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Fibulin-3 negatively regulates chondrocyte differentiation

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ABSTRACT

Fibulin-3 is a member of the fibulin family that has been newly recognized as extracellular matrix proteins. We assessed the effects of fibulin-3 overexpression on chondrocyte differentiation using the clonal murine cell line ATDC5. The ATDC5-FBLN3 stably expressing fibulin-3 protein was spindle-shaped cell compared to the ATDC5-mock with plump cell. The cell growth in the ATDC5-FBLN3 was accelerated in comparison to that in the ATDC5-mock. The ATDC5-FBLN3 was not stained by Alcian blue, nor was there any cartilage aggregate formed after the induction of chondrogenic differentiation. The expression of type II collagen, aggrecan, and type X collagen was completely suppressed in ATDC5-FBLN3 even after the induction of differentiation. The overexpression of fibulin-3 reduced the expression of *Sox5* and *Sox6*, while it maintained the expression of *Sox9*. These findings suggest that fibulin-3 may play an important role as a negative regulator of chondrocyte differentiation.

The complex multistep process of the development of skeletal elements through endochondral ossification is controlled by a variety of positive and negative regulators. Bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), fibroblast growth factors (FGFs), Indian hedgehog (Ihh), Wnts, and parathyroid hormone-related peptide (PTH-rP) positively regulate chondrocyte differentiation. At the transcription level, transcription factors such as Sox9, Sox5, Sox6, Runx2/Cbfa1, and DEC1 are critical for the induction and progression of chondrocyte differentiation [1–4]. In contrast, negative regulators such as epidermal growth factor (EGF), splicing factor 3b subunit 4 (SF3b4), retinoic acid receptor, aZfp60, c-fos, nuclear factor E2 p45-related factor 2 (Nrf 2), Notch, matrix metalloprotease-2 (MMP-2), and AP-2α suppress or delay chondrocyte differentiation [5–13]. The interplay of positive and negative regulators is essential to control chondrogenesis.

Fibulins are a newly recognized family of extracellular matrix glycoproteins with the distinctive features of a fibulin-type C-terminal domain preceded by tandem calcium-binding (cb) EGF-like modules. To date, the fibulin family consists of six members which are numerically named fibulin-1 through fibulin-6 [14,15]. Fibulin family proteins have unique and partially overlapping expression pattern. Tropoelastin is a common ligand for fibulins. Immunohistochemical analysis demonstrated that fibulins are localized in tissue rich in elastic fiber, such as lung, perichondrium, and blood vessels [14]. Fibulin-5$^{-/-}$ mice exhibited disrupted and disorganized elastic fibers throughout the body, suggesting that fibulin-5 may be involved in elastogenesis by tethering elastic fibers onto cell surface integrins [16,17]. Fibulin-4$^{-/-}$ mice exhibited almost complete loss of elastic fibers and perinatal lethality [18]. Fibulin-3$^{-/-}$ mice developed multiple large hernias and pelvic prolapse due to the loss of the integrity of elastic fibers in fascia connective tissue [19]. All these results indicate a distinct role of fibulin-3, -4, and -5 in elastic fiber formation. The resource of the fibulins considered to be primordial vascular smooth muscle cells, developing cartilage cells, endothelial cells of the artery [14,15,20–23]. Fibulin-3 was initially identified as a gene highly up-regulated in senescent and Werner syndrome fibroblasts [20]. Fibulin-3 is highly homologous to fibrillin which is the major causative gene of Marfan syndrome [15]. Genetic linkage and molecular analysis has associated a missense mutation (R345W) in fibulin-3 with heritable macular degenerative disorders, so-called Malattia Leventinese and Doyne honeycomb retinal dystrophy [21].

Fibulin-3 is highly expressed in cartilaginous tissue [14,22]. However, no direct evidence for the role of fibulin-3 underlying chondrocyte differentiation is available in the literature to date. The purpose of this study is to explore the function of fibulin-3 in chondrocyte differentiation.

Materials and methods

Construction of fibulin-3 expression vector and preparation of recombinant fibulin-3 protein. Using human EST clone: I.M.A.G.E. 6083389 (Invitrogen) as a template, a cDNA fragment corresponding

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to the entire open reading frame of fibulin-3 was amplified with the oligonucleotide primers 5′-AGCGGCCGCCACCATGTTGAAAGCCCTTTTCC-3′ and 5′-ATCTAGAAAATGAAAATGGC CCCAC-3′ by means of PCR, thus adding a NotI site at the 5′ end, XbaI site at the 3′ end and the termination codon substituted with a glycine codon. This fragment was cloned into NotI and XbaI sites in an expression vector p3xFLAG-CMV14 (Sigma–Aldrich) to generate p3xFLAG-FBLN3, in which human fibulin-3 cDNA was connected to a triple repeat of the FLAG tag sequence at the 3′ end. To generate recombinant fibulin-3 protein, p3xFLAG-FBLN3 was then cut off at the NotI and SphI sites and cloned into the NotI and SphI sites of vector pFastBac1 (Invitrogen, Tokyo, Japan). Using this plasmid, pFB-FBLN3, the human fibulin-3 protein connected to the FLAG tag at the C-terminus was expressed in a Bac-To-Bac Baculovirus expression system (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. The transfected Sf9 cells were cultivated as a source of recombinant baculovirus and the conditioned medium was substantially infected with freshly cultured Sf9 cells. After a 72-h incubation at 28 °C, transfected Sf9 cells were harvested and suspended in 50 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40 supplemented with one tablet per 50 ml of complete protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan), and then they were sonicated. After centrifugation, the soluble fraction was collected and applied to an anti-FLAG M2 affinity column (Sigma–Aldrich, Tokyo, Japan). Following washing with Tris-buffered saline (TBS) (pH 7.4), the fibulin-3-3xFLAG protein was eluted from the column with 100 mM glycine–HCl (pH 3.5) and neutralized with 1 M Tris–HCl (pH 8.0).

Cell culture. The mouse embryonal carcinoma-derived chondrogenic cell line ATDC5 was purchased from Cell Bank, RIKEN BioResource Center. The ATDC5 cells were cultured in the medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 5% fetal bovine serum, 10 μg/ml human transferring (Roche Diagnostics), and 3×10^{-8} M sodium selenite (Sigma–Aldrich) at 37 °C in a humidified atmosphere of 5% CO_2 in air, as described [24]. For the induction of chondrogenesis, human recombinant insulin (Roche Diagnostics) at 10 μg/ml was added in the medium. To generate stable fibulin-3 expressing ATDC5 cells (ATDC5-FBLN3), p3xFLAG-FBLN3 was transfected to ATDC5 cells using Lipofectamine™ 2000 Reagent (Invitrogen), followed by culture in the selective medium containing 500 ng/ml G418 (Nacalai Tesque). To generate control clones of stable transfectants without fibulin-3 insert (ATDC5-mock), p3xFLAG vector was transfected to ATDC5 cells. For the analysis of cell growth of the ATDC5 cells, the cell lines were plated at a cell density of 1×10^4 cells in 12-well plates in the maintenance medium supplemented with human insulin. The cells were harvested after washing each plate three times with PBS. The cell number for each dish was counted with a cell counter.

Western blotting. We performed Western blotting using cell lysate and serum-free culture medium. The cell pellets were lysed in solubilization buffer. After centrifugation, the supernatant fluid was mixed to 6xSDS sample buffer and boiled for 5 min. Thereafter, the samples were separated in 10% SDS–PAGE, transferred onto poly vinylidene difluoride membranes (Millipore). After blocking the membrane with TBS-T containing 5% of non-fat dried milk, the membrane was immersed in the first antibody solution (a mouse monoclonal anti-β-actin antibody or anti-Flag M2 monoclonal antibody, Sigma–Aldrich) overnight at 4 °C. After washing with TBS-T, the membranes were incubated with HRP-conjugated anti-IgG antibody (GE Healthcare). The bands were visualized using the ECL plus Western blotting detection system (GE Healthcare) and detected by LAS-1000plus (Fuji film).

Alcian blue staining. On the predetermined day, the plates were washed two times with PBS, fixed with 95% methanol at −20 °C for 2 min, and stained with 0.1% Alcian blue (Muto Pure Chemicals) in 0.1 M HCl for 2 h. After rinsed three times with distilled water, the results were recorded with a digital camera. The dye was extracted with 6 M guanidine–HCl overnight, and the total optical density of extracted dye was measured using a spectrophotometer at 620 nm.

Total RNA extraction and quantitative real-time polymerase chain reaction. After washing each plate three times with cold PBS, the total RNA was extracted from the ATDC5 cells using ISOGEN (Nippon Gene), and 1.0 μg of RNA was reverse-transcribed with the First Strand cDNA Synthesis Kit (Roche Diagnostics). TaqMan Universal PCR Master Mix and TaqMan® Gene Expression Assays were used to analyze the genes including β-actin, Sox9, Sox5, Sox6, type II collagen, type X collagen, and aggrecan. Real-time quantitative PCR amplifications were performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The C_t value of β-actin was used as an endogenous reference for normalization. Standard curves were generated using cDNA samples from ATDC5-mock on day 14 after induction of chondrogenic differentiation. The relative expression levels of each target gene were indicated by calculating the ratio for those from ATDC5-mock on day 14. Assays were performed in triplicate and repeated three times.

Northern blot analysis. The total RNA was extracted from the 1.0×10^7 ATDC5 cells using ISOGEN (Nippon Gene), Poly A^+ RNA was purified with an mRNA extraction kit (GE Healthcare), separated by agarose gel electrophoresis, and blotted onto a nylon (Hybond-N; GE Healthcare). Hybridization with β-actin cDNA was performed under the standard conditions described previously [20].

Statistics and analysis. The data were analyzed using the StatView statistical software program (version 5.0; SAS Institute). Statistical significance was set at a value of $p < 0.01$. Association with the variables was determined by *t*-test.

Results

Cell morphological and growth curve analysis

ATDC5 cells display a number of characteristics of committed chondroprogenitor cells and undergo insulin-induced chondrocyte differentiation, which resembles chondrocyte differentiation *in vivo* [24]. Thereafter, to investigate the effect of fibulin-3 on chondrocyte differentiation, we first isolated the subclones of the ATDC5 cell lines which stably express fibulin-3 protein (ATDC5-FBLN3). Among twelve isolated subclones, the three clones: clone#5, #10, and #12 expressed various amounts of fibulin-3 protein by a Western blotting (Fig. 1A). Therefore we employed these three clones for the following experiments as ATDC5-FBLN3. Meanwhile we generated three independent control clones of stable transfectants without the fibulin-3 insert (ATDC5-mock). It was found that the cell morphology of ATDC5-FBLN3 was drastically changed compared to the ATDC5-mock (Fig. 1B and C) and that ATDC5-FBLN3 was spindle-shaped although the proliferating ATDC5-mock cells were plump. Furthermore, we found that FBLN3 protein is indeed secreted into the culture medium at a concentration of 1 μg/ml to the medium (Fig. 1D), and that the cell proliferation in the ATDC5-FBLN3 was accelerated compared to that of ATDC5-mock (Fig. 1E).

The effect of fibulin-3 overexpression on production of proteoglycan and cartilage nodule formation

We next examined the effect of fibulin-3 overexpression on production of proteoglycan and cartilage nodule formation. As shown in Fig. 2A, the ATDC5-mock underwent progressive differentiation over the 28-day period in medium containing insulin, as evidenced by increased formation of cartilage aggregates and increased Alcian

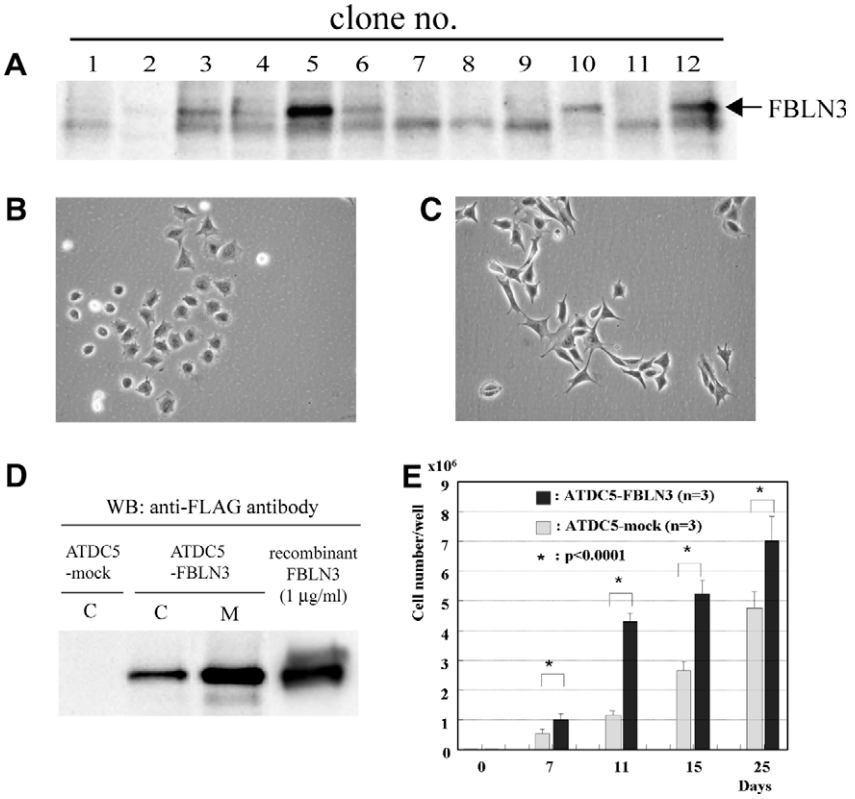

Fig. 1. Isolation of ATDC5-FBLN3. (A) ATDC5-FBLN3 which stably express FBLN3 was isolated. Among twelve isolated subclones, cell lysates from clone#5, #10, and #12 were revealed to contain the various amount of FBLN3 by a Western blot analysis. FBLN3: fibulin-3 protein. Microscopic findings showed that ATDC5-FBLN3 had spindle-shaped cytoplasm (C) although proliferating ATDC5-mock had plump cytoplasm (B). (D) Western blotting of cellular lysate and serum-free culture medium collected after 12-h incubation showed that fibulin-3 is indeed a secretory protein. C, cellular lysate; M, medium. To indicate the concentration of the fibulin-3 protein in the culture medium, recombinant fibulin-3 protein was applied at the concentration of 1 μg/ml into the far right lane. (E) Cell growth of ATDC5-mock and ATDC5-FBLN3. The cell proliferation in ATDC5-FBLN3 was accelerated in comparison to that of ATDC5-mock (gray box: ATDC5-mock, closed box: ATDC5-FBLN3). Independent three clones were examined, respectively. $^{*}p < 0.0001$.

blue staining. In contrast, the ATDC5-FBLN3 was not stained by Alcian blue, nor was there any cartilage aggregate formed. A quantitative determination of the extent of proteoglycan production showed that the overexpression of fibulin-3 resulted in the complete suppression during the induction of chondrogenesis (Fig. 2B). These results suggested that the overexpression of fibulin-3 protein suppressed cartilage aggregate formation and proteoglycan production despite induction.

The effect of the addition of exogenous recombinant fibulin-3 protein on ATDC5 differentiation

We presumed that the suppression of chondrogenic differentiation by fibulin-3 may cause a paracrine effect on the ATDC5 cells. Therefore we then generated recombinant fibulin-3 protein, and examined the effect of exogenous fibulin-3 protein on ATDC5 differentiation, adding this recombinant protein at a concentration of 1 μg/ml to the medium, which is the corresponding amount to secreted fibulin-3 protein in ATDC5-FBLN3 (Fig. 1D). The cartilage aggregates formation and production of proteoglycan in the ATDC5 cells were significantly suppressed (Fig. 2C and D). These results suggest that the suppression of chondrogenic differentiation by fibulin-3 did cause a paracrine effect on the ATDC5 cells.

The effect of fibulin-3 on chondrocyte differentiation at the molecular level

The effect of fibulin-3 on chondrocyte differentiation at the molecular level was further examined by quantitating the expression level of the chondrocyte marker gene: type II collagen, aggrecan, and type X collagen. Because the β-actin expression levels are not affected by either fibulin-3 or by chondrocyte differentiation (Fig. 3A), the β-actin was chosen as reference gene. As a result, the overexpression of fibulin-3 completely prevented the induction of type II collagen, while ATDC5-mock highly expressed type II collagen after induction of differentiation (Fig. 3B). Similar results were obtained with two other matrix genes: aggrecan and type X collagen genes (Fig. 3C and D). These results confirmed the overexpression of fibulin-3 indeed suppress chondrocyte differentiation.

The effect of fibulin-3 on expression of Sox9, Sox5, and Sox6 in ATDC5 cells

To understand the molecular mechanism of the inhibition of chondrocyte differentiation by fibulin-3, we examined the effect of fibulin-3 overexpression on the expression profiles of the Sox family of transcription factors such as Sox9, Sox5, and Sox6 which have been identified as key transcription factors required for early chondrogenesis [1,4,25]. Increased expression of Sox9 was observed in ATDC5-FBLN3 after stimulation with insulin, while the time in which the expression level of Sox9 reached to the peak level seemed to be one week later in ATDC5-FBLN3 than that in ATDC5-mock (Fig. 4A). The expression level of Sox5 in the ATDC5-mock increased at the beginning and early stage of chondrogenic differentiation (day 2), reached a peak expression level at day 4, and thereafter decreased. In contrast, the low expression level of Sox5 in the ATDC5-FBLN3 was stably observed before and after chondrogenic induction (Fig. 4B). The expression level of Sox6 in the ATDC5-mock was increased at the beginning and early stage of chondrogenic differentiation (day 4), and continued to increase

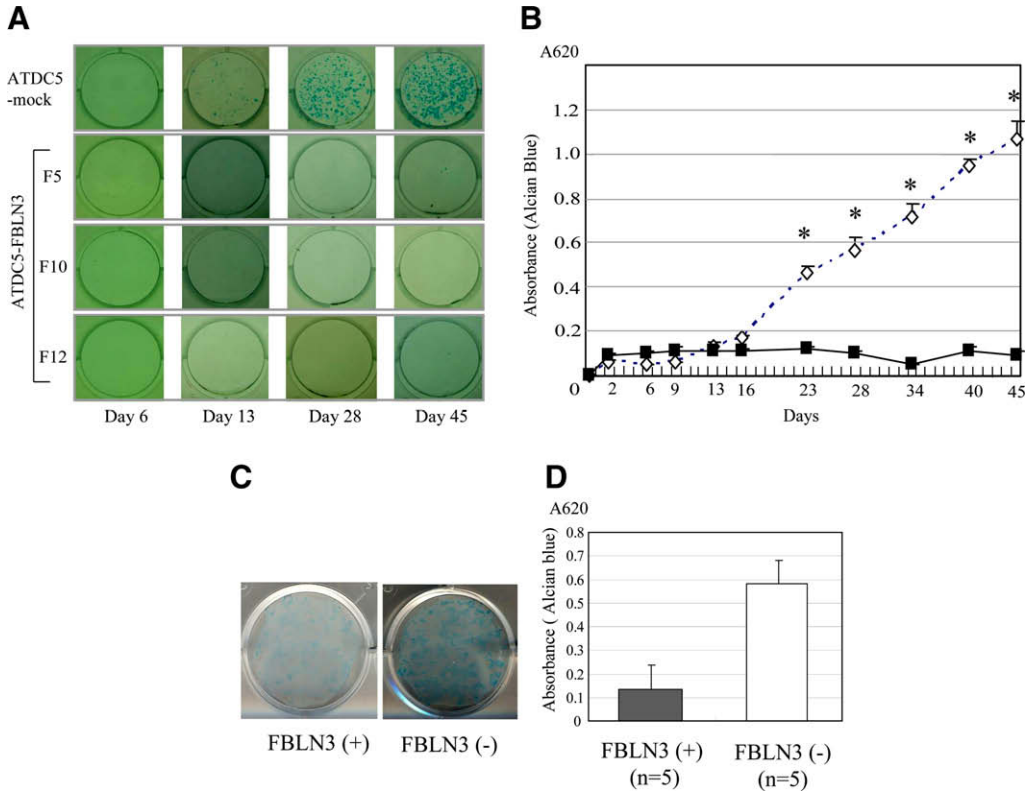

Fig. 2. The effect of fibulin-3 overexpression on cartilage nodule formation and production of proteoglycan. (A) ATDC5-mock underwent progressive differentiation in medium containing insulin, as evidenced by increased formation of cartilage aggregates and increased Alcian blue staining beginning at day 13. In contrast, ATDC5-FBLN3 was not stained by Alcian blue, nor were there any cartilage aggregates formed. (B) The quantitative determination of the extent of proteoglycan production showed the overexpression of fibulin-3 to result in the complete suppression in Alcian blue staining during the induction of chondrogenesis (open diamond: ATDC5-mock, closed square: ATDC5-FBLN3). Independent three clones were examined. $^{*}p < 0.0001$. (C, D) The effect of exogenous fibulin-3 protein on ATDC5 differentiation was examined by addition of the recombinant protein at a concentration of 1 μg/ml to the medium. The cartilage aggregates formation and production of proteoglycan were significantly suppressed at day 21 after induction of differentiation (C, D).

during the late stage of the differentiation. On the other hand, the expression levels of Sox6 in ATDC5-FBLN3 were completely suppressed during chondrogenic differentiation (Fig. 4C). These results demonstrated the overexpression of fibulin-3 reduce the expression of *Sox5* and *Sox6*, while maintaining the expression of *Sox9*.

Discussion

There have recently been several reports which describe the presence of fibulin-3 protein in cartilaginous tissue in the mouse embryo. Immunohistochemical analysis demonstrated fibulin-3 to be localized in the perichondrium in E15 mouse embryos [14]. An *in situ* analysis showed fibulin-3 to be found in the condensing mesenchymes, thus giving rise to bone and cartilage as well as in developing bone structures of the cranial and the axial skeleton [22]. During mouse embryogenesis, a very prominent expression of fibulin-2 was shown to be seen during the early stages of chondrogenesis in cartilaginous tissue [23]. These results suggest that fibulin-3 may thus play a possible role in organizing bone and cartilage development. However there have been no direct experimental studies which show the effect of fibulin-3 on chondrocyte differentiation.

In the present study, we demonstrated that fibulin-3 is a negative regulator of chondrocyte differentiation: the overexpression of fibulin-3 suppressed chondrocyte differentiation by inhibition of cartilage nodule formation, proteoglycan production, and matrix gene expression, and the overexpression of fibulin-3 selectively maintained the expression of *Sox9* but suppressed the expression of *Sox5* and *Sox6*. These results provide functional evidence showing that fibulin-3 is an important negative regulator in chondrocyte differentiation.

It was found that the cell morphology of ATDC5-FBLN3 was drastically changed. The ATDC5-FBLN3 cells were spindle-shape cells although proliferating ATDC5-mock cells were usually plump cells. The actin cytoskeleton is a primary determinant of cell shape. Recently, several interconnected transduction pathways and a number of signaling molecules which control actin cytoskeleton reorganization have been identified. In adherent cells, cell–matrix adhesions connect the extracellular matrix with the actin cytoskeleton and transmit forces in both directions. Interestingly, overexpression of the fibulin-3 in olfactory ensheathing cell (OEC) induced the more spindle-shape morphology with extremely long processes, compared to the control cultures of OEC. Furthermore, these effects of fibulin-3 were only present on matrigel, laminin, and collagen substrates but not PLL-coated cover slip [26]. These results suggest that possible interactions of fibulin-3 with these substrate components are influencing the cell morphology.

It was also found that the cell proliferation in ATDC5-FBLN3 was accelerated compared to that in ATDC5-mock. There are a few reports that indicate the effect of fibulin-3 on cell proliferation, but the findings remain controversial. The first evidence was that fibulin-3 is up-regulated in the senescent and Werner syndrome fibroblast, as well as in quiescent young fibroblasts. However paradoxically, a microinjection of the fibulin-3 gene into a Werner syndrome fibroblast consistently stimulated rather than inhibited DNA synthesis [20]. The effect of fibulin-3 on the cell proliferation may occur in a context specific manner and depends on the cell type.

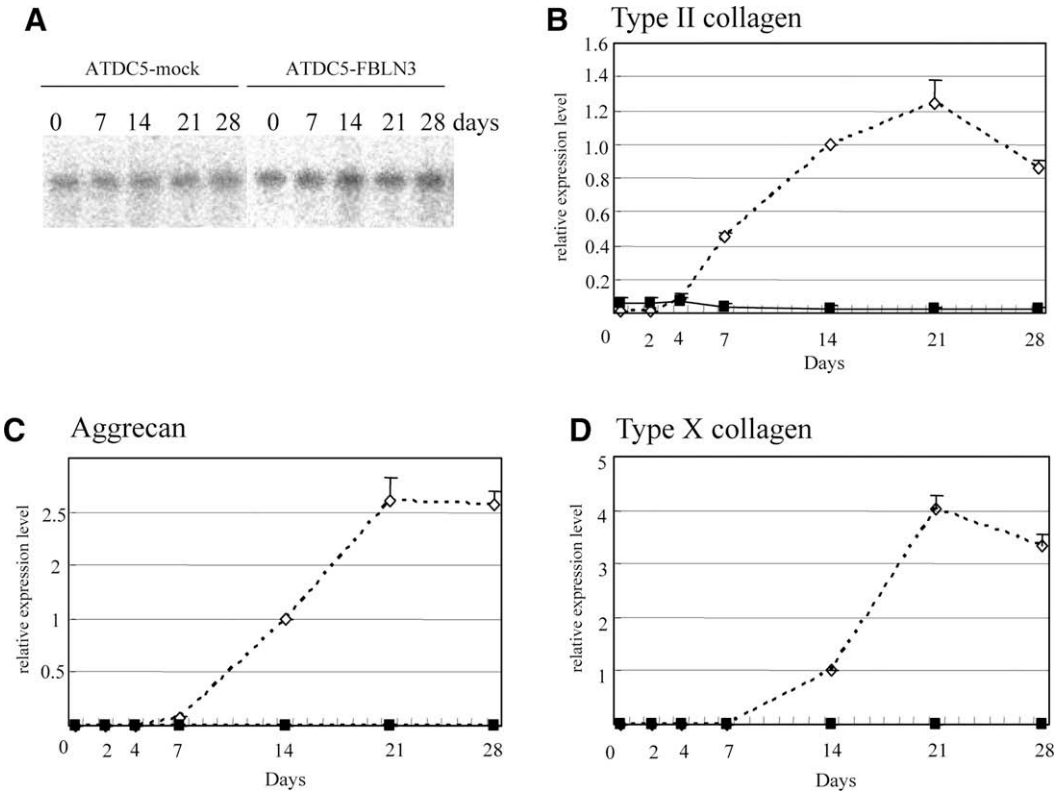

Fig. 3. The effect of fibulin-3 on chondrocyte differentiation at the molecular level. (A) The β-actin expression level detected with Northern blot analysis was not affected by either fibulin-3 or by chondrocyte differentiation. Thus, the β-actin was chosen as reference gene for the multiplex real-time PCR analysis. Overexpression of fibulin-3 completely prevented the induction of (B) type II collagen (C) aggrecan and (D) type X collagen genes, while ATDC5-mock highly expressed these three genes after induction of differentiation (open diamond: ATDC5-mock, closed square: ATDC5-FBLN3). Independent three clones were examined.

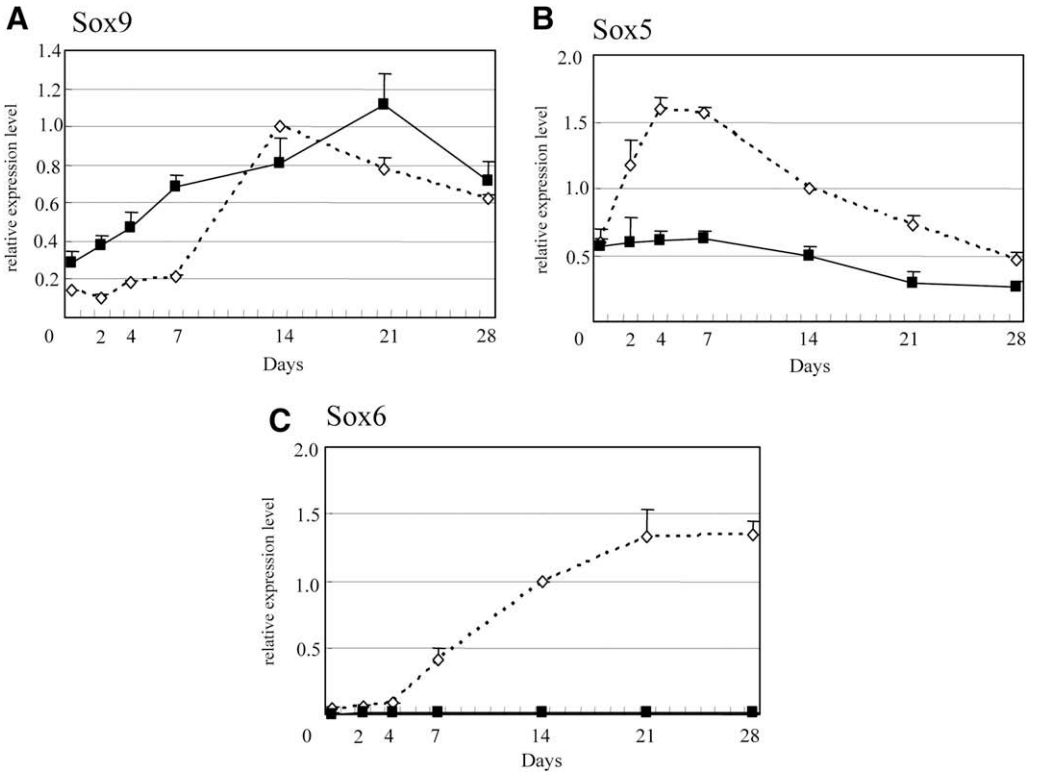

Fig. 4. The effect of the overexpression of fibulin-3 on expression of Sox9, Sox5, and Sox6 genes in ATDC5 cells. The overexpression of fibulin-3 maintained the expression of (A) Sox9, while reducing the expression of (B) Sox5 and (C) Sox6 after induction of differentiation (open diamond: ATDC5-mock, closed square: ATDC5-FBLN3). Independent three clones were examined.

The process of chondrogenesis occurs in stages beginning with mesenchymal cell recruitment and migration, proliferation and condensation, which are regulated by mesenchymal–epithelial cell interactions [4]. The aggregation of chondroprogenitor mesenchymal cells into precartilage condensations represents one of the earliest events in chondrogenesis [4]. For the condensation of chondroprogenitor mesenchymal cells, cell–cell adhesion through molecules such as neural cadherin (N-cadherin), neural cell adhesion molecule (N-CAM), and tenascin C are required [4]. In the present study, it appeared that the overexpression of fibulin-3 protein led to the suppression of aggregation of chondroprogenitor mesenchymal cells, and was followed by the reduced synthesis of proteoglycan despite induction of differentiation. Therefore, the overexpression of fibulin-3 in ATDC5 cells may alter the function of N-cadherin, N-CAM, and tenascin C.

Among several intracellular factors, Sox9 is a key molecule in early chondrogenesis. Sox9 is required for mesenchymal condensation and subsequent chondroblast differentiation. Sox9 forms heterodimers with two additional Sox family members, Sox5 and Sox6, and activates the transcription of Col2α1 gene by a binding enhancer [25]. Our study demonstrated that the overexpression of fibulin-3 selectively maintained the expression of Sox9 but suppressed the expression of Sox5 and Sox6. A reduction of Sox5 and Sox6 may contribute to the suppression of chondrocyte differentiation in ATDC5-FBLN3.

The mechanisms by which expression of fibulin-3 suppresses the expression of Sox5 and Sox6 are not yet clear. Recently, fibulin-3 protein has been demonstrated to interact with the tissue inhibitor of metalloproteinases-3 (TIMP-3). TIMP-3 is a matrix-bound inhibitor of matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase-10 (ADAM-10), ADAM-17, a disintegrin and metalloproteinase with thrombospondin motif-4 (ADAMTS-4), and ADAMTS-5 [27]. Furthermore, ADAMs regulate the proteolytic cleavage of transmembrane receptor protein: BMP receptor [28], TGF-β receptor [29], FGF receptor [30], Wnt receptor [31], EGF receptor [32], Notch [33], IGF receptor [34], N-CAM [35], and N-cadherin [36], all of which are well-known as positive or negative regulators of the chondrogenesis [2–6,11]. These results suggest that fibulin-3 may suppress the chondrogenic differentiation through TIMP-3 and ADAMs by modulating the cleavage of transmembrane proteins which regulate chondrogenic differentiation. Reduced differentiation signal from the transmembrane protein due to fibulin-3 may lead to the reduction of transcriptional activity of Sox5 and Sox6. Further investigation is therefore warranted.

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