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Gas6, a new regulator of chondrogenic differentiation from mesenchymal cells

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Abstract

The mesenchymal cell line C3H10T1/2 can be preferentially induced toward chondrogenesis by culturing as a micromass in the presence of bone morphogenetic protein 2. To screen new regulator genes for chondrogenic differentiation, we performed differential display polymerase chain reaction and identified growth arrest-specific 6 (Gas6) as a gene that was clearly downregulated by this induction of chondrogenic differentiation. Blockage of Gas6 mRNA expression by siRNA remarkably enhanced the chondrogenic differentiation, while stimulation with recombinant Gas6 inhibited the mRNA expressions of type II collagen (Col2a1) and aggrecan. Gas6 signaling activated the phosphorylation of ERK1/2, SAPK/JNK, and Akt, but not p38 MAPK. These results suggest that Gas6 negatively regulates chondrogenic differentiation, at least through the MAPK pathway.

Keywords: Gas6; Chondrogenesis; Differentiation; C3H10T1/2 cell; MAPK

The first step of chondrogenesis involves cell proliferation followed by condensation of undifferentiated mesenchymal cells [1]. The mesenchymal condensation is dependent on signaling that occurs upon cell–cell and cell–matrix contact, and is regulated by several adhesion molecules, including cadherins, integrins, and extracellular components [2–5]. During in vitro chondrogenesis, mesenchymal cells undergo spontaneous differentiation into chondrocytes when cultured at high densities, such as those attained in micromass cultures [6]. The cellular interaction optimization under micromass culture conditions contributes to the induction of mesenchymal chondrogenesis. Many studies have revealed roles for transforming growth factor-beta (TGF-β) superfamily members, including the bone morphogenetic proteins (BMPs), in commitment to the chondrogenic lineage. For example, the observed loss of cartilage following inactivation of BMPs in Noggin transgenic mice indicates that signals for cartilage production are exclusively reinforced by multiple BMPs [7,8]. Sox9, one of the earliest markers expressed during precartilaginous condensation, is promoted by BMP signaling [9,10]. These studies indicate that the BMP pathway is required for chondrogenic differentiation.

The mesenchymal cell line C3H10T1/2 is pluripotent and the cells have been reported to differentiate into myoblasts, osteoblasts, adipocytes, and chondrocytes [11,12]. Moreover, their responses to BMP-2 determine their differentiation into several cell lineages. BMP-2 causes dose-dependent differentiation of these cells into adipocytes (low concentrations) or chondrocytes and osteoblasts (high concentrations) [13]. Furthermore, Smad1, a BMP-2 signaling mediator, participates in osteogenic, but not chondrogenic, differentiation of C3H10T1/2 cells [14].

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Specifically, C3H10T1/2 cells can be preferentially induced toward chondrogenesis by culturing as a micromass in the presence of BMP-2 [3,4].

In this study, we performed a wide differential display polymerase chain reaction (PCR) to screen for candidate genes that regulate chondrogenic differentiation using C3H10T1/2 cells grown in micromass cultures in the presence or absence of BMP-2. As a consequence, we identified growth arrest-specific 6 (Gas6) as a gene that was downregulated by BMP-2 treatment. Gas6 is a γ-carboxylated glutamic acid (Gla) protein that has been shown in other systems to act through tyrosine kinase signaling pathways [15]. We investigated whether Gas6 regulates the process of differentiation from mesenchymal cells during chondrogenesis.

Materials and methods

Materials. Human recombinant BMP-2 and rat recombinant Gas6 (rGas6) were kindly provided by Astellas Pharma Inc. (Tokyo, Japan) and Discovery Research Laboratories, Shionogi & Co., Ltd. (Osaka, Japan), respectively. An antibody against Sox9 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), while antibodies against Akt, phospho-Akt (Ser473), extracellular signal-related kinase (ERK) 1/2, phospho-ERK1/2 (Thr202/Tyr204), stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phospho-SAPK/JNK (Thr183/Tyr185), p38 mitogen-activated protein kinase (MAPK), and phospho-p38 MAPK were purchased from Cell Signaling Technology Inc. (Beverley, MA).

Cell culture. The murine mesenchymal cell line C3H10T1/2 was purchased from the American Type Culture Collection (Rockville, MD). The cells were plated as monolayer cultures and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The micromass culturing technique was modified from Ahrens et al. [6]. Aliquots (10 μl) of cells at a density of 1×10^7 cells/ml were placed in 24-well plates, allowed to adhere for 2 h at 37 °C and 5% CO_2 and maintained in Ham's F12 medium containing 10% FBS with or without BMP-2.

Alcian blue staining. Micromass cultures in 24-well plates were rinsed twice with phosphate-buffered saline (PBS), fixed for 10 min in Kahle's solution and stained with 1% Alcian blue (pH 1.0) overnight at room temperature. After rinsing, the cultures were examined using a substance microscope (SMZ-U; Nikon, Tokyo, Japan) and photographed. Stained cell cultures were solubilized with 6 M guanidine hydrochloride overnight at room temperature, and the optical densities of the extracted dye were measured at 620 nm.

Differential display. Total RNAs were obtained from cells grown in micromass cultures with or without BMP-2 for 24 h and compared by differential display PCR conducted using a GeneFishing DEG system (Seegene Inc., Seoul, Korea) [16]. To synthesize cDNAs, 3 μg of total RNA was reverse-transcribed using dT-ACP1 (5′-CTGTGAATGCT GCGACTACGATXXXXX$(T)_{18}$-3′) with M-MLV (Invitrogen Corp., Carlsbad, CA). PCR amplification was performed using the reaction mixture and the following primer pairs: arbitrary ACP5 (5′-GTCTACC AGGCATTCGCTTCATXXXXXAGTGCGCTCG-3′); dT-ACP2 (5′-C TGTGAATGCTGCGACTACGATXXXXX$(T)_{15}$-3′). DNA fragments were isolated using a gel extraction kit (Qiagen Inc., Valencia, CA) and sequenced according to the manufacturer's directions.

Real-time reverse transcription (RT)-PCR. To synthesize cDNAs, 1 μg of total RNA was reverse-transcribed using oligo(dT) primers with M-MLV. Real-time RT-PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), and amplified using QuantiTect SYBR Green PCR Master Mix (Qiagen). For each gene, a set of primers was designed using sequences obtained from GenBank or published by other authors (Supplementary Table 1). All reactions were run in triplicate. The gene expression levels in each sample were determined by the comparative Ct method (after validation assays for each gene primer set), using the β-actin gene as an endogenous control. The ground condition (control) was set as 1 and expression data are presented as bar graphs of the mean values and SD.

Immunoblotting. Cells were lysed in a Triton-based lysis buffer (1% Triton X-100, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% protease inhibitor), scraped off the dish and centrifuged at 15,000 rpm at 4 °C for 10 min. The supernatants were collected as total cell lysates. Equal amounts of the cell lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane. After sequential incubation of the membranes with primary antibodies and horseradish peroxidase-conjugated secondary antibodies, the bound antibodies were visualized using an ECL system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

RNA interference. Cells were plated in 6-well plates and maintained in DMEM containing 10% FBS and antibiotics. After 24 h, transfection of a small interfering RNA (siRNA; 50 nM) was performed using Lipofectamine 2000 (5 μg/well; Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were transferred to 24-well plates for micromass culture and incubated with or without BMP-2. After 24 h, the mRNA expressions of differentiation markers were analyzed by real-time RT-PCR. Alcian blue staining was performed after 3 days. The sequence of the sense-strand Gas6 siRNA was GAGAGGUGUUCGAGAACGA with dTdT overhangs. The control siRNA (non-silencing siRNA) was UUCUCCGAACGUGUCACGU with dTdT overhangs (Qiagen).

Results

Characterization of chondrogenesis of C3H10T1/2 cells

To induce chondrogenesis, C3H10T1/2 cells were treated with BMP-2 (100 ng/ml) in micromass cultures and stained for sulfated proteoglycans using Alcian blue to assess chondrogenic differentiation. The treated cells clearly showed positive staining compared to control cells without BMP-2 treatment (Fig. 1A). Next, we performed semi-quantitative RT-PCR to analyze BMP-2-induced chondrogenesis by assessing the expressions of chondrogenesis-associated genes. Type II collagen, the most abundant protein in the extracellular matrix of cartilage, consists of two alternatively spliced isoforms, namely the IIA form expressed in chondroprogenitor mesenchymal cells and the IIB form expressed in differentiating and mature chondrocytes [17]. Collagen type IIA mRNA expression was detected in the monolayer and control micromass cultures, while collagen type IIB and aggrecan mRNA expressions were induced at day 1 of BMP-2 treatment and markedly increased after 3 days (Fig. 1B). In addition, the level of Sox9 protein, a key regulator of chondrogenic differentiation, was increased at day 1 of BMP-2 treatment (Fig. 1C). These results suggest that C3H10T1/2 cells grown in micromass cultures start to show a chondrocytic phenotype after 24 h of BMP-2 treatment.

Identification of Gas6 as a gene downregulated by BMP-2

To explore new regulatory genes for chondrogenic differentiation, we performed differential display PCR using cells grown in micromass cultures with or without BMP-2 for 24 h, when chondrogenesis was initiated as described

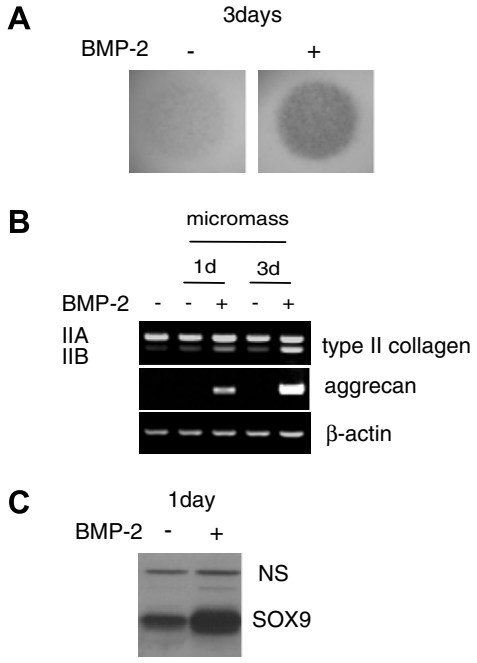

Fig. 1. Characterization of chondrogenesis of C3H10T1/2 cells. (A) Alcian blue staining of C3H10T1/2 cells grown in micromass cultures in the presence of BMP-2 for 3 days. (B) RT-PCR analysis of type II collagen (IIA and IIB forms) and aggrecan. Total RNA was extracted from non-treated control cells (day 0) and cells incubated with or without BMP-2 for 1 and 3 days. β-Actin was used as a loading control. (C) Western blotting for Sox9 protein. Cell lysates were prepared after 1 day of BMP-2 treatment.

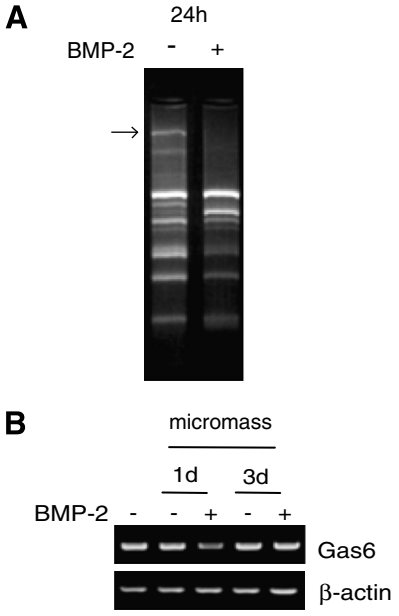

Fig. 2. Identification of Gas6 as a gene downregulated by BMP-2. (A) Differential display PCR was performed using cells grown in micromass cultures with or without BMP-2 for 24 h. Arrow, a band of the expressed genes amplified with the ACP5 primer is decreased by BMP-2. (B) Confirmation of the Gas6 expression pattern by RT-PCR. Total RNA was extracted from non-treated control cells (day 0) and cells grown in micromass cultures with or without BMP-2 for 1 and 3 days. β-Actin was used as a loading control.

above. We analyzed the sequences of genes whose expressions were either upregulated or downregulated by the induction of chondrogenesis, and identified a differentially expressed gene product whose sequence showed 95% identity to the 3′-untranslated region of Gas6 (GenBank Accession No.: BC005444). The Gas6 DNA fragment was present at high levels in untreated cells and not apparent in treated cells (Fig. 2A). RT-PCR amplification with specific primers confirmed that Gas6 mRNA expression was decreased by BMP-2 treatment for 1 day, although the effect was not obvious after 3 days (Fig. 2B). These results suggest that Gas6 is downregulated during the initiation of chondrogenic differentiation.

Promotion of chondrogenesis following blockage of Gas6 expression by siRNA

To examine whether inhibition of Gas6 induction enhances chondrogenic differentiation, we blocked endogenous Gas6 mRNA expression using a siRNA. In comparison to a non-silencing siRNA, the Gas6 siRNA blocked Gas6 mRNA expression by approximately 80% (data not shown) and remarkably enhanced the mRNA expressions of Col2a1 and aggrecan (Fig. 3A), while the mRNA expressions of osteocalcin and PPARγ remained unaffected. We also observed similar results using siRNAs created with another sequence of Gas6 (data not shown). We further assessed the chondrogenic differentiation by staining with Alcian blue, and found that the staining increased in parallel with the BMP-2 concentration (0–100 ng/ml) (Fig. 3B). The Gas6 siRNA also increased the intensity of Alcian blue staining. Quantitative analysis of the Alcian blue staining revealed that the Gas6 siRNA induced an approximately 50% increase in the proteoglycan production (Fig. 3B). These results suggest that chondrogenic differentiation is promoted by inhibition of Gas6 mRNA expression.

Inhibition of chondrogenesis by Gas6

Next, we examined whether Gas6 inhibits chondrogenic differentiation. After culture of C3H10T1/2 cells in monolayers in the presence or absence of rat recombinant Gas6 (rGas6) for 24 h, the cells were replated in micromass cultures and incubated with or without BMP-2 for 24 h. The presence of Gas6 suppressed the upregulated Col2a1 and aggrecan mRNA expressions in response to the induction of chondrogenesis, but had no significant effects on the mRNA expressions of osteocalcin and PPARγ (Fig. 4A). These results suggest that addition of Gas6 inhibits chondrogenic differentiation of mesenchymal cells.

Effects of Gas6 on intracellular signaling in C3H10T1/2 cells

To clarify the function that Gas6 inhibits during chondrogenic differentiation, we investigated its effect on the phosphorylation of MAPKs and Akt in C3H10T1/2 cells.

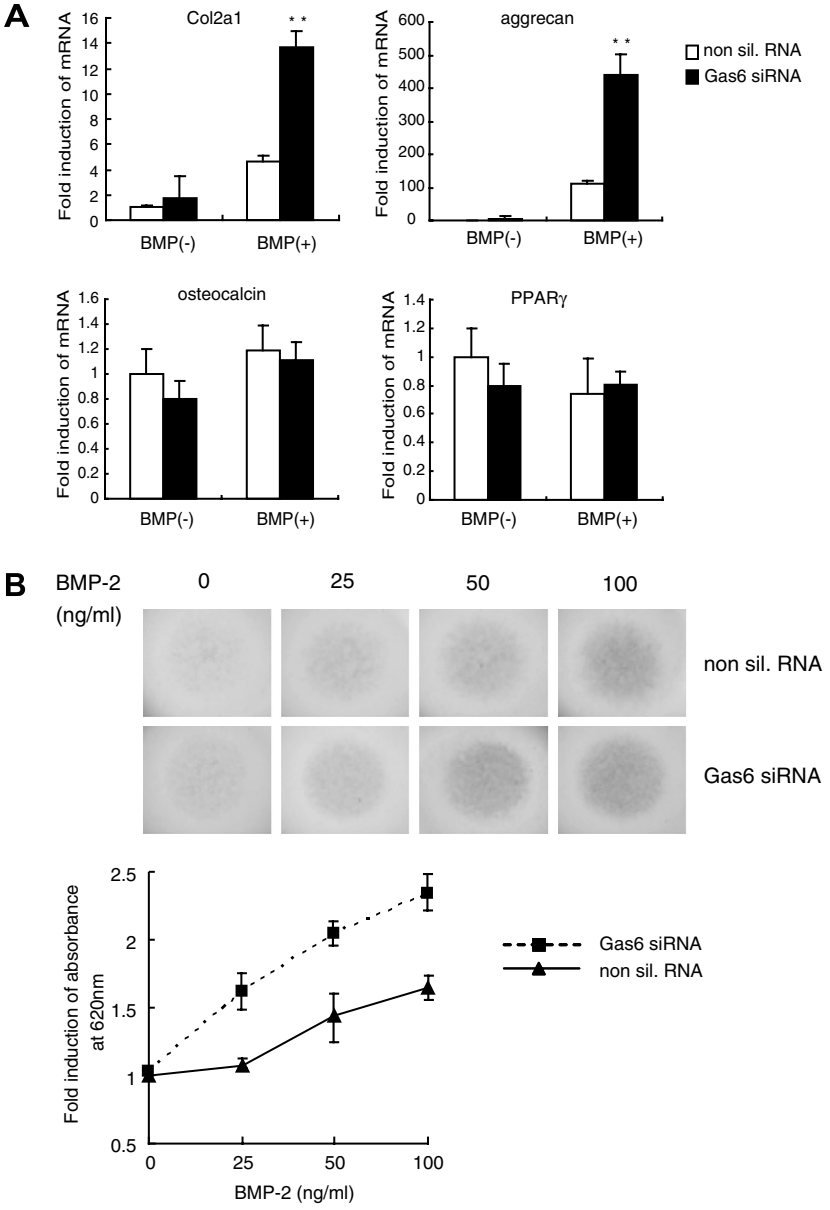

Fig. 3. Promotion of chondrogenesis by siRNA-mediated blockage of Gas6 expression. (A) At 24 h after transfection of a Gas6 siRNA or non-silencing siRNA into monolayer cultures of C3H10T1/2 cells, the cells were replated in micromass cultures and incubated with or without BMP-2 for 24 h. Total RNAs were extracted and analyzed for their mRNA expressions of differentiation markers by real-time RT-PCR. The mRNA levels were compared with those in the non-silencing siRNA-transfected and BMP(−) cells, and normalized by β-actin. The data represent means ± SD ($n = 3$). $^{**}P < 0.01$, Student's *t*-test. (B) Cells transfected with the Gas6 siRNA or non-silencing siRNA were treated with BMP-2 (0, 25, 50 or 100 ng/ml) for 3 days and stained with Alcian blue. Chondrogenesis in the presence of the non-silencing siRNA (▲) or Gas6 siRNA (■) was quantified by measuring the absorbance of the Alcian blue dye at 620 nm. The absorbance of the non-silencing siRNA-transfected cells in the absence of BMP-2 was set as 1. The data represent means ± SD ($n = 3$). Similar results were obtained in three independent experiments.

Gas6 was reported to have mitogenic and anti-apoptotic effects through MAPK and Akt pathways in multiple systems [18–21]. rGas6 induced transient phosphorylation of ERK1/2 that peaked at 5 min and slight phosphorylation of SAPK/JNK at 5 min. In contrast, p38 MAPK phosphorylation was not affected by the stimulation until 60 min, while phosphorylation of Akt increased at 5 min and continued until 60 min (Fig. 4B). These results suggest that Gas6 controls the MAPK and Akt pathways, which are the major intracellular signaling pathways.

Discussion

In the present study, we have shown that Gas6 mRNA expression decreases in the early phase of BMP-2-induced chondrogenic differentiation of C3H10T1/2 cells grown in micromass cultures, and moreover, that forcible inhibition of Gas6 mRNA expression enhances this process. Furthermore, we have demonstrated that exogenous Gas6 inhibits chondrogenic differentiation and activates ERK1/2, SAPK/JNK and Akt, but not p38 MAPK, in undifferentiated

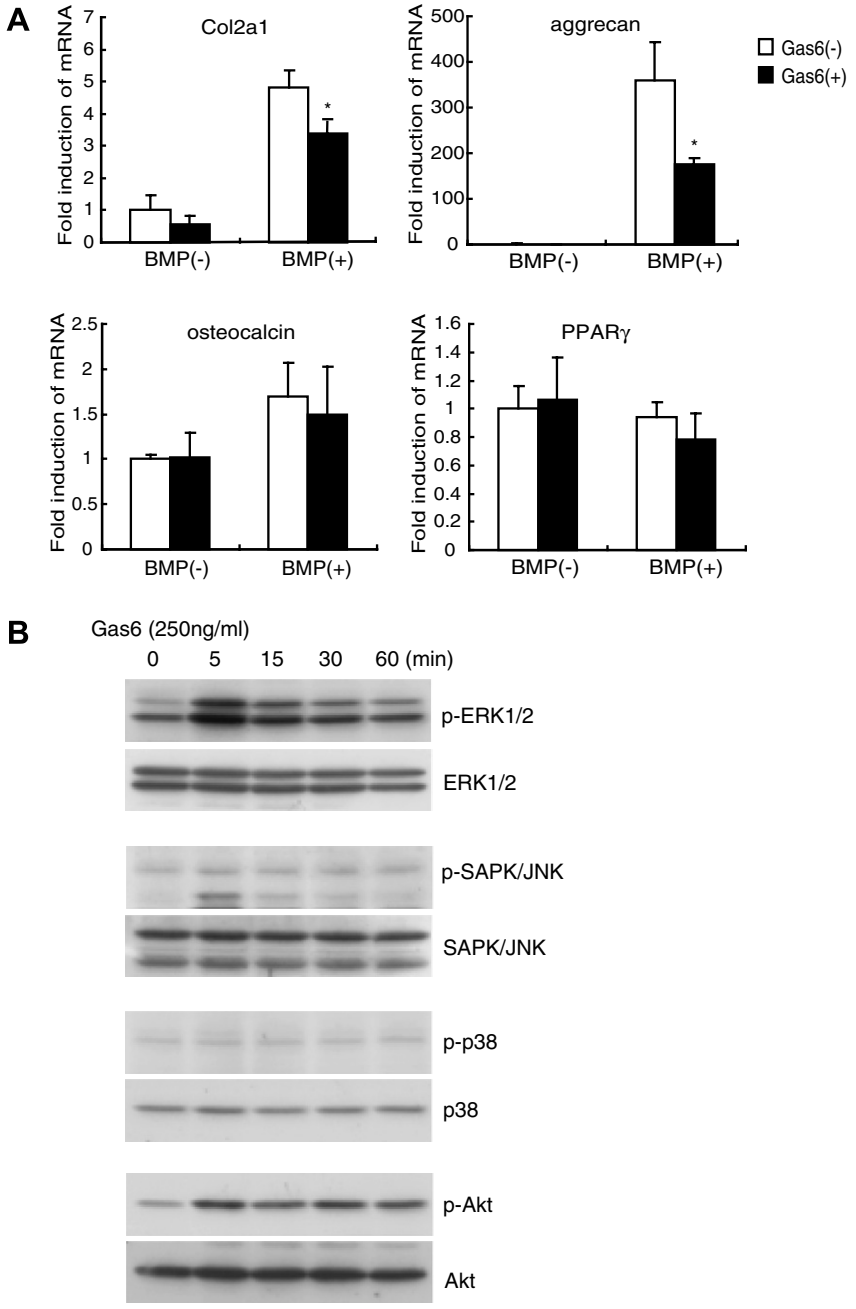

Fig. 4. Inhibition of chondrogenesis by Gas6. (A) After culture of C3H10T1/2 cells in monolayers in the presence or absence of rGas6 for 24 h, the cells were replated in micromass cultures and incubated with or without BMP-2 for 24 h. Total RNAs were extracted and analyzed for their mRNA expressions of differentiation markers by real-time RT-PCR. The mRNA levels were compared with those in the Gas6(−) and BMP(−) cells, and normalized by β-actin. The data represent means ± SD ($n = 3$). $^{*}P < 0.05$, Student's t-test. (B) Cells were cultured in serum-free medium for 6 h and then stimulated with rGas6 for 0, 5, 15, 30 or 60 min. Cell lysates were immunoblotted with phospho-ERK1/2, phospho-SAPK/JNK, phospho-p38 MAPK, and phospho-Akt antibodies. Each membrane was then stripped and probed with ERK1/2, SAPK/JNK, p38 MAPK, and Akt antibodies, respectively.

mesenchymal cells. These results represent the first evidence that Gas6 functions as a molecule that can down-regulate chondrogenesis.

Gas6 is a ligand for Axl, Sky, and Mer among the receptor tyrosine kinases (RTKs) [22], and its interactions with these receptors have been implicated in reversible cell growth arrest, survival, proliferation and cell adhesion in cell- and tissue-specific manners [23,24]. Gas6 is ubiquitously expressed in bone marrow, bone and cartilage as well as connective tissues [20,25,26]. Gas6 and Axl were reported to be present in human cartilage and to regulate chondrocyte growth and survival [26], whereas the functions of Gas6 in bone and cartilage formation have remained unknown. We further confirmed that Axl is expressed in C3H10T1/2 cells and that Gas6 stimulates its phosphorylation (data not shown).

The MAPK and Akt pathways are mainly involved in the downstream of Gas6 signaling through RTKs and

produce mitogenic and survival effects depending on the cellular background [18–21]. RTKs are crucial regulators of ERK activity and also potentially of other MAP kinases. Inhibition of chondrogenesis by epidermal growth factor (EGF) was reported to involve upregulation of ERK and suppression of p38 activity through tyrosine phosphorylation of EGF receptor [27]. On the other hand, the BMP and fibroblast growth factor (FGF) pathways act antagonistically to regulate chondrocyte proliferation, since FGF signaling through the ERK pathway inhibits hypertrophic differentiation, and has opposite effects to BMP [28,29]. The Gas6 signaling pathway may also regulate BMP-induced chondrogenesis by mediating the MAPK pathway.

Interestingly, activation of Axl was reported to inhibit the osteogenic differentiation of vascular pericytes, similar to its effects on mesenchymal cells [30]. It was suggested that the Gas6–Axl interaction prevents cell death occurring before the onset of calcification. Similarly, cell survival was promoted when primary human chondrocytes were grown in high-density cultures in serum-free medium containing recombinant Gas6 [26]. Apoptosis is associated with the transition from cartilage to bone during endochondral ossification, and therefore Gas6 may play similar roles to osteocalcin and matrix Gla protein (MGP), which modulate differentiation into mature chondrocytes and calcification during late chondrogenesis [31]. Axl and Tyro3 were recently identified as potent mediators of osteoarthritis pathogenesis in a genome-wide study, and increased expression of Gas6 was detected in chondrocyte clusters in osteoarthritic cartilage [32]. Further studies are required to fully define the mechanism by which the downstream components of Gas6 signaling mediate chondrogenic differentiation. In vivo studies of Gas6 will provide additional evidence to clarify the functional roles of Gas6 in chondrogenesis and articular cartilage degeneration.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.035.

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