

Activation of epidermal growth factor receptor gene is involved in transforming growth factor- β -mediated fibronectin expression in a chondrocyte progenitor cell line

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Abstract. Chondrocytes produce many types of ECM to maintain elasticity and plasticity in articular cartilage of revolute joints. Both transforming growth factor β (TGF- β) and bone morphogenetic proteins (BMPs) induce extracellular matrix proteins such as type II α 1 collagen and aggrecan during chondrogenic differentiation *in vitro*. However, differences in the matrix gene expression pattern by the stimulation of TGF- β s and BMPs remains unclear. In the present study, we created a customized PCR-based ECM array to investigate the pattern of ECM expression genes in the chondrocyte progenitor cell line ATDC5, that was stimulated by TGF- β s or BMPs. *Fibronectin* (*Fn*) expression was drastically induced after TGF- β stimulation, but not BMP-4. *Epidermal growth factor receptor* (*Egfr*) gene was also significantly activated in TGF- β 1-induced chondrogenic differentiation as compared to BMP-4-mediated differentiation. Furthermore, EGFR-knockdown assay of the cells showed decreasing *Fn* expression during TGF- β 1-induced chondrogenic differentiation. These data indicated that *Egfr* gene activation by TGF- β is involved in the differences in the expression of cellular matrix genes such as *Fn*, as compared to the expression pattern induced by BMPs.

Introduction

Cartilage absorbs shock and provides a smooth articulating surface for proper joint mobility. Articular cartilage is

comprised of chondrocytes and their surrounding hydrated proteoglycans such as aggrecan and collagen. Chondrocytes maintain cartilage homeostasis through a balance of the extracellular matrix (ECM) synthesis and the degradation, although they occupy <5% of the total tissue volume (1).

Previous studies have shown that several growth factors are implicated in chondrogenesis, such as fibroblast growth factors (2,3), insulin-like growth factor (4-6), TGF- β (7-9), and bone morphogenetic proteins (BMP) (3,10,11). The TGF- β family, in particular, is strongly associated with chondrogenic differentiation.

TGF- β s signal through heteromeric TGF- β s receptors composed of type I and type II serine/threonine kinases. The receptor associated Smads, Smad2 and 3, was phosphorylated by activated type I receptors after heteromeric receptor complex formation. The phosphorylated Smads associates with Smad4, translocate to the nucleus, and act as transcription factors. In contrast, BMPs transduce the signal via type I and type II BMP receptors complex. Then, Smad1, 5, and 8, the BMPs signal-associated Smads, are phosphorylated by the activated type I BMP receptor, and regulate gene expression with Smad4 (12). The TGF- β s also appear to signal through the MAPK pathway via the MAPKKK, TAK1 (TGF- β activated kinase), and its associated partner TAB1 (TAK1 binding protein) (13,14).

TGF- β is a multifunctional cytokine that is crucially involved in multiple processes throughout development, ECM synthesis, cell proliferation, differentiation, and tissue repair. Baker *et al* reported that, in cartilage, TGF- β 1 promotes cartilage matrix synthesis and induces the activity of tissue inhibitor of metalloproteinase-3 (TIMP-3), which inhibits matrix metalloproteinases, aggrecanases, and TNF- β -converting enzyme, all of which are implicated in articular cartilage degradation and joint inflammation (15). In contrast, BMPs and their receptors are required for chondrogenesis and are expressed throughout the growth plate and perichondrium (16,17). BMPs, but not TGF- β promote differentiation at a late stage, which are required for the induction of type X

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collagen, and leads to ossification (18,19). Though TGF- β s and BMPs have different characteristics, they both induce major cartilage matrix synthesis such as aggrecan and type IIa1 collagen through different pathways, and they are both important for chondrogenic regulation.

In the present study, we demonstrated that TGF- β , but not BMP-4, induces *Fibronectin* (*Fn*) expression during the chondrogenic differentiation of ATDC5 cells. Furthermore, expression of the EGFR ligands, *Tgf- α* and *Hb-egf*, and *Egf* receptor (*Egfr/ErbB1*) significantly increased during the TGF- β -induced chondrogenic differentiation. The induction of *Fn* gene by TGF- β stimulation was disturbed during chondrogenic differentiation in the EGFR-knocked-down ATDC5 cells. These data indicated that the EGFR signaling cascade is associated with FN synthesis during TGF- β -induced chondrogenic differentiation.

Materials and methods

Cell cultures. ATDC5 (RIKEN cell bank; Tsukuba, Japan) was purchased from the RIKEN cell bank. ATDC5 cells were maintained in a 1:1 mixture of DME and Ham's F-12 (DME/F12) medium (Wako, Osaka, Japan) containing 5% FBS (Nichirei Bioscience, Tokyo, Japan) and TS, which consisted of 10 μ g/ml human transferrin (T; Roche Diagnostics, Mannheim, Germany), and 3 \times 10⁻⁸ M sodium selenite (S; Sigma, St. Louis, MO, USA), as previously described (11,20,21). The medium was replaced every other day. In some experiments, ATDC5 cells were plated at a density of 6 \times 10⁴ cells per 35-mm dish and cultured in DME/F12 medium containing 5% FBS and TS. On day 3 after the cells were plated, the culture medium was replaced by DME/F12 medium containing 5% FBS, TS, and either 10 μ g/ml human insulin (I; Roche Diagnostics) or human recombinant BMP-4 (R&D Systems, Minneapolis, MN, USA) or human recombinant TGF- β 1 (R&D Systems). The medium and the ligands were replaced every other day.


Western blotting. The experiments were terminated by aspirating the culture medium and washing the cells three times with ice-cold PBS. Cell lysates were prepared in 0.5 M Tris lysis buffer (pH 6.8) containing 2% sodium dodecyl sulfate, 2-mercaptoethanol, and 5% glycerol and then subjected to sonication. They were stored at -80°C until use. For Western blot analysis, equal amounts of proteins were pooled from triplicate samples and separated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred to nitrocellulose membranes. Immunoblots were blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20 and then probed overnight with primary antibodies (1:1000 dilution). Western blotting was performed by using rabbit monoclonal antibodies against EGFR (Cell Signaling Technology, Danvers, MA, USA) and mouse monoclonal antibodies against β -actin (Sigma), followed by incubation with anti-rabbit IgG or anti-mouse IgG. The results were visualized by using the ECL Plus detection kit (Pharmacia) and quantified with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). Where indicated, the blots were stripped according to the ECL Plus protocol prior to re-probing.

Table I. Primer used for qRT-PCR.

Gene	Primer
<i>Collagen IIa1</i>	
Forward	5'-CAGGATGCCCGAAAATTAGGG-3'
Reverse	5'-ACCACGATCACCTCTGGGT-3'
<i>Aggrecan</i>	
Forward	5'-CCTGCTACTTCATCGACCCC-3'
Reverse	5'-AGATGCTGTTGACTCGAACCT-3'
<i>ErbB1</i>	
Forward	5'-GCCATCTGGGCCAAAGATACC-3'
Reverse	5'-GTCTTCGCATGAATAGCCAAT-3'
<i>ErbB2</i>	
Forward	5'-CCAAGGCACAAGTAACAGGCT-3'
Reverse	5'-CCACGGTGTGAGGGCAAT-3'
<i>ErbB3</i>	
Forward	5'-AAGTGACAGGCTATGTACTGGT-3'
Reverse	5'-GCTGGAGTTGGTATTGTAGTTCA-3'
<i>Tgfa</i>	
Forward	5'-CACTCTGGGTACGTGGGTG-3'
Reverse	5'-CACAGGTGATAATGAGGACAGC-3'
<i>Hb-egf</i>	
Forward	5'-TGCCGTCGGTGATGCTGAACT-3'
Reverse	5'-GGTTCAGATCTGTCCCTTCCAAGTC-3'
<i>Fibronectin</i>	
Forward	5'-TTCAAGTGTGATCCCCATGAAG-3'
Reverse	5'-CAGGTCTACGGCAGTTGTCA-3'
<i>Hprt 1</i>	
Forward	5'-TCAGTCAACGGGGGACATAAAA-3'
Reverse	5'-GGGGCTGTACTGCTTAACCAG-3'

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, La Jolla, CA, USA), and first-strand cDNA was synthesized from 1 μ g of RNA by using Superscript III (Invitrogen). The Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time RT-PCR was performed in a 25- μ l reaction volume containing 15 μ l of the SYBR Green qPCR Supermix Universal (Invitrogen), 1 μ l of the RT reaction mixture, and 200 nM primers. The primer sequences are listed in Table I. All primers generated PCR fragments with the correct sizes, and non-specific products were not generated; thus, the specificity of the real-time RT-PCR was confirmed (data not shown). The cycle threshold (Ct) value for each gene of interest was measured for each RT sample.

Lentiviral shRNA-mediated knockdown of EGFR in ATDC5 cells. To produce ATDC5 cells in which the expression of the *Egfr* gene is suppressed during long-term culture, a lentivirus-mediated RNA interference-knockdown system was applied

 SPANDIDOS PUBLICATIONS DC5 cells. An HIV-based self-inactivating lentiviral vector (CSII-CMV-MCS-IRES-Bsd) and two packaging vectors (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev) were obtained from Dr Hiroyuki Miyoshi (RIKEN BioResource Center, Japan) (22). A human H1-RNA pol III promoter (-217 to +1) DNA fragment was amplified by polymerase chain reaction. To drive the expression of a small hairpin RNA shRNA, the H1 promoter was into the U3 region of the 3'-long terminal repeat (LTR) in the original lentiviral vector, as described previously (23), in order to drive the expression of a small hairpin RNA (shRNA). In the modified shRNA expression vector, termed CSII-CMV-IRES-Bsd-H1, *Bam*HI and *Eco*RI sites were introduced into the 3' end of the H1 promoter, allowing the insertion of the shRNA template. The original algorithm (B-Algo™) from B-Bridge International (Tokyo, Japan) was used to design specific shRNA target sequences. The target sequence of the mouse *Egfr* gene for knockdown was 5'-GGGAAATGCTCTTTATGAA-3', corresponding to the 324-342 nucleotide positions of the mouse *Egfr* coding sequence (GenBank accession no. NM_207655). The targeting sequence for EGFP-shRNA, (5'-CTACAACAGCCACAACGTC-3') was selected as a negative control. To construct the shRNA expression cassette, two complementary oligonucleotides were synthesized, annealed, and inserted between the *Bam*HI and *Eco*RI sites just downstream of the H1 promoter. The shRNA cassette features a TTCAAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTT terminator at the 3'-end as described previously (24). The lentiviral vector also carries a blasticidin-resistant gene as a transduction selection marker. Lentiviral particles were generated by using a standard transfection procedure. After transduction of the transgenes, a pool of blasticidin-resistant ATDC5 was used to analyze the gene expression downstream of the EGFR-dependent signal during chondrogenic differentiation.

RT² Profiler Customized PCR array system. The expression levels of 42 chondrogenic-related mouse genes were examined by using the customized RT² Profiler PCR array (SuperArray Bioscience, Frederick, MD, USA). The TRIzol reagent (Invitrogen) and Superscript III (Invitrogen) were used to isolate total RNA from the wild-type ATDC5 cells and ATDC5 cells that were infected with shRNA lentivirus vector against *Gfp* (sh*Gfp*/ATDC5) or *Egfr* (sh*Egfr*/ATDC5). cDNA was synthesized from 1 μg of RNA by using a SuperScript RT II enzyme (Invitrogen). The Applied Biosystems 7300 real-time PCR system and RT² Profiler PCR array system were used to perform PCR, according to the manufacturer's instructions. The mRNA expression level of each gene in sh*Egfr*/ATDC5 was normalized against the housekeeping gene expression (*Gusb*, *Hprt1*, *Hspcb*, *Gapdh*, and *Actb*), and then compared with the data obtained from sh*Gfp*/ATDC5. Individual RNA samples from the cells in each group were subjected to quantitative RT-PCR to confirm the results. The primers used for the RT² Profiler Customized PCR array are listed in Table II.

Statistical analysis. The results are presented as mean ± SE. The two groups were compared by using the Student's t-test.

Table II. Primers used for RT² profiler customized PCR array system.

<i>CollagenIa1</i>	<i>Coll1a1</i>
<i>CollagenIIa1</i>	<i>Col2a1</i>
<i>CollagenIIIa1</i>	<i>Col3a1</i>
<i>CollagenIVa2</i>	<i>Col4a2</i>
<i>CollagenVa1</i>	<i>Col5a1</i>
<i>CollagenVIIa1</i>	<i>Col7a1</i>
<i>CollagenIXa1</i>	<i>Col9a1</i>
<i>CollagenXa1</i>	<i>Col10a1</i>
<i>CollagenXIa1</i>	<i>Col11a1</i>
<i>CollagenXIIa1</i>	<i>Col12a1</i>
<i>CollagenXVIIa1</i>	<i>Col17a1</i>
<i>CollagenXVIIIa1</i>	<i>Col18a1</i>
<i>Aggrecan</i>	<i>Acan</i>
<i>Biglycan</i>	<i>Bgn</i>
<i>Brevican</i>	<i>Bcan</i>
<i>Chondroadherin</i>	<i>Chad</i>
<i>Colin</i>	<i>Corin</i>
<i>Decolin</i>	<i>Dcn</i>
<i>Fibronectin</i>	<i>Fn</i>
<i>Filamin α</i>	<i>Flna</i>
<i>Filamin β</i>	<i>Flnb</i>
<i>Filamin C, γ</i>	<i>Flnc</i>
<i>Glypican1</i>	<i>Gpc1</i>
<i>Glypican2</i>	<i>Gpc2</i>
<i>Glypican3</i>	<i>Gpc3</i>
<i>Glypican3</i>	<i>Gpc3</i>
<i>Glypican4</i>	<i>Gpc4</i>
<i>Glypican5</i>	<i>Gpc5</i>
<i>Glypican6</i>	<i>Gpc6</i>
<i>Gelsolin</i>	<i>Gsn</i>
<i>Necdin</i>	<i>Ndn</i>
<i>Osteoglycan</i>	<i>Ogn</i>
<i>Syndecan1</i>	<i>Sdc1</i>
<i>Syndecan2</i>	<i>Sdc2</i>
<i>Syndecan3</i>	<i>Sdc3</i>
<i>Syndecan4</i>	<i>Sdc4</i>
<i>Chondroitin sulfate synthase 1</i>	<i>Chsy1</i>
<i>Chondroitin sulfate synthase 3</i>	<i>Chsy3</i>
<i>Hyaluronan synthase1</i>	<i>Has1</i>
<i>Hyaluronan synthase 2</i>	<i>Has2</i>
<i>Hyaluronan synthase 3</i>	<i>Has3</i>
<i>Versican</i>	<i>Vcan1</i>
<i>Elastin</i>	<i>Eln</i>

Analysis of variance with Bonferroni's post-hoc test was used for multiple comparisons. Statistical significance was established at $p < 0.05$.

Results

Chondrocyte-related ECM gene expression after treatment with TGF-β1 and BMP-4. We analyzed the gene expression

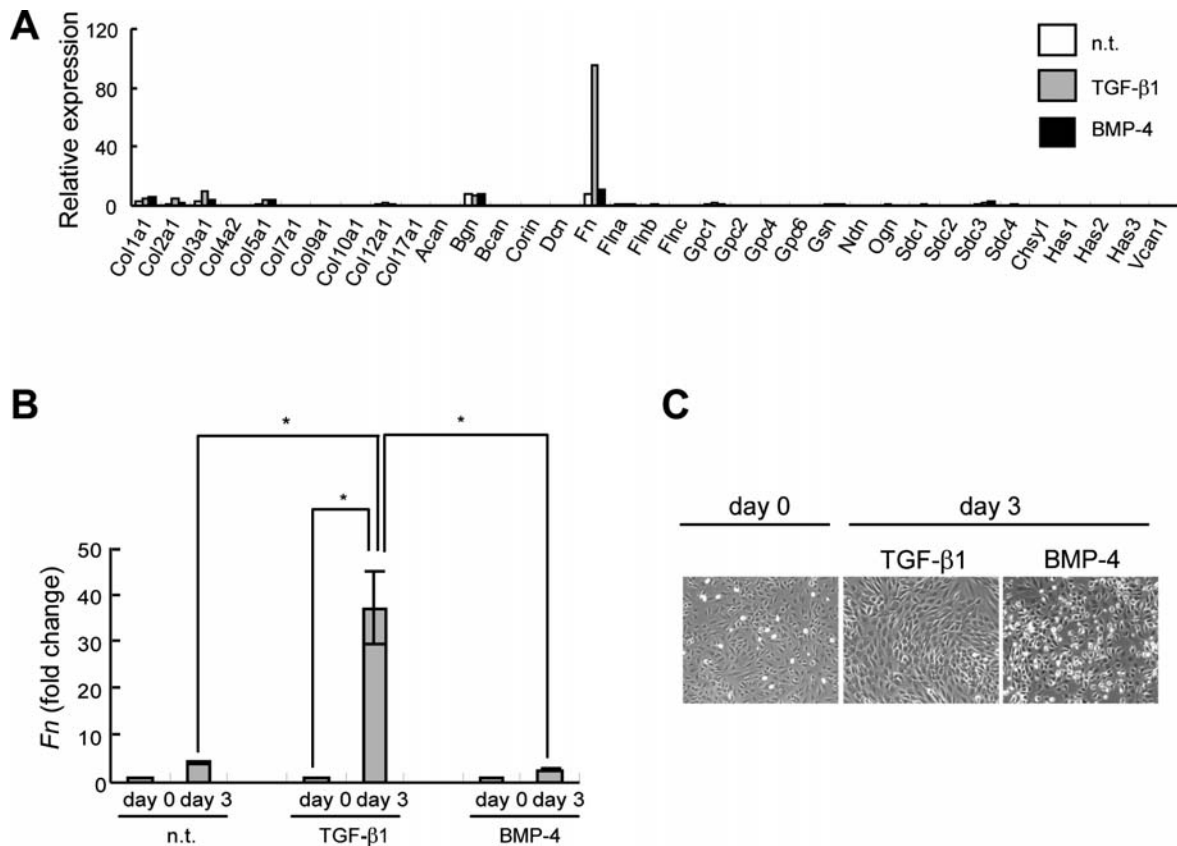


Figure 1. Comparison between chondrocyte-related genes by treatment with TGF- β 1 and BMP-4. The expression of chondrocyte-related genes in ATDC5 cells were assessed by qRT-PCR in ATDC5 cells. (A) Expression levels of chondrocyte-related ECM genes after treatment with TGF- β 1 or BMP-4 for 3 days. Expression values shown are relative to the average value of housekeeping genes (*Gusb*, *Hprt1*, *Hspcb*, *Gapdh*, and *Actb*). (B) The expression of *Fibronectin* during chondrogenic differentiation induced by TGF- β 1 or BMP-4 for 3 days. The results are normalized to *Hprt1* and presented as a fold change relative to the expression of day 0. (C) Morphological findings of ATDC5 cells by treatment with TGF- β 1 or BMP-4 for 3 days. n.t., no treatment.

profiles of chondrocyte-related ECM genes during chondrogenic differentiation of ATDC5 cells treated with TGF- β 1 or BMP-4. We performed qRT-PCR to analyze the mRNA expression levels of the chondrogenic-related genes, and we assessed the expression of chondrocyte-related ECM genes by using an RT² Profiler PCR array system customized for profiling ECM genes. We compared the expression levels of genes in 3-day-differentiated cells treated with TGF- β 1 or BMP-4. *Fibronectin* (*Fn*) was greatly up-regulated in TGF- β 1-treated cells, as compared to untreated cells or BMP-4-treated cells (Fig. 1A). The significantly higher expression levels of *Fn* in TGF- β 1-treated cells was confirmed by qRT-PCR with another specific primer set for the *Fn* gene (Fig. 1B). TGF- β 1-treated ATDC5 cells adopted, a spindle-shaped phenotype, while BMP-4-treated cells displayed a round phenotype on day 3 after differentiation (Fig. 1C).

TGF- β 1, but not BMP-4, strongly up-regulates the gene expression of EGFR in ATDC5 cells. In mesangial cells from the kidney, TGF- β treatment stimulates the processing and release of HB-EGF which in turn transactivates the EGFR-mediated ERK and p38 MAPK cascades that regulate *Fn* gene transcription (25). Therefore, we examined whether or not the expression of the *ErbB* gene family, including *Egfr*, is dependent on TGF- β 1 or BMP-4 signal during chondrogenic differentiation. We performed qRT-PCR to analyze the

mRNA expression levels of *Egfr*, and *ErbB2*, *ErbB3* and *ErbB4* in ATDC5 cells treated with TGF- β 1 or BMP-4. TGF- β , but not BMP-4, strongly up-regulated the gene expression of *Egfr* (Fig. 2A). The up-regulation of *ErbB2* and *B3* was significantly less than the up-regulation of *Egfr* in TGF- β 1-treated cells. *ErbB4* was not detected after stimulation by either ligand (data not shown).

To determine if the activation of the *Egfr* gene is dependent on the concentration of TGF- β 1, ATDC5 cells were stimulated with four different concentrations of TGF- β 1 (0, 0.1, 1, and 10 ng/ml) for 3 days. We found that *Egfr*, along with the major chondrocyte-specific ECM genes such as *CollagenIIa1* (*Colla1*) and *aggrecan* (*Acan*), was up-regulated in a TGF- β 1 dose-dependent manner (Fig. 2B). Western blot analysis showed that the EGFR protein level was also induced by TGF- β 1 (Fig. 2C). We also analyzed the expression of EGFR ligands, *TGF- α* and *Hb-egf*, after treatment with TGF- β 1 and BMP-4 during chondrogenic differentiation. *TGF- α* and *Hb-egf* were more strongly expressed during induction with TGF- β 1 than with BMP-4 (Fig. 2D). Thus, these findings indicate that TGF- β 1-induced chondrogenic cells could activate their own EGFR signaling cascade by the autocrine secretion of TGF- α and HB-EGF.

A knockdown assay by shRNA against Egfr. To investigate whether EGFR affects chondrogenic differentiation with

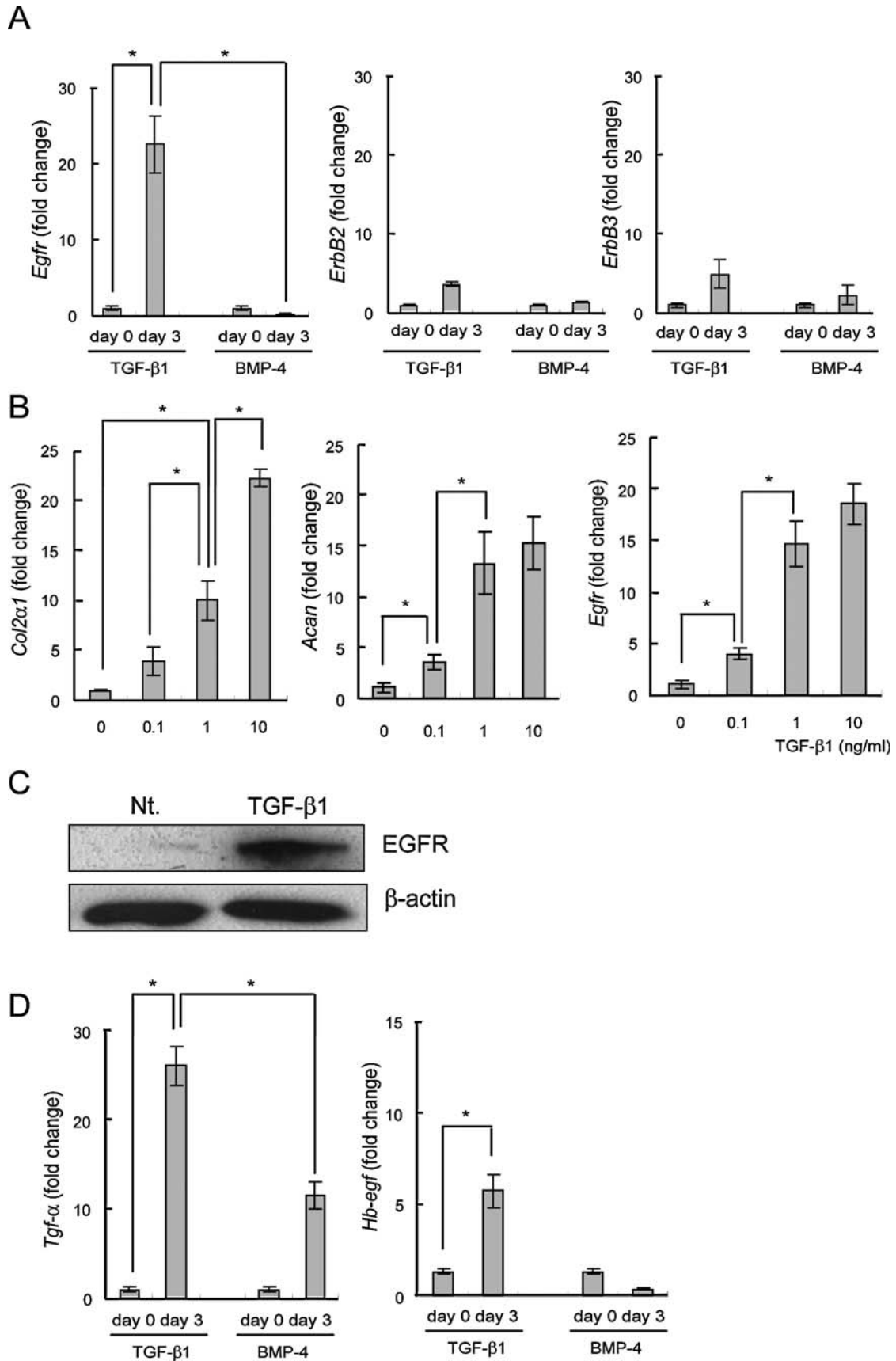


Figure 2. *Egfr* was induced during chondrogenic differentiation by treatment with TGF-β1 but not BMP-4. (A) ATDC5 differentiated into chondrocytes by TGF-β1 (10 ng/ml) or BMP-4 (200 ng/ml) treatment in ATDC5 cells. The gene expression levels of *Egfr* and *ErbB2*, *3*, and *4* were detected by quantitative real-time RT-PCR (qRT-PCR). *ErbB4* was not detected by qRT-PCR. The results are normalized to *Hprt1* and presented as a fold change relative to the expression of day 0. (B) To examine dose effects, four concentrations of TGF-β1 (0, 0.1, 1, and 10 ng/ml) were added to ATDC5 cells. The mRNA expression levels of *CollagenIa1* (*Colla1*), *Aggrecan* (*Acan*), and *Egfr* were detected by qRT-PCR. (C) Western blots of ATDC5 cells were probed for EGFR. Antibodies against β-actin were used as a loading control. (D) The gene expression levels of EGF ligands (*Tgf-α* and *Hb-egf*) during chondrogenic differentiation by the treatment with TGF-β1 or with BMP-4 were detected by qRT-PCR. n.t., no treatment.

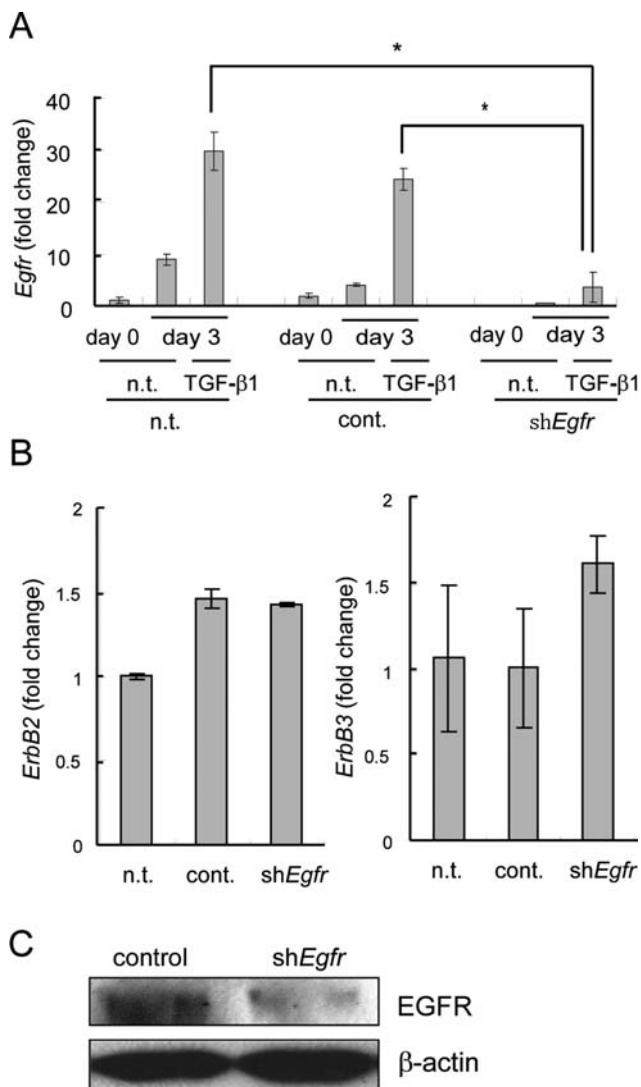


Figure 3. A knockdown assay by shRNA against *Egfr*. A knockdown assay of *Egfr* mRNA is performed to see the function of EGFR during chondrogenesis. (A) Gene expression of *Egfr* was compared between sh*Egfr*/ATDC5 and sh*Gfp*/ATDC5 using qRT-PCR. The results are normalized to *Hprt1* and presented as a fold change relative to the expression of day 0. The expression of *Egfr* in a comparison between sh*Egfr*/ATDC5 and sh*Gfp*/ATDC5 on day 3 of TGF- β 1-induced differentiation. (B) Gene expression levels of *ErbB2* and *ErbB3* were compared between sh*Egfr*/ATDC5 and sh*Gfp*/ATDC5. (C) Western blots of ATDC5 were probed for EGFR. Antibodies against β -actin were used as a loading control. n.t., no treatment.

TGF- β 1 treatment, we performed an *Egfr* knockdown assay with lentivirus vectors carrying two shRNAs targeting *Egfr* (lenti-sh*Egfr*) or green fluorescent protein (lenti-sh*Gfp*), which was used as a control. We infected ATDC5 cells with each of lenti-shRNA and assessed the *Egfr* suppression by RT-PCR and Western blotting. *Egfr* gene expression in ATDC5 cells was more effectively down-regulated by infection with lenti-sh*Egfr* than by infection with lenti-sh*Gfp* (Fig. 3A). In contrast, the gene expression levels of *ErbB2* and *ErbB3* were not down-regulated in sh*Egfr*/ATDC5 (Fig. 3B). Western blotting revealed that the protein level of EGFR was significantly decreased in sh*Egfr*/ATDC5 (Fig. 3C).

EGFR knockdown leads to the down-regulation of Fn during chondrogenic differentiation induced by TGF- β 1 treatment.

We used the RT² Profiler Customized PCR array system to examine the effect of EGFR knockdown on the expression of ECM and matrix metalloprotease genes in TGF- β 1-treated parental ATDC5 cells, sh*Egfr*/ATDC5 or sh*Gfp*/ATDC5. sh*Egfr*/ATDC5 had a lower expression of four ECM genes, *CollagenXa1* (*ColXa1*), *CollagenXVIIa1* (*ColXVIIa1*), *Acan*, and *Fn*, in ECM genes as compared to parental ATDC5 cells and sh*Gfp*/ATDC5 (Fig. 4A). The expression level of *Fn* was significantly lower in sh*Egfr*/ATDC5 than in sh*Gfp*/ATDC5 in a time-dependent manner with TGF- β 1 treatment (Fig. 4B). These data suggest that the TGF- β signaling could regulate certain ECM genes, such as FN via EGFR signaling during chondrogenesis.

Discussion

In our study, we focused on the differences in the pattern of ECM genes induced by TGF- β 1 and BMP-4. The customized RT-PCR array analysis showed that there was a definite difference in *Fn* gene expression. FN is a large dimeric glycoprotein that functions primarily as a connecting molecule in the ECM by both cell-matrix and matrix-matrix interactions. FN is involved in cell adhesion, migration, and ECM accumulation in processes such as embryogenesis, wound healing, blood coagulation, host defense, and metastasis. Various isoforms are produced from a single FN gene, and full length recombinant FN reduces spreading and promotes the condensation and chondrogenesis of limb mesenchymal cells (26). FN expression is increased in areas of cellular condensation that are decreased as cyto-differentiation proceeds (27,28). According to recent studies, isoform switching of *Fn* mRNA is important for chondrogenic differentiation (29-31) and that TGF- β 1 treatment during chondrogenic differentiation of ATDC5 cells increases the expression of FN isoforms that lack the extra domain A and B during chondrogenic differentiation of ATDC5 cells (32,33). Regulation of both quantity and quality of FN by TGF- β 1 could be involved in the early stage of chondrogenesis.

It was previously reported that HB-EGF and EGFR transactivation are involved in TGF- β -mediated FN expression in mesangial cells, however, the new protein synthesis induced by TGF- β did not participate in induction of FN expression (25). In contrast, our study found that EGFR expression in ATDC5 cells is induced by TGF- β treatment and that this expression is crucial for *Fn* gene expression. We believe that differences in the cell types are responsible for the different results of these two studies. The study that *Fn* mRNA is stabilized by TGF- β -dependent protein synthesis in cardiac fibroblasts supports this speculation (34).

Our study also showed that the knockdown of EGFR expression in the chondrogenic ATDC5 cells reduces the expression levels of several ECM genes such as *ColXa1*, *ColXVIIa1*, and *Acan* as well as *Fn* (Fig. 4A). It has been even reported that expression of TGF- α , a member of the EGFR ligand family, in mouse lungs caused extensive pulmonary fibrosis without inflammation (35). A recent study indicated that EGF receptor tyrosine kinase inhibitors diminish TGF- β -induced pulmonary fibrosis (36). Amphiregulin, another member of the EGFR ligand family, is involved in

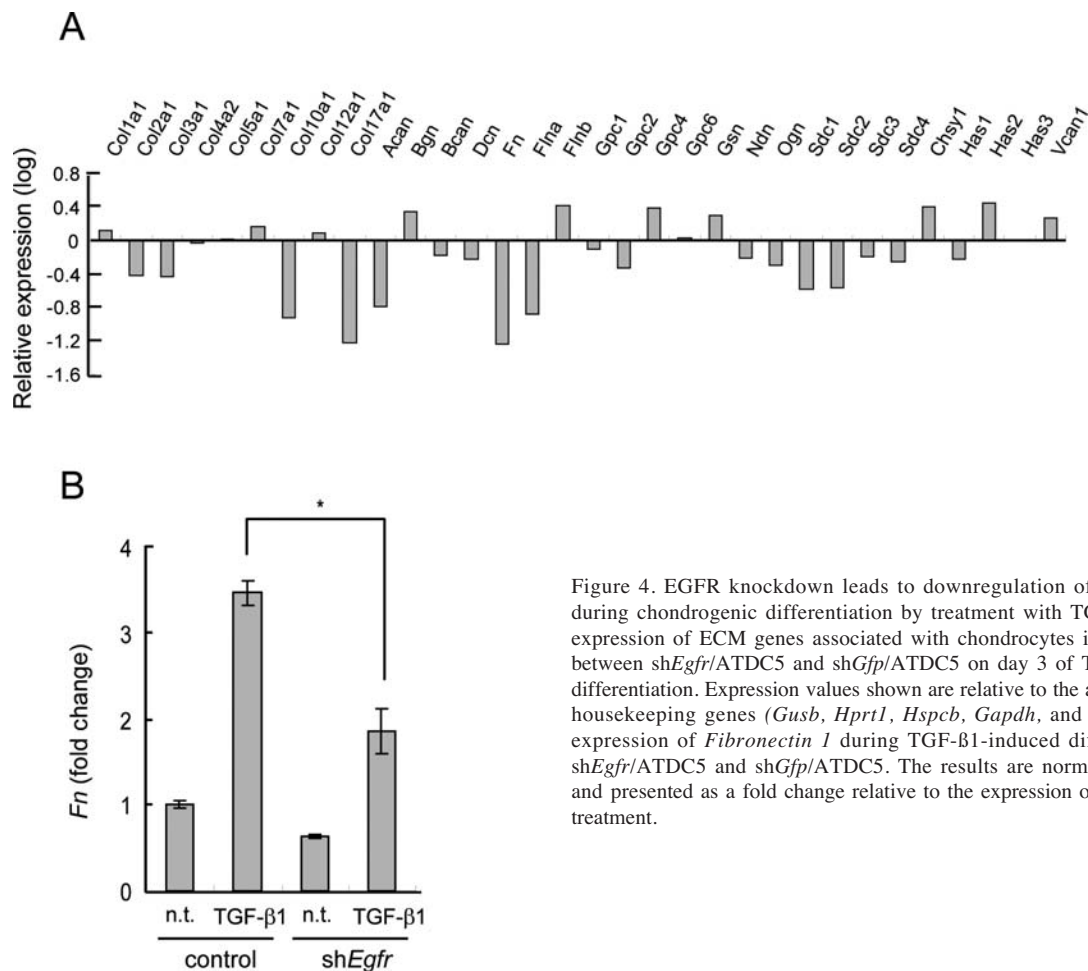


Figure 4. EGFR knockdown leads to downregulation of *Fibronectin 1* during chondrogenic differentiation by treatment with TGF-β1. (A) The expression of ECM genes associated with chondrocytes in a comparison between *shEgfr*/ATDC5 and *shGfp*/ATDC5 on day 3 of TGF-β1-induced differentiation. Expression values shown are relative to the average value of housekeeping genes (*Gusb*, *Hprt1*, *Hspcb*, *Gapdh*, and *Actb*). (B) The expression of *Fibronectin 1* during TGF-β1-induced differentiation of *shEgfr*/ATDC5 and *shGfp*/ATDC5. The results are normalized to *Hprt1* and presented as a fold change relative to the expression of day 0. n.t., no treatment.

the development of mouse liver fibrosis (37). Other studies showed that TGF-α, HB-EGF, or EGFR plays a critical part in pancreatic fibrosis formation (38-40). These findings suggest that EGFR signaling is important in extracellular matrix production as with cell growth and survival.

In conclusion, the expression of EGFR and its ligands is required for the TGF-β-mediated expression of ECM genes such as *Fn* during the chondrogenic differentiation of ATDC5 cells. BMP-4, which induces late-stage chondrocyte differentiation, did not induce the *Egfr* gene products that regulate ECM genes. We concluded that EGFR signaling in chondrocytes might prevent mineralization and subsequent ossification such that chondrocytes continue to produce articular cartilage-specific ECM.

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References

- Kuettner KE, Aydelotte MB and Thonar EJ: Articular cartilage matrix and structure: a minireview. *J Rheumatol* 27: S46-S48, 1991.
- Karsenty G: Genetics of skeletogenesis. *Dev Genet* 22: 301-313, 1998.
- Niswander L and Martin GR: FGF-4 and BMP-2 have opposite effects on limb growth. *Nature* 361: 68-71, 1993.
- Takigawa M, Okawa T, Pan H, *et al*: Insulin-like growth factors I and II are autocrine factors in stimulating proteoglycan synthesis, a marker of differentiated chondrocytes, acting through their respective receptors on a clonal human chondrosarcoma-derived chondrocyte cell line, HCS-2/8. *Endocrinology* 138: 4390-4400, 1997.
- Avruch J: Insulin signal transduction through protein kinase cascades. *Mol Cell Biochem* 182: 31-48, 1998.
- White MF and Yenush L: The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. *Curr Top Microbiol Immunol* 228: 179-208, 1998.
- Denker AE, Nicoll SB and Tuan RS: Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-beta 1. *Differentiation* 59: 25-34, 1995.
- Chimal-Monroy J and Díaz de León L: Differential effects of transforming growth factors beta 1, beta 2, beta 3 and beta 5 on chondrogenesis in mouse limb bud mesenchymal cells. *Int J Dev Biol* 41: 91-102, 1997.
- Johnstone B, Hering TM, Caplan AI, Goldberg VM and Yoo JU: In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 238: 265-272, 1998.
- Denker AE, Haas AR, Nicoll SB and Tuan RS: Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. *Differentiation* 64: 67-76, 1999.
- Shukunami C, Ohta Y, Sakuda M and Hiraki Y: Sequential progression of the differentiation program by bone morphogenetic protein-2 in chondrogenic cell line ATDC5. *Exp Cell Res* 241: 1-11, 1998.
- Kawabata M, Imamura T, Inoue H, *et al*: Intracellular signaling of the TGF-β superfamily by Smad proteins. *Ann NY Acad Sci* 886: 73-82, 1999.

13. Yamaguchi K, Shirakabe K, Shibuya H, *et al.*: Identification of a member of the MAPKKK family as a potential mediator of TGF- β signal transduction. *Science* 270: 2008-2011, 1995.
14. Shibuya H, Yamaguchi K, Shirakabe K, *et al.*: TAB1: an activator of the TAK1 MAPKKK in TGF- β signal transduction. *Science* 272: 1179-1182, 1996.
15. Baker AH, Edwards DR and Murphy G: Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci* 115: 3719-3727, 2002.
16. Retting KN, Song B, Yoon BS and Lyons KM: BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. *Development* 136: 1093-1104, 2009.
17. Yoon BS and Lyons KM: Multiple functions of BMPs in chondrogenesis. *J Cell Biochem* 93: 93-103, 2004.
18. Minina E, Schneider S, Rosowski M, Lauster R and Vortkamp A: Expression of Fgf and TGF- β signaling related genes during embryonic endochondral ossification. *Gene Expr Patterns* 6: 102-109, 2005.
19. Fujii M, Takeda K, Imamura T, *et al.*: Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. *Mol Biol Cell* 10: 3801-3813, 1999.
20. Shukunami C, Akiyama H, Nakamura T and Hiraki Y: Requirement of autocrine signaling by bone morphogenetic protein-4 for chondrogenic differentiation of ATDC5 cells. *FEBS Lett* 469: 83-87, 2000.
21. Watanabe H, de Caestecker MP and Yamada Y: Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor- β -induced aggrecan gene expression in chondrogenic ATDC5 cells. *J Biol Chem* 276: 14466-14473, 2001.
22. Miyoshi H, Blomer U, Takahashi M, Gage FH and Verma IM: Development of a self-inactivating lentivirus vector. *J Virol* 72: 8150-8157, 1998.
23. Wiznerowicz M and Tronto D: Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 77: 8957-8961, 2003.
24. Qin XF, An DS, Chen IS and Baltimore D: Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci* 100: 183-188, 2003.
25. Uchiyama-Tanaka Y, Matsubara H, *et al.*: Involvement of HB-EGF and EGF receptor transactivation in TGF- β -mediated fibronectin expression in mesangial cells. *Kidney Int* 62: 799-808, 2002.
26. White DG, Hershey HP, Moss JJ, Daniels H, Tuan RS and Bennett VD: Functional analysis of fibronectin isoforms in chondrogenesis: Full-length recombinant mesenchymal fibronectin reduces spreading and promotes condensation and chondrogenesis of limb mesenchymal cells. *Differentiation* 71: 251-261, 2003.
27. Dessau W, von der Mark H, von der Mark K and Fischer S: Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. *J Embryol Exp Morphol* 57: 51-60, 1980.
28. Kulyk WM, Upholt WB and Kosher RA: Fibronectin gene expression during limb cartilage differentiation. *Development* 106: 449-455, 1989.
29. Gehris AL, Oberlender SA, Shepley KJ, Tuan RS and Bennett VD: Fibronectin mRNA alternative splicing is temporally and spatially regulated during chondrogenesis in vivo and in vitro. *Dev Dyn* 206: 219-230, 1996.
30. Gehris AL, Stringa E, Spina J, Desmond ME, Tuan RS and Bennett VD: The region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. *Dev Biol* 190: 191-205, 1997.
31. Bennett VD, Pallante KM and Adams SL: The splicing pattern of fibronectin mRNA changes during chondrogenesis resulting in an unusual form of the mRNA in cartilage. *J Biol Chem* 266: 5918-5924, 1991.
32. Han F, Adams CS, Tao Z, *et al.*: Transforming growth factor- β 1 (TGF- β 1) regulates ATDC5 chondrogenic differentiation and fibronectin isoform expression. *J Cell Biochem* 95: 750-762, 2005.
33. Han F, Gilbert JR, Harrison G, *et al.*: Transforming growth factor-beta1 regulates fibronectin isoform expression and splicing factor SRp40 expression during ATDC5 chondrogenic maturation. *Exp Cell Res* 313: 1518-1532, 2007.
34. Moriguchi Y, Matsubara H, Mori Y, *et al.*: Angiotensin II-induced transactivation of epidermal growth factor receptor regulates fibronectin and transforming growth factor-beta synthesis via transcriptional and posttranscriptional mechanisms. *Circ Res* 84: 1073-1084, 1999.
35. Korfhagen TR, Swantz RJ, Wert SE, *et al.*: Respiratory epithelial cell expression of human transforming growth factor-alpha induces lung fibrosis in transgenic mice. *J Clin Invest* 93: 1691-1699, 1994.
36. Hardie WD, Davidson C, Ikegami M, *et al.*: EGF receptor tyrosine kinase inhibitors diminish transforming growth factor-alpha-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 294: 1217-1225, 2008.
37. Perugorria MJ, Latasa MU, Nicou A, *et al.*: The epidermal growth factor receptor ligand amphiregulin participates in the development of mouse liver fibrosis. *Hepatology* 48: 1251-1261, 2008.
38. Sandgren EP, Luetkeke NC, Palmiter RD, Brinster RL and Lee DC: Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell* 61: 1121-1135, 1990.
39. Means AL, Ray KC, Singh AB, *et al.*: Overexpression of heparin-binding EGF-like growth factor in mouse pancreas results in fibrosis and epithelial metaplasia. *Gastroenterology* 124: 1020-1036, 2003.
40. Blaine SA, Ray KC, Branch KM, Robinson PS, Whitehead RH and Means AL: Epidermal growth factor regulates pancreatic fibrosis. *Am J Physiol Gastrointest Liver Physiol* 297: G434-G441, 2009.