direct application of hMSCs may not be appropriate or adequate for the tissue engineering of permanent articular cartilage. (1) Adult MSCs are known to be involved in fracture healing, a process that includes the formation of cartilaginous tissues that undergo hypertrophy and are eventually replaced by bone to repair the fracture[2,9,11,19]. This phenomenon suggests that hMSCs may be programmed for the formation of transient cartilage destined to form bone, and unless appropriately controlled, may not be a ready precursor of permanent, articular cartilage. (2) In the developing embryonic limb bud, articular cartilage cells are derived from undifferentiated mesenchymal condensation cells at very early stage of joint formation, as a result of formation of the inter-zone[2,20,21]. However, once synovial joint formation

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is completed, articular cartilage cells are no longer derived from mesenchymal condensation cells[18,22], while chondrocytes derived from the remaining mesenchymal condensations will undergo hypertrophy and endochondral ossification[22,23]. This developmental pathway also suggests that hMSCs in adult bone marrow may not be able to differentiate to articular cartilage in a simple manner. (3) Regenerated articular cartilage must be composed completely of articular chondrocytes in order to be applicable for the treatment of diseased joints. Even a small component of bone in the regenerated tissue could induce and worsen the arthritic conditions. Given that hMSCs, isolated using currently available methods, do not consist of a homogeneous cell population[9,11], and the ability of hMSCs to differentiate into osteoblasts[10,12–14,16,24], modification of hMSCs is likely to be necessary for tissue engineering of cartilage[3].

Microscopic observation of the OA articular cartilage frequently shows a heterogeneous mixture of reparative cartilage[2]. It is also known that degenerated OA articular cartilage can regenerate after high tibial valgus osteotomy[25]. These reparative reactions suggest that articular chondrocytes may secrete bioactive factors that can induce articular cartilage formation from progenitor cells, including hMSCs, even in the osteoarthritic joint. Thus, we have hypothesized that identification and characterization of secreted proteins from articular cartilage may lead to the isolation of an articular cartilage inducible factor(s). It is conceivable that transfection of such gene(s) or simple addition of the protein(s) might enable hMSCs to contribute to the tissue engineering of articular cartilage. Based on this idea, we have performed a comprehensive analysis of gene-expression profiles in human articular cartilage and 30 other normal human tissues by means of a cDNA microarray consisting of 23,040 human genes[26]. We targeted those genes that are strongly expressed only in articular cartilage, and those genes whose products are secreted. One of the genes that met both of our criteria was Scrapie Responsive Gene 1 (*SCRG1*). The expression of *SCRG1* in the cartilage is also confirmed in 8–12 week human fetus[27].

SCRG1 is a transcript originally discovered through identification of the genes associated with or responsible for the neurodegenerative changes observed in transmissible spongiform encephalopathies[28]. *SCRG1* transcript is detected in the brain, heart and spinal cord, and its sequence is highly conserved in humans, mice and rats. *SCRG1* encodes a 98 amino acid, cysteine-rich, 10 kDa protein with signal peptide in its N-terminal, and has been suggested to be associated with the secretory pathway of neuronal cells[29–31]. The exact function of *SCRG1* is still unknown.

Glucocorticoid (GC) hormones play crucial roles in regulating gene transcription. Dexamethasone (Dex) is one of the synthetic GCs, and the molecular mechanism of Dex-mediated transcriptional regulation has been identified in the past decade. Dex mainly acts by binding to homodimeric glucocorticoid receptor (GR) and transactivates target genes via a glucocorticoid response element[32,33]. Dex is known to induce *in vitro* chondrogenesis by hMSCs through molecular mechanisms that are yet unknown[34–36].

In this study, we show that *SCRG1* was expressed specifically in human articular cartilage. Expression of *SCRG1* was induced by Dex through the activation of GR. Forced expression of *SCRG1* suppressed growth of hMSCs and stimulated *in vitro* chondrogenesis, suggesting its potential role in mesenchymal chondrogenesis and possible application to tissue engineering of articular cartilage.

Material and methods

HUMAN TISSUE SAMPLES AND MSCs

Articular cartilage and bone marrow were obtained from the knee joints and femoral heads of four patients (aged from 47 to 81 years) undergoing total joint arthroplasty with the approval of the Institutional Review Board of George Washington University Medical School (Washington, D.C.). Articular cartilage was shaved off from the removed joints. Bone marrow derived hMSCs were isolated and cultured as described previously[24,37].

CELL LINES

Human mesenchymal progenitor cell line, hMPC 32F, was established as described[36]. Murine C3H10T1/2 clone cells 8 (passage 10) and monkey COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). C3H10T1/2 cells were cultured and frozen stocked at passage 11 in dimethyl sulfoxide freeze medium (IGEN International, Gaithersburg, MD) until use. All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO BRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS; GIBCO BRL), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 25 ng/ml of Fungizone (GIBCO) under a humidified atmosphere of 5% CO_2 at 37 °C. Culture medium was changed every 3–4 days.

RNA ISOLATION AND SEMI-QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Articular cartilage samples were digested with 0.25% trypsin (GIBCO BRL) in PBS for 20 min, and further digested with 1 mg/ml of collagenase II (Worthington, Lakewood, NJ) in DMEM containing 10% FBS at 37 °C for 16 h. The digest was filtered with a cell strainer (BD Biosciences, San Jose, CA) to collect chondrocytes. The chondrocytes and hMSCs were extracted with Trizol (Invitrogen, Carlsbad, CA) to isolate total RNA. Total RNAs of human brain, heart, and spinal cord were obtained from BD Biosciences. A 2 μg aliquot of total RNA was reverse-transcribed for single-stranded cDNAs using oligo $(dT)_{12-18}$ primer and Superscript II (Invitrogen). Semi-quantitative RT-PCR was carried out as described previously[26,38,39]. The primer sets (forward and reverse) used are given below. *SCRG1*: 5′-TTG CTC TGC TGC CCA AAA GAC G-3′ and 5′-GAA ATC AGG AAT GGT GTT CTC CAG-3′; mouse *collagen type I (α1)*: 5′-ACA CTG GTA GAG ATG GTG CTC-3′ and 5′-TCT CCA GAG GGA CCC TTT TCA-3′; mouse *collagen type II (α1)*: 5′-GCC TCG CGG TGA GCC ATG ATC-3′ and 5′-GAG GGC CAG GAG GTC CTC TGG-3′; and mouse *aggrecan*: 5′-GAG AGT TCT CAC GCC AGG TTT G-3′ and 5′-TTG CCA GGG GGA GTT GTA TTC-3′. PCR reactions were optimized for the number of cycles to ensure product intensity within the linear phase of amplification. The PCR product of *SCRG1* was sequenced by MTR Scientific, LLC (Ljamsville, MD). Some of the results were analyzed by NIH Image-J software (http://rsbweb.nih.gov/ij/).

GENERATION OF ANTIBODY FOR SCRG1 PEPTIDE

An immunogenic peptide sequence KISFVIPCNNQ-COOH (human SCRG1 amino acid sequence: 88–98) was synthesized by Tufts University core peptide facility (Department of Physiology, Tufts Medical School, Boston, MA). KLH conjugation, immunization and bleeding of

rabbits were performed by Zymed Laboratories Inc. (South San Francisco, CA). IgG fractions were isolated by ammonium sulfate precipitation from both pre-immune and immune sera.

WESTERN BLOTTING AND IMMUNOPRECIPITATION

For Western blotting, cell pellets were washed twice with ice-cold phosphate-buffered saline (PBS), lysed with immunoprecipitation buffer (50 mM Tris–HCl, pH 7.4; 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediamine tetraacetate) containing protease and phosphatase inhibitor mixture, incubated on ice for 30 min, homogenized, and centrifuged at $14,000 \times g$ for 15 min. The supernatant was collected, and protein concentrations were determined using the BIO-RAD Protein Assay (Hercules, CA). For immunoprecipitation, 1.5 ml aliquots of culture medium were incubated with 10 μl of anti-SCRG1 antiserum, followed by addition of protein G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and samples were prepared following the manufacturer's protocol. Equal amounts of protein extracts were fractionated by 18% Ready Gel Tris–HCl gel (BIO-RAD), electroblotted onto Hybond-P membrane (Amersham Biosciences, Piscataway, NJ), probed with ECL anti-rabbit IgG (Amersham Biosciences), and immunoblotted using the ECL Western blotting kit and Hyperfilm according to the manufacturer's protocol (Amersham Biosciences). Gels were stained with Coomassie blue using Collidal Blue staining kit (Invitrogen) and dried with DryEase Mini-Gel Drying system (Invitrogen).

IMMUNOHISTOCHEMISTRY

Fresh articular cartilage specimen was washed with PBS, frozen and embedded in Tissue-Tek O.C.T. (Sakura Finetek U.S.A. Inc., Torrance, CA) and cryosectioned at 12 μm thickness. Sections were fixed with 100% ethanol at 4°C for 15 min, and then rehydrated through graded ethanol washes. For SCRG1 detection, purified IgG preparations derived from pre-immune serum and anti-SCRG1 antiserum were used as primary antibodies at 1/1000 dilution in Tris-buffered saline with 0.05% of Tween-20 (TBS-T). Immunohistochemistry was performed using Histostain-SP kit for DAB (Zymed Laboratories) following the manufacturer's protocol.

Gene transfected cells were cultured on Labtek Permanox chamber slides (Nunc, Naperville, IL) for 48 h, washed with PBS and fixed with methanol for 10 min, preincubated with blocking solution (4% bovine serum albumin in TBS) for 1 h at room temperature, and incubated for 2 h at room temperature with anti-SCRG1 IgG diluted 1:1000 in blocking solution. Immunostaining was visualized with a donkey anti-rabbit Alexa Fluor 488 conjugated IgG (Molecular Probes, Eugene, OR) and observed using a Leica DM IL microscope (Leica Cameras Inc., Northvale, NJ).

CELL PROLIFERATION ASSAY

The PCR amplified full-length cDNA sequences of *GFP* and *SCRG1* cDNA were inserted into pcDNA5/FRT vector (Invitrogen) and designated as pcDNA-*GFP* and pcDNA-*SCRG1*, respectively. Human MSCs and C3H10T1/2 cells were transfected with pcDNA-*GFP* vector or pcDNA-*SCRG1* using human MSC nucleofector kit (Amaxa Biosystem, Gaithersburg, MD)[40]. Briefly, 1×10^6 of trypsinized hMSCs was suspended with 100 μl of Human MSC Nucleofector Solution and 2 μg of plasmid DNA. The suspension was electroporated with the Amaxa Nucleofector U-23 program, and then maintained in DMEM with 20% FBS overnight. The next day, the transfected cells were cultured in DMEM with 10% steroid-stripped FBS (ss-FBS; charcoal/dextran treated fetal bovine serum; Hyclone, Logan, UT) containing 0.1 mg/ml (hMSCs) or 0.4 mg/ml (C3H10T1/2 cells) of G418 (GIBCO BRL) for a week. C3H10T1/2 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 1 week of culture, 1.8×10^4 of viable, transfected cells were plated in 24 well plates with DMEM containing 10% ss-FBS and 10 μg/ml of G418 for further analysis. Counting of viable cells was performed either by direct cell counting after Trypan Blue staining (Bio WHITTAKER, Watersville, MD) or by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's suggestions.

IN VITRO CHONDROGENESIS

For C3H10T1/2 cells, the high-density micromass culture technique was carried out according to Denker *et al.*[41] C3H10T1/2 cells were used at passage 11 from frozen stocks of less than 3 months. One day after transfection with either pcDNA-*GFP* vector or pcDNA-*SCRG1* using human MSC nucleofector kit, cells were trypsinized, counted, and plated as 10 μl drops at 1×10^7 cells/ml in Ham's F12 medium with 10% of ss-FBS onto 24-well polystyrene tissue culture dish. After adherence for 1.5 h at 37°C and 5% CO_2, the cells were fed 1 ml of Ham's F12 medium with 10% ss-FBS and 100 ng/ml of human recombinant bone morphogenetic protein-2 (BMP-2) (R&D system, Minneapolis, MN). The culture medium was changed every 3–4 days until further analysis.

For hMSCs, the pellet culture was performed with hMPC 32F as previously described[24,42]. Briefly, 0.25×10^6 cells were pelleted by centrifugation and maintained in a serum-free medium containing 0.1 μM DEX, 50 μg/ml L-ascorbate 2-phosphate, 40 μg/ml L-proline, 100 μg/ml sodium pyruvate (Sigma), ITS-Premix (BD Biosciences, Bedford, MA), and 10 μg/ml transforming growth factor-β3 (TGF-β3, R&D Systems, Inc., Minneapolis, MN). In selected cultures, the GC antagonist, RU-36846 (Sigma), was added at 10 mM to the medium.

HISTOLOGICAL ANALYSIS OF CHONDROGENESIS

The presence of sulfated proteoglycans indicative of cartilage formation was detected by alcian blue staining[43]. Micromass cultures of C3H10T1/2 cells were washed in PBS, fixed in 2% acetic acid in ethanol for 15 min and rehydrated with graded ethanol. Sulfated cartilage matrix was visualized by overnight staining with 0.5% Alcian blue 8GX (pH 1) (Sigma, St. Louis, MO). Alcian blue was quantified by measuring absorbance of the extracted dye at 595 nm as described previously[44]. Each experiment was repeated at least three times using triplicate samples, and results presented as mean ± S.D.

STATISTICAL ANALYSIS

Numerical data were analyzed for statistical significance using Student's *t* test with a confidence level of 95% ($P < 0.05$). All values are reported as mean ± S.D.

Results

SCRG1 EXPRESSION IN ARTICULAR CARTILAGE

Our previous microarray data suggested that *SCRG1* is expressed at a very high level only in articular cartilage[26]. On the other hand, *SCRG1* has also been suggested to be expressed in spinal cord, brain and heart[29]. To better access the human tissue expression profile of *SCRG1*, we performed semi-quantitative RT-PCR to compare the expression of *SCRG1* among the tissues purported to express *SCRG1* [Fig. 1(A)]. Our results showed that articular cartilage and spinal cord expressed *SCRG1* at the highest level. Interestingly, the level of *SCRG1* expression in hMSCs was approximately the same as that in brain and heart tissues.

LOCALIZATION OF SCRG1 *IN VITRO* AND *IN VIVO*

To assess the distribution of SCRG1 protein in human articular cartilage, we prepared antisera against a SCRG1 peptide sequence predicted to be hydrophilic and antigenic. To confirm the specificity of the anti-SCRG1 antibody, we performed Western blotting using extracts of C3H10T1/2 cells transfected with pcDNA5-*GFP* and pcDNA5-*SCRG1* constructs. As shown in Fig. 1(B), an immunoreactive 10 kDa band was detected in pcDNA5-*SCRG1* transfected cells, but not in pcDNA5-*GFP* transfected cells. Incubations with pre-immune serum also did not show any immunoreactive band in either pcDNA5-*GFP* or pcDNA5-*SCRG1* transfected cells (data not shown). Taken together, these results indicate the reactivity and specificity of the anti-SCRG1 antibodies. To examine whether SCRG1 was produced as a secreted protein, we performed immunoprecipitation of culture media of GFR-transfected and *SCRG1*-transfected C3H10T1/2 cells. As shown in Fig. 1(C), SCRG1 was detected only in the culture medium of *SCRG1* transfected cells, suggesting that SCRG1 is produced as a secreted protein.

To determine the subcellular localization of SCRG1 protein in mesenchymal cells, C3H10T1/2 cells transfected with pcDNA5-*GFP* and pcDNA-*SCRG1* were analyzed by fluorescence immunohistochemistry. The micrograph [Fig. 1(D)] showed that SCRG1 protein is localized in cytoplasmic granules, consistent with the secretory nature of SCRG1 protein production.

Enzyme-based immunohistochemistry revealed that SCRG1 protein was also present in human adult articular chondrocytes *in vivo* [Fig. 1(E, F)]. The immunostaining appeared to be localized to the pericellular matrix surrounding the articular chondrocytes, especially within the lacunae. Interestingly, SCRG1 protein was well detected in the intermediate zone of articular cartilage.

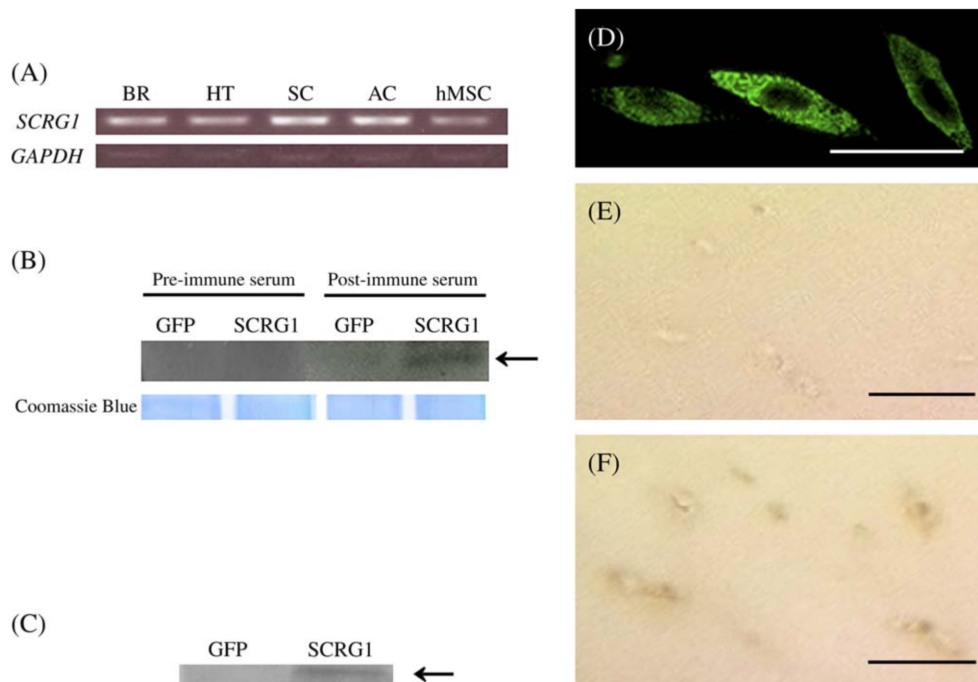

Fig. 1. SCRG1 expression *in vitro* and *in vivo*. (A) Semi-quantitative RT-PCR of *SCRG1* in human tissues. RT-PCR was performed using RNAs of human brain, heart, spinal cord, articular cartilage and hMSCs. *GAPDH* was used as an internal control. Sequencing was performed to confirm the PCR products. Articular cartilage and spinal cord expressed *SCRG1* at the same level, indicating that articular cartilage was one of the highest *SCRG1*-expressing tissues in human. BR, brain; HT, heart; SC, spinal cord; AC, articular cartilage. (B) Western blotting of pcDNA5-*GFP* and pcDNA5-SCRG1 transfected C3H10T1/2 cells. Ectopic expression of SCRG1 in C3H10T1/2 cells was detected by Western blotting (pre-immune IgG used as control). The arrow indicates the presence of 10 kDa SCRG1 band only in the SCRG1 transfected cultures upon reaction with immune IgG. (C) Immunoprecipitation of culture medium of *GFP* vector or *SCRG1* transfected cells, showing the presence of SCRG1 (arrow) within culture medium of only the SCRG1 transfected cells. (D) Immunofluorescence of SCRG1 transfected C3H10T1/2 cells showing the localization of the protein in cytoplasmic granules. (E,F) SCRG1 immunohistochemistry of human articular cartilage. Antiserum directed against immunogenic peptide sequence of SCRG1 protein (KISFVIPCNNQ-COOH) was used. Human adult articular cartilage was stained either with pre-immune serum (E) or anti-SCRG1 serum (F). Both articular chondrocytes and the extracellular matrix surrounding their lacunae were positively stained. Magnification: bar = 25 μm (D) and 100 μm (E,F).

SCRG1 EXPRESSION DURING CHONDROGENESIS *IN VITRO*

To investigate whether *SCRG1* was expressed during mesenchymal chondrogenesis, we performed RT-PCR of *SCRG1* using chondrogenic pellet culture of hMSCs. As is shown in Fig. 2(A), the expression of *SCRG1* was induced in a time dependent and Dex/TGF-β3 dependent manner. By Day 21, the expression of *SCRG1* increased to a level similar to that detected in articular cartilage. To analyze the dependence of the *SCRG1* induction on Dex and/or TGF-β3, these two reagents were added separately to the culture medium. RT-PCR analysis revealed that addition of Dex alone could induce *SCRG1* expression [Fig. 2(B)], i.e., the induction was not enhanced by addition of TGF-β3 to Dex supplemented medium. To investigate whether this induction was mediated by the GR, we tested the effect of RU-36846 on the culture. RU-36846 completely inhibited the induction of *SCRG1* on Day 11; however, RU-36846 could not completely inhibit the induction on Day 21 [Fig. 2(C)].

SCRG1 EXPRESSION SUPPRESSED PROLIFERATION OF hMSCs

As articular cartilage is an avascular tissue, articular chondrocytes are not normally exposed to serum *in vivo*[2]. To determine whether the presence of serum could affect *SCRG1* expression during chondrogenesis, we added 10% FBS to the otherwise serum-free chondrogenic culture medium of hMSCs. As shown in Fig. 3(A), addition of FBS suppressed the induction of *SCRG1*. As one of the effects of serum is the enhancement of cell proliferation, we next tested the involvement of SCRG1 in the regulation of mesenchymal cell proliferation. MTS analysis revealed that forced expression of *SCRG1* in hMSCs suppressed cell growth in a time dependent manner [Fig. 3(B)]. We have also transfected C3H10T1/2 cells with *GFP* and *SCRG1* vectors using either Nucleofector or Lipofectamine 2000, and assayed cell growth rate by direct cell counting and MTS assay, and obtained similar results (data not shown). These results suggest that *SCRG1* may play a role in the regulation of mesenchymal cell growth.

SCRG1 EXPRESSION STIMULATED CHONDROGENESIS *IN VITRO*

We next investigated the effect of forced *SCRG1* expression during the early events of mesenchymal chondrogenesis *in vitro*. High-density micromass cultures of mesenchymal cells, including limb bud cells and murine C3H10T1/2 cells, have been shown to undergo chondrogenesis *in vitro*[41,45–48]. In this study, C3H10T1/2 cells were transfected either with pcDNA-*GFP* or pcDNA-*SCRG1*, and treated with 100 ng/ml of BMP-2. In this culture system, most chondrogenic cell condensations eventually developed into cartilaginous nodules, which could be identified by alcian blue staining. After 5 days of culture, both transfected groups showed small round cells with metachromatic borders, whose morphology resembled early chondrocytes in culture as described previously[41,49]. Although chondrogenesis was seen in both *GFP* vector and *SCRG1* transfected cell cultures, transfection of *SCRG1* resulted in a significant increase in alcian blue staining when compared with *GFP* vector transfected controls [Fig. 4(A, B)]. Although less evident as on Day 5, the difference in alcian

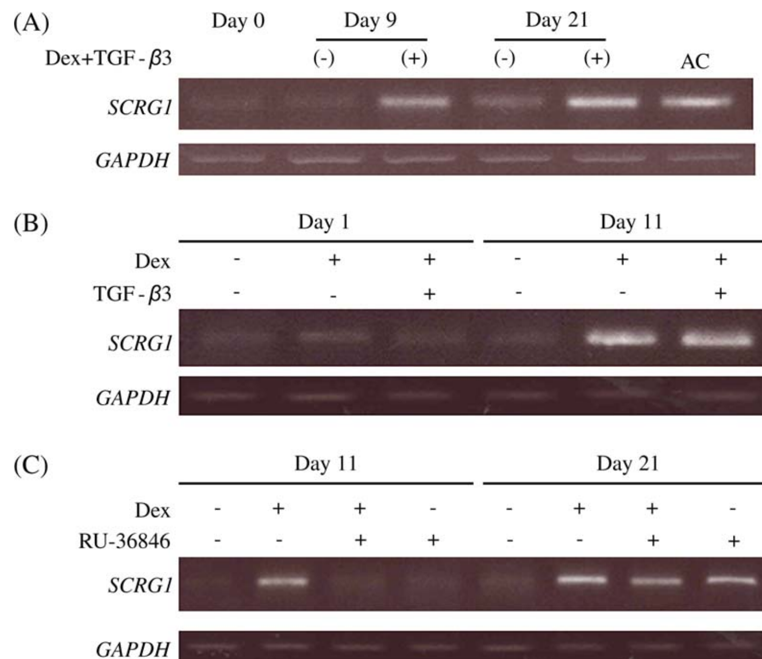

Fig. 2. RT-PCR analysis of *SCRG1* expression in *in vitro* chondrogenic pellet culture. (A) Expression levels of *SCRG1* are dependent on time and on Dex and TGF-β treatment as shown in the Day 0, Day 9 and Day 21 samples. Note that the level of expression of *SCRG1* on Day 21 is similar to that in articular cartilage. AC, articular cartilage. (B) Dex treatment is sufficient to induce *SCRG1* expression. Either Dex alone or Dex/TGF-β3 was added to the culture medium. RT-PCR revealed that addition of Dex alone could induce *SCRG1* expression on Day 11, and that this induction was not further enhanced by the addition of TGF-β3. (C) Dex-mediated induction of SCRG1 is modulated via GR action. Co-treatment with the glucocorticoid inhibitor, RU-36846, completely inhibited Dex induced *SCRG1* expression by Day 11. However, by Day 21, RU-36846 was less effective.

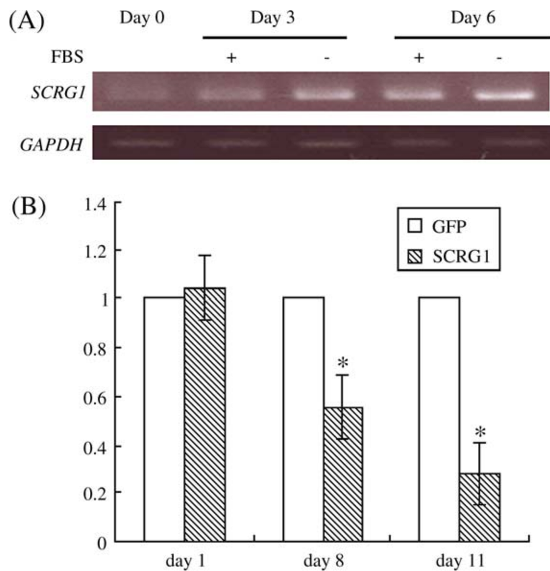

Fig. 3. Effect of *SCRG1* on the proliferation of hMSCs. (A) *SCRG1* expression in hMSCs is modulated by the presence of serum in culture medium. Cells were cultured in chondrogenic culture medium either with or without 10% ss-FBS. Addition of FBS suppressed induction of *SCRG1* in hMSCs. (B) *SCRG1* suppresses hMSC proliferation. Forced expression of *SCRG1* in hMSCs suppressed cell growth in a time dependent manner, based on MTS assay. $n = 6$; $^*P < 0.05$, when compared with GFP.

blue staining between *GFP* and *SCRG1* transfected cells persisted on Day 14 [Fig. 4(A, B)]. In the absence of BMP-2, neither *GFP* vector nor *SCRG1* transfected micromass culture showed alcian blue staining (data not shown), suggesting that the action of SCRG1 alone may not be able to induce chondrogenesis *in vitro*. The role of SCRG1 could be the enhancement of cartilage development, perhaps in the synthesis and deposition of extracellular matrix.

SCRG1 mediated enhancement of chondrogenesis was also confirmed by RT-PCR analysis of the expression of chondrocyte markers. As shown in Fig. 4(C), expression of pre-chondrocyte markers (collagens type I and type IIA) was slightly reduced, while that of differentiated chondrocyte markers (collagen type IIB and aggrecan) was slightly elevated. These changes were apparent especially during the early stages of chondrogenic differentiation. (Note that gene-expression levels of collagen type IIA, the alternatively spliced mRNA isoform associated with pre-cartilage mesenchyme, were undetectable on Days 10 and 14.)

Discussion

We report here that a GC inducible gene *SCRG1*, which was expressed normally at high levels in articular cartilage, could suppress cell growth and stimulate *in vitro* chondrogenesis in high-density micromass culture of mesenchymal cells, such as C3H10T1/2 cells. Evidence for the high level of expression of *SCRG1* in articular cartilage was obtained through comparison of *SCRG1* transcript levels between *SCRG1*-expressing tissues [Fig. 1(A)]. It has been previously shown that *SCRG1* mRNA is expressed at the highest level in spinal cord. Using a membrane blotted with poly (A)+ RNA from 50 human tissues, the level of *SCRG1* transcript was shown to be more than 2 fold higher than any other tissues[29]. On the other hand, our previous work comparing 31 human tissues using microarray suggested that articular cartilage expressed *SCRG1* at the highest level[26]. To better access which tissue is in fact expressing *SCRG1* at the highest level, we have performed here semi-quantitative RT-PCR of five human tissues, articular cartilage, spinal cord and other *SCRG1*-expressing tissues. As shown in Fig. 1(A), comparable highest level of *SCRG1* expression was seen in articular cartilage and spinal cord, indicating that articular cartilage was indeed among the highest *SCRG1*-expressing tissues in human.

Immunofluorescence analysis of *SCRG1* transfected cell showed that SCRG1 protein is localized in cytoplasmic granules in C3H10T1/2 cells. Western blotting showed an immunoreactive protein of about 10 kDa in size, and immunoprecipitation analysis showed that this protein was actually secreted into media. These properties of SCRG1 protein in mesenchymal cells are similar to those observed for neuronal cells[31]. In addition, immunohistochemistry of human adult articular cartilage, as shown in Fig. 1(E), showed the presence of SCRG1 protein in the extracellular matrix within the lacunae of articular chondrocyte. Taken together, these observations suggest that SCRG1 protein was secreted *in vivo* by articular chondrocytes and deposited within their immediate extracellular matrix.

Dex is known to induce *in vitro* chondrogenesis in mesenchymal progenitor cells[7,11,34,35,50], but its exact molecular mechanisms are still unknown. We have shown here that *SCRG1* expression was induced in a Dex-dependent manner during *in vitro* chondrogenic pellet culture of hMSCs. This *SCRG1* induction by Dex is mediated via GR during the earlier stage of chondrogenesis (Day 11). In the later stage of chondrogenesis (Day 21), the Dex antagonist, RU-36846, failed to inhibit *SCRG1* expression completely. These findings suggest that *SCRG1* expression is regulated by the Dex-GR pathway during the early stages of chondrogenesis.

Dex is known to suppress cell growth during pre-chondrogenic and chondrogenic period of murine multipotential mesenchymal cell line ATDC5, but the exact molecular mechanisms are not known[50]. We have also recently observed similar growth suppression by addition of Dex to undifferentiated hMSCs (Derfoul *et al.*, in preparation). Our finding here that SCRG1 is Dex-inducible and is capable of suppression of cell proliferation of hMSCs and C3H10T1/2 cells suggests that *SCRG1* may be one of the key molecules in Dex-mediated mesenchymal cell growth suppression.

BMP-2 is a key inducer of chondrogenesis for *in vitro* chondrogenesis system in micromass culture of C3H10T1/2 cells[41]. In cultures of both C3H10T1/2 cells and hMSCs, BMP-2 up-regulates Sox-9 in a dose dependent manner[51–53]. Sox-9 is a high mobility group domain transcription factor expressed in all cartilage primordia during embryogenesis, and is believed to play a key regulatory role in chondrogenesis[54]. We have shown here that SCRG1 expression alone could not induce chondrogenesis, but in the presence of BMP-2, *SCRG1* transfected cells yielded significant increase in alcian blue staining, compared to control, *GFP* vector transfected cells. These data suggest that SCRG1 can enhance events downstream of BMP-2 action. This effect was not mediated through BMP-2 modulation of the Sox-9 pathway, since RT-PCR analysis showed no detectable differences in Sox-9 level in control, *SCRG1* or *GFP* vector transfected micromass cultured cells. SCRG1 enhancement of chondrogenesis is thus likely to act either independent of Sox-9 or downstream of Sox-9 activation. Given that *SCRG1* was induced as early as Day 3 in chondrogenic pellet culture [Fig. 3(A)], whereas Sox-9

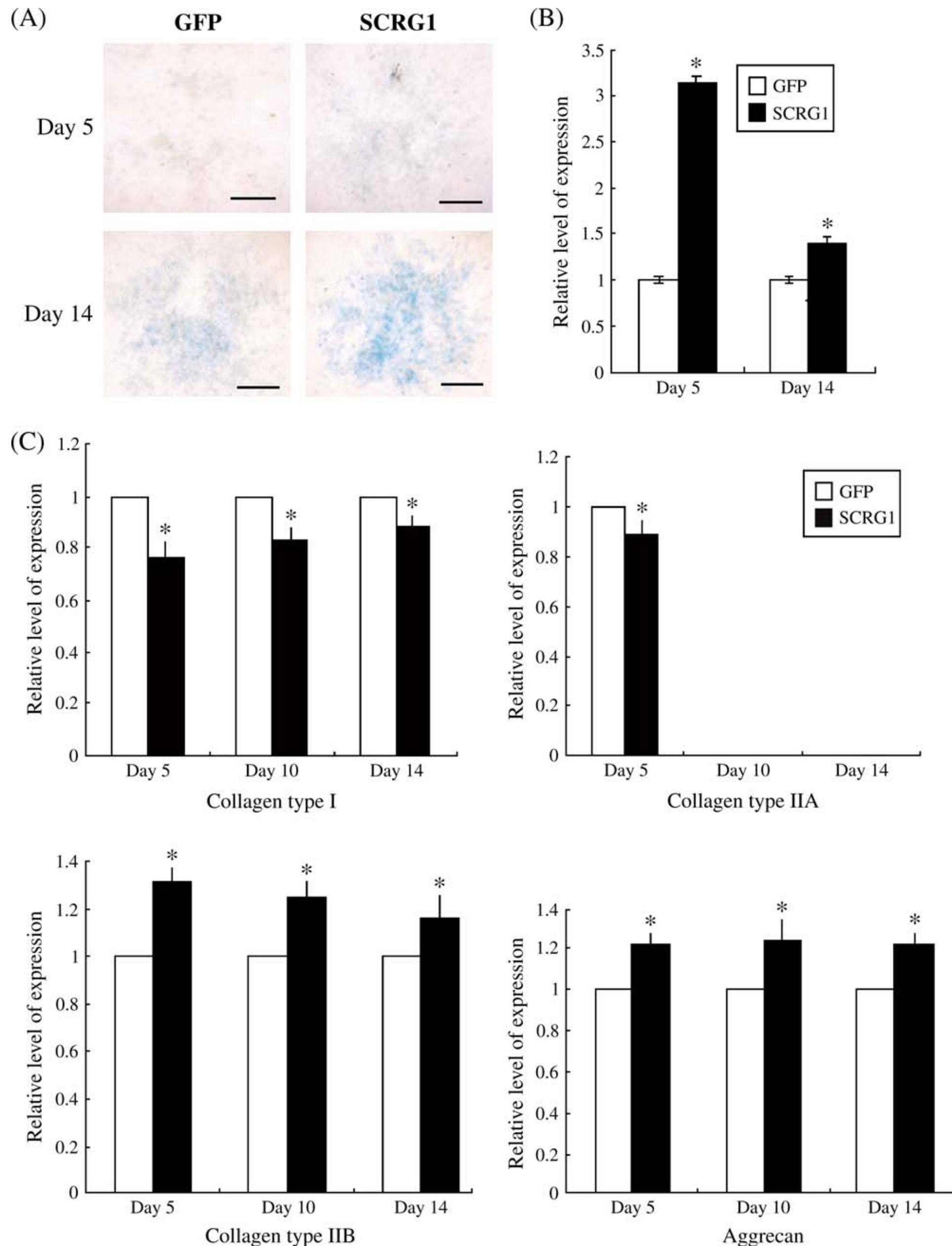

Fig. 4. Acceleration of *in vitro* chondrogenesis by *SCRG1* expression in high-density micromass cultures of C3H0T1/2 cells. (A) Alcian blue staining of cartilage matrix. In control cultures transfected with GFP construct, matrix produced by the micromass culture became alcian blue positive by Day 5, and was highly evident by Day 14. *SCRG1* transfected micromass culture appeared more strongly stained than the control throughout the same time course. Scale bar: 1 mm. (B) Quantitation of alcian blue staining. Significant increase in alcian blue staining was observed in SCRG1-transfected cultures compared to the control. $n = 6$; *, $P < 0.05$ when compared with GFP. (C) Gene expression analyzed by semi-quantitative RT-PCR. The level of pre-chondrocyte markers (collagen type I and type IIA) decreased, while the level of differentiated chondrocyte markers (collagen type IIB and aggrecan) increased upon forced expression of *SCRG1*. These changes were apparent especially during the early stages of chondrogenic differentiation. (Note that gene-expression levels of collagen type IIA, the alternatively spliced mRNA isoform associated with pre-cartilage mesenchyme, were undetectable on Days 10 and 14.) Expression levels were normalized to GFP transfected C3H10T1/2 cells at each time points. $n = 3$; $^*P < 0.05$, when compared with GFP.

was not induced until after Day 7 in the same culture system[55], the *SCRG1* mediated enhancement of chondrogenesis is likely to be independent of Sox-9 pathway, rather than acting downstream in Sox-9 mediated chondrogenesis.

Concerning the time course of the effect of *SCRG1* forced expression on chondrogenesis, it is noteworthy that culture Day 9 corresponds approximately to the time point when pellet cultures of hMSCs start to chondrify and become alcian blue positive, i.e., the early phase of chondrogenesis. Similarly, in the C3H10T1/2 micromass cultures, *SCRG1* also appears to play a stimulatory role in accelerating early chondrogenesis. Given that in both systems, other effectors, i.e., DEX or BMP-2, are required, the exact

mechanism of action of forced expression of *SCRG1* on other early phase chondrogenesis, particularly related to the other effectors, remains to be elucidated.

Another issue related to *SCRG1* stimulation of chondrogenesis in micromass cultures of C3H10T1/2 cells is that alcian blue staining increased considerably higher than aggrecan gene expression [Fig. 4(B, C)]. The most likely explanation is that gene-expression level was assessed by non-quantitative RT-PCR, which may not directly correlate to the level of sulfated proteoglycan produced, detectable by alcian blue staining. Other possibilities may include increased level of sulfation itself, and the production of other sulfated proteoglycans apart from aggrecan. In any event, the acceleration of chondrogenesis in early chondrogenesis (Day 5) is also supported by the changes in the expression level of collagen type I and collagen type IIB.

Although Dex can induce *in vitro* chondrogenesis in pellet culture of mesenchymal stem cells, it is not a specific chondrogenic differentiation factor. Dex can induce hMSCs to differentiate into osteoblasts, myoblasts and adipocytes depending on specific culture conditions[35]. As for chondrogenesis, the effect of Dex differs depending on the differentiation stage of the chondroprogenitor cells[50]. Thus, identifying chondrogenesis-specific genes downstream of Dex action would be of great value to cartilage tissue engineering as well as provide insights into the biology of skeletal development.

It is also well known that clinical use of GCs has severe side effects, including growth retardation and osteoporosis. Repeated articular injection of Dex potentially worsens OA by accelerating cartilage degradation[56]. These side effects severely limit the application of Dex for skeletal tissue engineering. Ideally, if *SCRG1* could produce the chondrogenic effect of Dex without its side effects, *SCRG1* should be of great utility in gene-based cartilage tissue engineering. Further investigation of the role in chondrogenesis should shed light on the molecular mechanisms involved in skeletal tissue development, repair and tissue engineering.

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