Fibronectin regulates proteoglycan production balance in transforming growth factor-β1-induced chondrogenesis

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Abstract. Transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) induce a cartilage-specific extracellular matrix (ECM) gene, *aggrecan*, in a chondrogenic cell line, ATDC5. The results of our recent study show that TGF-β1, but not BMP-4, strongly induces an ECM gene, *fibronectin*, during chondrogenesis. However, the role of fibronectin in chondrogenesis is unclear. In the current study, our results showed that TGF-β1, but not BMP-4, led to versican-dominant proteoglycan production during chondrogenesis of ATDC5 cells. siRNA-mediated reduction of *fibronectin* and interference in the liaison between fibronectin and integrins by the Arg-Gly-Asp-Ser (RGDS) peptide increased *aggrecan* expression, and decreased *versican* expression by TGF-β1 stimulation. These data suggest that fibronectin is a critical mediator for TGF-β-specific production balance of 2 major proteoglycans, aggrecan and versican, during chondrogenesis.

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Introduction

Cartilage is a firm connective tissue with high water-holding capacity found in many parts of animal bodies, including the joints, ears, nose, trachea, and intervertebral discs. Cartilage is composed of chondrocytes which produce copious extracellular matrix (ECM), including type I or II collagen fibres, proteoglycans, and elastin fibres. Cartilage is classified into 3 types, elastic cartilage, hyaline cartilage and fibrocartilage, which differ in the relative amounts of the above-mentioned main components. In the ECM, aggrecan is a major and specific proteoglycan in cartilage (1), and it contributes to the gel-like property as a shock absorber. *Aggrecan*-null mice exhibit perinatal lethal dwarfism with little ECM in the cartilage and deficient chondrocyte differentiation (2).

ATDC5 cells are derived from a mouse teratocarcinoma and represent a well-characterized chondrogenic cell line (3-5). After confluent growth on the culture plate, ATDC5 cells form cartilaginous nodules and produce cartilage-related ECM such as type II collagen and aggrecan. Recent reports have revealed that transforming growth factor (TGF)-β, bone morphogenetic protein (BMP)-4, and growth/differentiation factor (GDF)-5 rapidly induce a chondrogenic marker such as type II collagen or aggrecan in confluent ATDC5 cells (4,6,7), indicating that the TGF-β superfamily plays a critical role in chondrogenesis.

The TGF-β and BMP pathways each have a specific intracellular signaling route and regulate different genes. TGF-β causes complex formation of type I and II TGF-β receptors and kinase activation of type I receptors. The activated type I receptors mainly phosphorylate TGF-β pathway-specific Smads such as Smad2 and Smad3, and lead to oligomerization with Smad4, which is termed a common Smad. The Smad complex translocates to the nucleus and specifically activates the target genes. On the other hand, BMP stimulates type I BMP receptors via complex formation with type II BMP receptors. The activated type I BMP receptors stimulate BMP pathway-specific Smads such as Smad1, Smad5 and Smad8, and form a complex with Smad4. The BMP-specific Smad complex stimulates its own target genes (8).

Both TGF-β1 and BMP-4 induce *aggrecan* and type II collagen expression in ATDC5 cell differentiation assays, while chondrocyte terminal differentiation is stimulated by BMP signaling and is inhibited by the TGF-β signaling pathway (9). Our previous study also showed that the shape of TGF-β1-induced chondrogenic ATDC5 cells is different from that of BMP-4-induced cells (10), suggesting that the TGF-β-induced chondrogenic fate is distinct from the BMP-induced one. In this study, we investigated the role of fibronectin, which was identified as an ECM component that greatly increased with TGF-β treatment but not with BMP treatment in ATDC5 cells (10). Consequently, it was clarified that fibronectin plays the role of a critical mediator in balancing proteoglycan production during TGF-β1-induced chondrogenesis.

Materials and methods

Cell cultures. Chondrogenic progenitor ATDC5 cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). ATDC5 cells were maintained in a Dulbecco's modified Eagle's medium/Ham's F-12 medium (Wako, Osaka, Japan) containing 5% fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan) with 10 μg/ml human transferrin (Roche Diagnostics, Mannheim, Germany) and 3×10^{-8} M sodium selenite (Sigma, St. Louis, MO, USA), (1,3-5,10). The medium was replaced every 3 days. To induce differentiation, ATDC5 cells were plated on a culture dish (Iwaki, Chiba, Japan) at a density of 3×10^4/ml. On Day 3 after the cells were plated, the culture medium was replaced by a maintenance medium containing 200 ng/ml human recombinant BMP-4 (R&D Systems, Minneapolis, MN, USA) or 10 ng/ml human recombinant TGF-β1 (R&D Systems) for 3 days.

Quantitative reverse transcription-polymerase chain reaction. Total-RNA was extracted from cells using the TRIzol reagent (Invitrogen, La Jolla, CA, USA). A total of 1 μg of total-RNA was reverse transcribed into cDNA using the SuperScript III enzyme (Invitrogen). The Applied Biosystems 7500 real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) was used for quantitative reverse transcription-PCR (qRT-PCR). qRT-PCR was performed using the SYBR-Green ER qPCR SuperMix (Invitrogen) according to the manufacturer's protocol. The primer sequences used were as follows: *fibronectin* forward, 5'-TTCAAGTGTGATCCCCATGAAG-3' and reverse, 5'-CAGGTCTACGGCAGTTGTCA-3'; *aggrecan* forward, 5'-CCTGCTACTTCATCGACCCC-3' and reverse, 5'-AGATGCTGTTGACTCGAACCT-3'; *versican* forward, 5'-TTTTACCCGAGTTACCAGACTCA-3' and reverse, 5'-GG AGTAGTAGTTGTTACATCCGTTGC-3'. All the primers generated PCR fragments of appropriate sizes, and non-specific products were not generated (data not shown). After amplification, the mRNA levels of these genes were normalized with those of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*) (primer sequences for *HPRT1*: forward, 5'-TCAGTCAACGGGGGACATAAA-3' and reverse, 5'-GGGGCTGTACTGCTTAACCAG-3').

Relative quantification. The relative *aggrecan/versican* expression ratio was calculated as follows: ratio = $2^{\Delta(\text{Ct } HPRT1 \text{ - Ct } aggrecan)}/2^{\Delta(\text{Ct } HPRT1 \text{ - Ct } versican)}$ (11,12); Ct, cycle threshold value.

Fibronectin knockdown assay. siTrio *fibronectin* 1, which was a mixture of 3 targeted small interference RNAs (siRNAs), and negative control siRNAs were purchased from B-Bridge International, Inc. (Sunnyvale, CA, USA). The transfection reagent RNAimax (Invitrogen, La Jolla, CA, USA) was used to transfect these siRNAs at the concentration of 10 nM in accordance with the manufacturer's instructions. ATDC5 cells were plated on a culture dish at a density of 3×10^4/ml. After 2 days, these siRNAs were transfected for 6 h. The medium was replaced on the day after transfection by a maintenance medium with or without TGF-β1 for 3 days.

Western blotting assay. Cell lysates were prepared using the M-PER Mammalian Protein Extraction reagent that was supplemented with protease inhibitors cocktail kit (both from Thermo Scientific, Rockford, USA). Cell lysates were run on a polyacrylamide gel, transferred to a methylcellulose membrane, and immunoblotted. The primary antibodies used in this study were as follows: mouse monoclonal antibodies against fibronectin 1 (BD Bioscience, San Jose, CA, USA) and β-actin (Sigma). After incubation with these primary antibodies, the membranes were incubated for 1 h at room temperature with the secondary antibody horseradish peroxidase-conjugated anti-mouse immunoglobulin (Promega, Madison, WI, USA).

RGD sequence blocking assay. RGD (Arg-Gly-Asp) sequence blocking assays were performed as described in earlier studies (13,14). ATDC5 cells were plated on a culture dish at a density of 3×10^4/ml. After 3 days, the culture medium was replaced and pre-treated for 30 min with 0.5 mM of Arg-Gly-Asp-Ser (RGDS) peptide or Arg-Gly-Glu-Ser (RGES) peptide (Sigma). Subsequently, differentiation was induced in cells by treatment with TGF-β1 for 3 days.

Statistical analysis. All the assays were independently performed 3 times. The results are presented as mean ± standard deviation (SD). The 2 groups were compared using the Student's t-test. Analysis of variance with the Dunnett's test was used for multiple comparisons. Data analyses were performed using the JMP 8 software (SAS Institute, Inc., Cary NC, USA). P-values <0.05 were considered statistically significant.

Results

The characteristics of TGF-β1-induced chondrogenic ATDC5 cells differ from those of BMP-4-induced chondrogenic ATDC5 cells in proteoglycan production. A recent study performed by our group showed that TGF-β1 treatment induced a much higher *fibronectin* expression than did BMP-4 treatment during chondrogenesis of ATDC5 cells (10). We further investigated differences between TGF-β1 and BMP-4 stimulation with respect to the gene expression of large proteoglycans by using qRT-PCR. *Aggrecan* expression levels increased by both TGF-β1 (7.4-fold) and BMP-4 (7.5-fold) stimulation. However, *versican* was more strongly induced by stimulation with TGF-β1 (16.2-fold) than by BMP-4 (2.1-fold) (Fig. 1A). Moreover, the ratio of *aggrecan/versican* expression was 1.3 for BMP-4 treatment and 0.2 for TGF-β1 treatment (Fig. 1B). These data indicate that TGF-β1 causes versican-dominant large proteoglycan production during chondrogenesis.
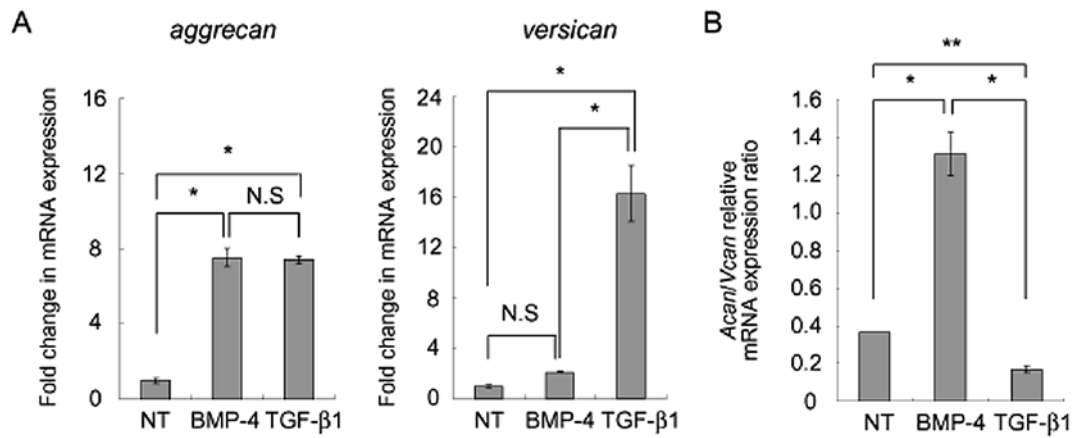
Figure 1. Effects of the TGF-β superfamily on proteoglycan gene expression during chondrogenesis. (A) The expression levels of the proteoglycan genes, *aggrecan* and *versican*, were analysed by qRT-PCR after treatment with BMP-4 or TGF-β1 for 3 days. *Aggrecan* and *versican* mRNA expression levels were normalized to *HPRT1* and are presented as fold-change relative to the mRNA expression in non-treated cells (NT). (B) *Aggrecan (Acan)/versican (Vcan)* relative expression ratios after BMP-4 or TGF-β1 treatment. The values (mean ± SD) are representative of three independent experiments. *P<0.001; **P<0.01. NS, not significant.
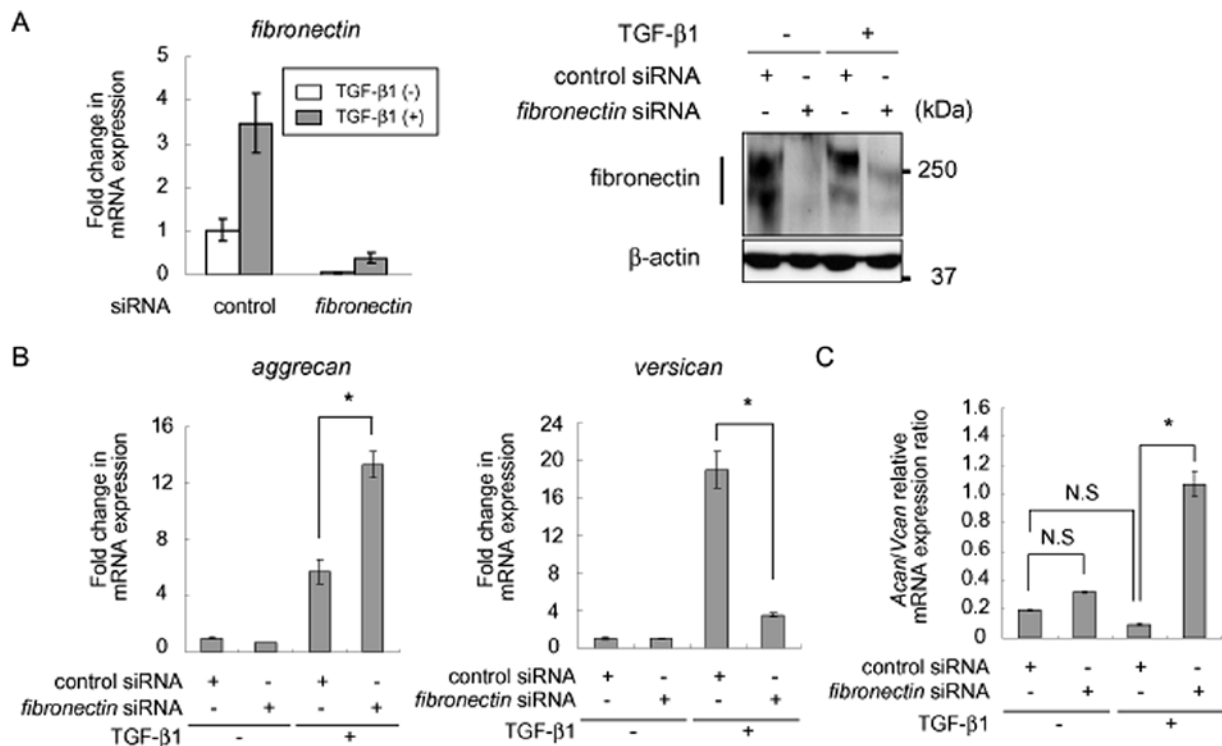

Figure 2. Fibronectin knockdown drastically changes the proteoglycan gene expression pattern of TGF-β1 induced chondrogenesis. (A) The efficiency of *fibronectin* knockdown by *fibronectin* siRNA was evaluated by comparing the mRNA and protein expression levels to those obtained using control siRNA with or without TGF-β1 treatment. The *fibronectin* mRNA expression level was analysed by qRT-PCR. These results were normalized to *HPRT1* and are presented as fold-change relative to the mRNA expression in control siRNA-treated cells without TGF-β1 stimulation. The fibronectin protein levels were analysed using Western blotting. β-actin was used as the loading control. (B) The effect of *fibronectin* knockdown on *aggrecan* and *versican* expression with TGF-β1 treatment was analysed using qRT-PCR. The *aggrecan* and *versican* mRNA expression levels were normalized to *HPRT1* and are presented as fold-change relative to the mRNA expression in control siRNA-treated cells without TGF-β1 stimulation. (C) *Aggrecan (Acan)/versican (Vcan)* relative expression ratios after treatment with siRNA, with or without TGF-β1 stimulation. The values (mean ± SD) are representative of three independent experiments. *P<0.001; **P<0.01. NS, not significant; NT, no treatment.

Fibronectin knockdown modulates large proteoglycan production in TGF-β1-treated ATDC5 cells. To examine whether fibronectin contributed to the induction of versican expression by TGF-β1 stimulation of chondrogenesis *in vitro, fibronectin*-knockdown ATDC5 cells were used for qRT-PCR. *Fibronectin* knockdown decreased *fibronectin* mRNA expression by

TGF-β1 stimulation; the expression was reduced to a level that was 89% lower than that obtained with control siRNA. The fibronectin protein levels were also sufficiently repressed by siRNA directed against *fibronectin* (Fig. 2A). Down-regulation of *fibronectin* increased *aggrecan* expression and decreased *versican* expression by TGF-β1 stimulation in chondrogenic
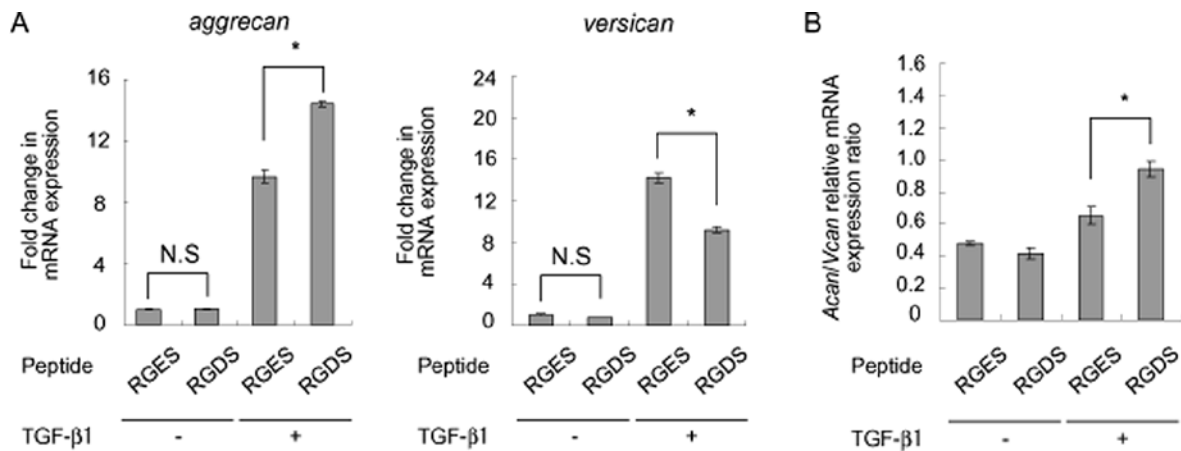
Figure 3. Blocking of the RGD sequence in fibronectin changes the proteoglycan gene expression pattern during TGF-β1-induced chondrogenesis. (A) ATDC5 cells were pre-treated with an RGDS or RGES peptide for 30 min. Subsequently, the cells were cultured with or without TGF-β1 for 3 days. The expression of the proteoglycan genes, *aggrecan* and *versican*, was analysed by qRT-PCR. *Aggrecan* and *versican* mRNA expression levels were normalized to *HPRT1* and are presented as fold-change relative to the mRNA expression in RGES-treated cells without TGF-β1 stimulation. (B) *Aggrecan (Acan)/versican (Vcan)* relative expression ratios after treatment with RGDS or RGES peptides, with or without TGF-β1 stimulation. The values (mean ± SD) are representative of three independent experiments. *P<0.001; **P<0.01. NS, not significant; RGDS, Arg-Gly-Asp-Ser; RGES, Arg-Gly-Glu-Ser.
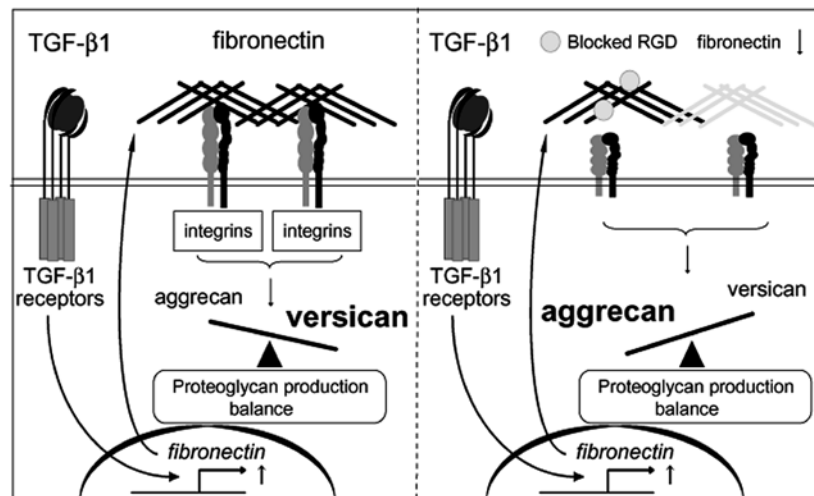

Figure 4. Representation of the role of fibronectin in TGF-β1-induced chondrogenesis. During chondrogenesis in ATDC5 cells, *fibronectin* expression is induced by TGF-β1 treatment. Thus, fibronectin production leads to versican-rich proteoglycan production balance via the fibronectin-integrin signal (left). However, *fibronectin* knockdown or the blocking of the RGD sequence in fibronectin by RGDS peptide leads to aggrecan-rich proteoglycan production balance in TGF-β1-treated ATDC5 cells (right). RGD, Arg-Gly-Asp.

differentiation of ATDC5 cells; the *aggrecan* expression levels were 2.3-fold higher and the *versican* expression levels were 5.4-fold lower than those obtained with treatment with control siRNA (Fig. 2B). Thus, the *aggrecan/versican* expression ratio for TGF-β1 treatment drastically increased with *fibronectin* knockdown (*fibronectin* siRNA, 1.1 vs. control siRNA, 0.1) (Fig. 2C). These data indicate that *fibronectin* may play an important role in controlling large proteoglycan production balance during TGF-β1-induced chondrogenesis.

Interference of fibronectin-integrins interaction with RGDS peptide increases aggrecan expression and the aggrecan/versican expression ratio in TGF-β1-induced chondrogenesis. Fibronectin mediates intracellular signaling via integrins as fibronectin receptors. The tripeptide RGD sequence in fibronectin is recognized by integrins, including α5β1, αvβ1, αvβ3

and αvβ5 (15,16). Adult articular chondrocytes express α5β1, αvβ3 and αvβ5 integrins (17). To investigate the role of the fibronectin-integrin pathway in large proteoglycan synthesis, we firstly used the RGDS peptide to block the interaction between fibronectin and integrins on mulitple sites and analysed its effect on TGF-β1-induced proteoglycan production balance. Blocking the RGD sequence increased *aggrecan* expression and decreased *versican* expression by TGF-β1 induction in the chondrogenic differentiation of ATDC5 cells; *aggrecan* expression levels were 1.5-fold higher and *versican* expression levels were 1.5-fold lower than those obtained after treatment with RGES peptide (Fig. 3A). The *aggrecan/versican* expression ratio in TGF-β1-treated cells increased on treatment with the RGDS peptide (RGDS peptide, 0.9 vs. RGES peptide, 0.6) (Fig. 3B). Moreover, we analysed the direct effect of integrin α5β1, which is one of the major integrins that

binds fibronectin (18), on the TGF-β1-induced proteoglycan expression pattern by using a neutralizing antibody against integrin α5β1. However, this antibody treatment did not affect the versican-dominant proteoglycan gene expression pattern by TGF-β1 stimulation (data not shown). These data suggest that the fibronectin-integrin pathway, except for the signaling via integrin α5β1, may be involved in controlling versican-dominant proteoglycan production balance.

Discussion

In this study, TGF-β1 induced versican expression more strongly than did BMP-4 during chondrogenesis of ATDC5 cells. It has also been reported that TGF-β2 or β3 induces chondrogenesis of mesenchymal stem cells derived from the bone marrow, with an increase in aggrecan and versican core protein synthesis (19). Versican is a large chondroitin sulfate proteoglycan expressed by human fibroblasts. The expression levels of versican are much lower than those of aggrecan in human articular cartilage, although the chondrocytes in human articular cartilage express mRNA for both *aggrecan* and *versican* (20). Bovine microarray data revealed that the *aggrecan* expression in articular cartilage is higher than that in the annulus fibrosus, whereas *versican* expression in articular cartilage is lower than that in the annulus fibrosus (21). These data suggest that increased expression of versican in the cartilage correlates with fibrosis and decreases the water-holding capacity, and TGF-βs, which induce *versican* expression, may be key molecules that increase the degree of fibrosis during chondrogenesis.

We recently showed that TGF-β1 strongly induces *fibronectin* expression in ATDC5 cells (10). Moreover, this study showed that *fibronectin* knockdown increases both *aggrecan* expression and the *aggrecan/versican* expression ratio after TGF-β1 stimulation. Integrin-mediated activation of members of the mitogen-activated protein kinase (MAP kinase) family regulates chondrocyte gene expression. Focal adhesion kinase (FAK) is one of the upstream mediators of MAP kinase, and FAK/Src signaling suppresses early chondrogenesis, including the induction of aggrecan, Sox6, and Ccn2 in mesenchymal cells by connective tissue growth factor gene regulation in micromass culture (22). On the other hand, *versican* expression is regulated by activator protein-1 (AP-1) and T cell factor (TCF) transcriptional factors (23). Moreover, fibronectin induces AP-1 activation through integrin α5β1-dependent Akt, extracellular signal-regulated kinase (ERK), and Jun-amino-terminal kinase (JNK) signaling pathways in endothelial cells (24). These reports indicate that fibronectin could regulate the expression of proteoglycans such as *aggrecan* and *versican* via the fibronectin-integrin-MAP kinase pathway. Meanwhile, it has been revealed that *fibronectin* knockdown induces mitochondria-dependent apoptosis in rat mesangial cells (25) and integrin α5β1, a major fibronectin receptor, mediates the survival signal in human chondrocytes (26). The result of our study, which were obtained from neutralizing antibody experiments, indicated that this antibody treatment did not affect the regulation of the proteoglycan genes expression pattern by TGF-β1 stimulation and was rather critical for survival in TGF-β1-induced chondrogenesis (data not shown). These data suggest that the fibronectin-integrin α5β1 signaling pathway may participate in cell survival rather than in proteoglycan synthesis during TGF-β1-associated chondrogenesis.

The amino acid sequence RGD found within fibronectin is also recognized by approximately half of all the known integrins such as αvβ1, αvβ3 and αvβ5 (15,16). These integrins may regulate the expression ratio of *aggrecan* to *versican* by TGF-β1 stimulation. To obtain more information about fibronectin-integrin-mediated proteoglycan synthesis, further studies on these integrin-mediated molecular pathways are required.

In conclusion, we showed that fibronectin is a key molecule for controlling proteoglycan production balance via the fibronectin-integrin signaling pathway during TGF-β1-induced chondrogenesis in ATDC5 cells (Fig. 4). Loss of TGF-β signaling results in susceptibility to cartilage damage; therefore, TGF-β replacement therapy is useful for cartilage maintenance and repair (9). However, TGF-β injection into a joint induces fibroplasia and fibrosis (27). If we further understand the regulation of proteoglycan production by fibronectin-mediated integrin signaling in chondrocytes, TGF-β replacement could be a more controllable therapy for articular cartilage disorder.

Acknowledgements

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