ARTICLE
Journal of Cellular Biochemistry 106:580–588 (2009)

Journal of Cellular Biochemistry

Transcriptional Regulation of Fibrillin-2 Gene by E2F Family Members in Chondrocyte Differentiation

Takuya Yanagino, Keizo Yuasa, Masami Nagahama, Yoshiko Matsuda, and Akihiko Tsuji*

Department of Biological Science and Technology, the University of Tokushima Graduate School, 2-1 Minamijosanjima, Tokushima 770-8506, Japan

ABSTRACT

Mutation in fibrillin-2, a major structural component of extracellular microfibrils in connective tissue, results in the autosomal dominant disease congenital contractural arachnodactyly. This genetic disease is characterized by dolichostenomelia and arachnodactyly, in addition to contractures of the large joints and abnormal pinnae formation, thus indicating the significance of fibrillin-2 in chondrogenesis. In this study, we investigated the transcriptional regulation of fibrillin-2 in chondrogenic differentiation. Although mRNA expression of fibrillin-1, a highly homologous protein to fibrillin-2, remained almost unchanged during chondrogenesis of mouse ATDC5 cells, fibrillin-2 mRNA expression varied. Fibrillin-2 was highly expressed at the early stage and declined progressively during differentiation. The 5′-flanking region of the fibrillin-2 gene contains potential binding sites for E2F, Runx, AP-2, and Sox transcription factors. The promoter activity of fibrillin-2 decreased markedly following deletion and mutagenesis of the E2F binding site between −143 and −136 bp. Overexpression of E2F1 resulted in a marked increase in its promoter activity, whereas expression of other transcription factors including AP-2α and Runx2 had no effect. The increase in promoter activity by E2F1 was completely suppressed by the coexpression of E2F4. E2F2 and E2F3 had positive effects on the promoter activity. Although ATDC5 cells expressed transcripts for the E2F family genes at all stages of differentiation, the expression profiles differed. E2F1 expression remained almost unchanged, whereas E2F4 expression increased markedly at the late stage of differentiation. These results indicated that coordinated expression of the E2F family is critical for the transcriptional regulation of fibrillin-2 during chondrogenesis. J. Cell. Biochem. 106: 580–588, 2009.

KEY WORDS: FIBRILLIN-2; EXTRACELLULAR MATRIX; E2F; TRANSCRIPTIONAL REGULATION; CHONDROGENESIS

Formation of the skeleton in vertebrates involves the differentiation of mesenchymal cells through cartilage to bone. This process, called endochondral ossification, is a coordinately regulated event involving multiple stages including condensation of the mesenchyme, commitment to the chondrogenic lineage, differentiation into chondrocytes, and finally apoptosis, to leave behind a scaffold for invasion by osteoblasts and osteoclasts. These events are regulated by the interplay of locally secreted signaling molecules and extracellular matrix (ECM) components along with specific transcription factors. It is well known that the ECM components not only form the external scaffold around which cells reside, but also control the action of chondrogenic triggering signals during chondrogenesis [Montero and Hurlé, 2007]. The fibrillin family consists of two members, fibrillin-1 and fibrillin-2, and represents the major structural component of extracellular filaments, referred to as microfibrils. Loss of function mutations in genes encoding the fibrillins causes skeletal abnormalities [Viljoen, 1994; Judge and Dietz, 2005]. Fibrillin-1 and fibrillin-2 are ~350 kDa cysteine-rich glycoproteins that essentially comprise epidermal growth factor domains interspersed with 8-cysteine motifs. The main difference between fibrillin-1 and fibrillin-2 lies in the unique hydrophobic sequence towards the amino terminus, which may act as a potential molecular hinge, where in fibrillin-1 and fibrillin-2 this sequence is proline- and glycine-rich, respectively [Handford et al., 2000; Kielty et al., 2002; Hubmacher et al., 2006]. It has been proposed that the two fibrillins have some divergent functional roles since the two fibrillin genes are expressed in partially overlapping but distinct patterns [Zhang et al., 1995; Quondamatteo et al., 2002]. During development, fibrillin-2 mRNA accumulates prior to tissue differentiation, decreasing rapidly thereafter [Zhang et al., 1995]. Subsequently, fibrillin-2 is found preferentially in limited elastic tissues such as cartilage. In contrast, fibrillin-1 mRNA levels increase at a gradual rate during development. Furthermore, mutation of the genes encoding fibrillin-1 and fibrillin-2 result in different autosomal dominant diseases such as Marfan syndrome and congenital contractural arachnodactyly

*Correspondence to: Prof. Akihiko Tsuji, Department of Biological Science and Technology, the University of Tokushima Graduate School, 2-1 Minamijosanjima, Tokushima 770-8506, Japan.
E-mail: tsuji@bio.tokushima-u.ac.jp

Received 17 July 2008; Accepted 21 November 2008 • DOI 10.1002/jcb.22029 • 2009 Wiley-Liss, Inc.
Published online 13 January 2009 in Wiley InterScience (www.interscience.wiley.com).

(CCA), respectively [Viljoen, 1994; Judge and Dietz, 2005]. This suggested that fibrillin-2 plays a restricted but important role in the maintenance of microfibrils. However, the transcriptional regulatory programs for fibrillins in skeletogenesis have remained obscure. Therefore, delineation of the function of fibrillin molecules in endochondral ossification would provide some significant information.

The E2 factor (E2F) family of transcription factors includes both transcriptional activators (E2F1, E2F2, and E2F3) and repressors (E2F4 and E2F5) [Stevens and La Thangue, 2003]. E2F1 transgenic mice exhibited delayed endochondral bone formation, which is characterized by reduced hypertrophic zones and disorganized growth plates [Scheijen et al., 2003]. In the chondrogenic cell line ATDC5, E2F1 overexpression strongly inhibited early- and late-stage differentiation, and was accompanied by diminished cartilage nodule formation as well as decreased type II collagen and type X collagen gene expression. Taken together, these data showed that E2F1 overexpression inhibited chondrocyte differentiation, thus resulting in delayed endochondral ossification.

In this study, we focused on the transcriptional regulation of fibrillin-2 during chondrogenic differentiation. These results showed that fibrillin-2 expression is regulated by E2Fs and that fibrillin-2 may play an important role in chondrocyte differentiation.

MATERIALS AND METHODS

CELL CULTURE

Mouse embryonal carcinoma cell line ATDC5 and mouse fibroblastic cell line C3H10T1/2 were obtained from Riken Cell Bank. ATDC5 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12) (Invitrogen) containing 5% fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulfate). C3H10T1/2 cells were cultured with high glucose (4.5 g/L) DMEM containing 10% FCS and antibiotics. Both cell lines were grown at 37°C under 5% CO_2. For chondrogenesis, ATDC5 cells were plated at a density of 0.8×10^5 cells/35-mm dish and cultured in maintenance medium for 3 or 4 days to reach confluence. These cells were then incubated in differentiation medium comprising DMEM/F12 supplemented with 5% FCS, antibiotics, 10 μg/ml of bovine recombinant insulin (Sigma), 10 μg/ml transferin (Sigma), and 30 nM sodium selenite (Sigma) for 27 days with medium changes every 3rd day.

REVERSE TRANSCRIPTION-PCR (RT-PCR) ANALYSIS

Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene) and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Wako) and a random hexamer according to the manufacturers' instructions. PCR was performed through 25–35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, followed by extension at 72°C for 1 min with specific primers (Table I). PCR products were subjected to agarose gel electrophoresis and subsequently detected using ethidium bromide staining.

5′-RACE ANALYSIS OF TRANSCRIPTION INITIATION SITES

The 5′ end of mouse fibrillin-2 was determined by 5′-RACE methods using the 5′-Full RACE Core Set (Takara Bio). First-strand DNA was synthesized from total cellular RNA (3 μg) isolated from ATDC5 cells using a 5′-phosphorylated fibrillin-2 specific primer (5′-TTGCGTCCGCATGCT-3′). The first PCR reaction was carried out using the 1st-primer set (5′-ATACTCCGAGGGCCCAACGTGTG-3′ and 5′-CCAGAGCGTCTTTGGTGGAGG-3′), and the nested PCR reaction was performed using the 2nd-primer set (5′-TGCGGTTC-TAGATTCCACTCC-3′ and 5′-AACCCAGCCACACGAAGTAGG-3′). Amplified products were analyzed by agarose gel electrophoresis, cloned using the pGEM-T-easy (Promega) vector and subsequently sequenced. Two independent amplifications were performed, and five clones from each product were sequenced to determine the transcription initiation sites.

CONSTRUCTION OF MOUSE FIBRILLIN-2 REPORTER PLASMIDS

A 1.3 kb fragment of the fibrillin-2 promoter was amplified from MSM mouse BAC clones (Riken) using the primer set 5′-CCCA-TGATATGAATTCCTTCTGGAC-3′ and 5′-AAGCTTCGCCGGCGC-CAAGAG-3′. The amplified PCR product was cloned into the pGEM-T-easy vector and the fidelity of the sequence was confirmed by DNA sequencing. The 1.3 kb insert was then cloned into the *Nhe*I and *Hin*dIII sites of the pGL3-Basic luciferase reporter gene vector (Promega), resulting in "F2-PR (−1306/+174)". Shorter promoter constructs F2-PR (−986/+174), F2-PR (−286/+174), and F2-PR (−95/+174) were produced by restriction enzyme digestion. Mutation of the E2F binding site ($^{-139}$TAGA^{-136}) was introduced by PCR and restriction enzyme digestion. PCR was performed using two sets of primers (5′-GCTAGCTTTGGTGACTTCTGCAGC-3′ and 5′-TCTAGAAATTACAAAAGTAACCCG-3′) and (5′-TCTAGACCC-CACCCGCTGGCGGTC-3′ and 5′-AAGCTTCGCCGGCGCCAAGAG-3′). Each product was cloned into pGEM-T-easy vector and digested with either *Pst*I and *Xba*I or *Xba*I and *Hin*dIII. Both digestion

TABLE I. Primer Sequences Used for RT-PCR and Expected Product Size

Gene	Primer sequence	Product size (bp)
Fibrillin-1	S: 5′-GAATGTGAGATCGGAGCACACAA-3′; As: 5′-CATCTGTGCAGTTTCCACCAC-3′	490
Fibrillin-2	S: 5′-GGCCGAATGGCAAAAGCTC-3′; As: 5′-CCCCCAACGCCAGGAGAAAAG-3′	257
E2F1	S: 5′-AGCCTTGGATCCCAGTCAATCC-3′; As: 5′-TCAGAAATCCAGAGGGGTCAGC-3′	289
E2F2	S: 5′-AACATCCAGTGGGTAGGCAG-3′; As: 5′-GGGAGCAACTCTGAATGAGC-3′	133
E2F3	S: 5′-TGCAGTCTGTCTGAGGATGG-3′; As: 5′-GGGTCTGTGTGTTTCCGTCTC-3′	351
E2F4	S: 5′-CGTCCAGAACAGCTGCTTGG-3′; As: 5′-GACAGGCACAGCCACAGGTG-3′	235
Type II collagen	S: 5′-CACACTGGTAAGTGGGGCAAGACCG-3′; As: 5′-GGATTGTGTTGTTTCAGGGTTCGGG-3′	173
Type X collagen	S: 5′-TTCTCCTACCACGTGCATGTG-3′; As: 5′-AGGCCGTTTGATTCTGCATT-3′	191
G3PDH	S: 5′-GGTGAAGGTCGGAGTCAACGGATTTGG-3′; As: 5′-CATGTGGGCCATGAGGTCCACCAC-3′	984

fragments were mixed and cloned into the *Pst*I and *Hin*dIII restriction sites of pGL3-F2-PR (−286/+174).

LUCIFERASE REPORTER ASSAY

C3H10T1/2 cells were plated at a density of 1.0×10^5 cells/35-mm dish. The following day, cells were cotransfected with a total of 2 μg of promoter construct and expression vectors for E2F1-4 [Yuasa et al., 2007] together with pCMV-β-galactosidase using FuGENE 6 (Roche). Two days following transfection, cells were lysed and subjected to the luciferase reporter assay. β-galactosidase and luciferase assays were performed as previously described [Koide et al., 2003]. β-galactosidase activity was measured to normalize the luciferase activity. As well, ATDC5 cells (2.0×10^5 cells/35-mm dish) were cotransfected by Lipofectamine 2000 (Invitrogen) and then collected after 24 h for luciferase reporter assay.

PREPARATION OF GST-FUSION PROTEINS AND NUCLEAR EXTRACTS FROM ATDC5 CELLS

Expression of GST-hE2F1 and GST-hDP1 fusion proteins was performed as described elsewhere [Smith and Johnson, 1988; Kwon et al., 2001]. GST-hE2F1 and GST-hDP1 fusion proteins were expressed in *Escherichia coli* BL 21 and purified using glutathione Sepharose 4B (GE Healthcare) according to the manufacturer's procedures.

Nuclear extracts of ATDC5 cells were prepared as described elsewhere [Yuasa et al., 2007]. Briefly, ATDC5 cells that had been undifferentiated or differentiated for 12 days or 24 days were washed with PBS and resuspended in hypotonic buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 2 μg/ml each of aprotinin, pepstatin A and leupeptin). Following vortexing, nuclei was pelleted and resuspended in hypertonic buffer (50 mM HEPES, pH 7.8, 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol, and 2 μg/ml each of aprotinin, pepstatin A and leupeptin). The mixture was incubated at 4°C for 30 min with rotation and then centrifuged at 15,000*g* for 15 min. The supernatant was used as the nuclear extract for gel mobility shift assay and Western blot analysis.

Protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad) with bovine serum albumin as the standard.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Complementary strands of each oligonucleotide (Fig. 5A) were annealed to prepare a double-stranded oligonucleotide probe. The probe was 5′-end-labeled using [γ-^{32}P] ATP (PerkinElmer Life & Analytical Sciences) and T4 polynucleotide kinase. For GST-fusion proteins, the binding reaction was performed in a 15 μl reaction mixture containing 20 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg salmon sperm DNA, 50 μg GST-hE2F1, 50 μg GST-hDP1, and ^{32}P-labeled oligonucleotide probe. Using nuclear extracts from ATDC5, the binding reactions were performed in gel shift binding buffer (20 mM HEPES, pH 7.8, 60 mM KCl, 1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg salmon sperm DNA, and ^{32}P-labeled oligonucleotide probe) with 5 μg of nuclear extracts in a total volume of 30 μl. Equal amounts of the extracts were incubated with 0.5 ng of radiolabeled probes in the absence or presence of unlabeled oligonucleotide competitor probe (100- or 200-fold) and 5 μg of antibody against E2F1 (sc-251) (Santa Cruz Biotechnology). The reaction mixture was incubated at room temperature for 30 min and then electrophoresed through a 4.5% polyacrylamide gel. Gels were dried and then exposed to X-ray film at −80°C for 20 h.

WESTERN BLOT ANALYSIS

Western blot analysis was performed by using standard procedures. Equal amounts of nuclear extracts (20 μg for E2F1 or 10 μg for E2F4) prepared from ATDC5 cells were electrophoresed and transferred to a nitrocellulose membrane (Bio-Rad). The blot was probed with primary antibodies directed against E2F1 (sc-251) or E2F4 (sc-866) (Santa Cruz Biotechnology). Bands were detected using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and revealed using the enhanced chemiluminescence detection system (Pierce).

RESULTS

THE EXPRESSION PATTERN OF FIBRILLIN-1 AND FIBRILLIN-2 DURING CHONDROGENESIS OF ATDC5 CELLS

ATDC5 cells were employed as a model for chondrogenesis in an effort to investigate the expression of fibrillin-1 and fibrillin-2 during chondrocyte differentiation. Confluent ATDC5 cells were treated with 10 μg/ml insulin to initiate differentiation (day 0), and thereafter collected at various time points for RT-PCR analysis (Fig. 1). The expression of type II collagen, which is a marker of early and mature chondrocytes, was detected at day 9 and peaked at day 15. Transcript levels of the hypertrophic chondrocyte marker type X collagen began to increase at day 15. Fibrillin-2 transcript levels were abundant in undifferentiated chondrocytes and progressively declined during differentiation, whereas the level of fibrillin-1 transcripts remained relatively constant. Thus, expression of fibrillin-2 is regulated at the transcriptional level and these results suggested a functional role of fibrillin-2 in chondrocyte differentiation.

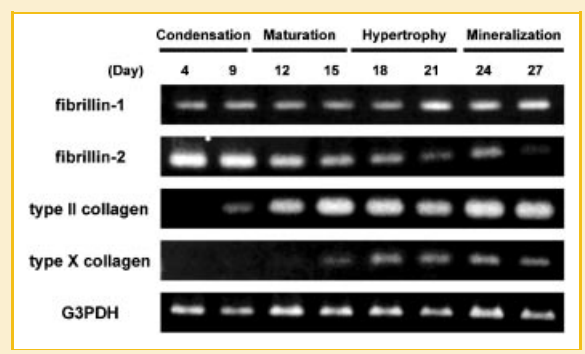

Fig. 1. Expression patterns of fibrillins during chondrocyte differentiation. ATDC5 cells were induced to differentiate by treatment with insulin (10 μg/ml) for 27 days, and total RNA was harvested at different times as indicated. The expression levels of fibrillins and chondrocyte marker genes type II collagen and type X collagen were analyzed by RT-PCR. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was also analyzed as a control.

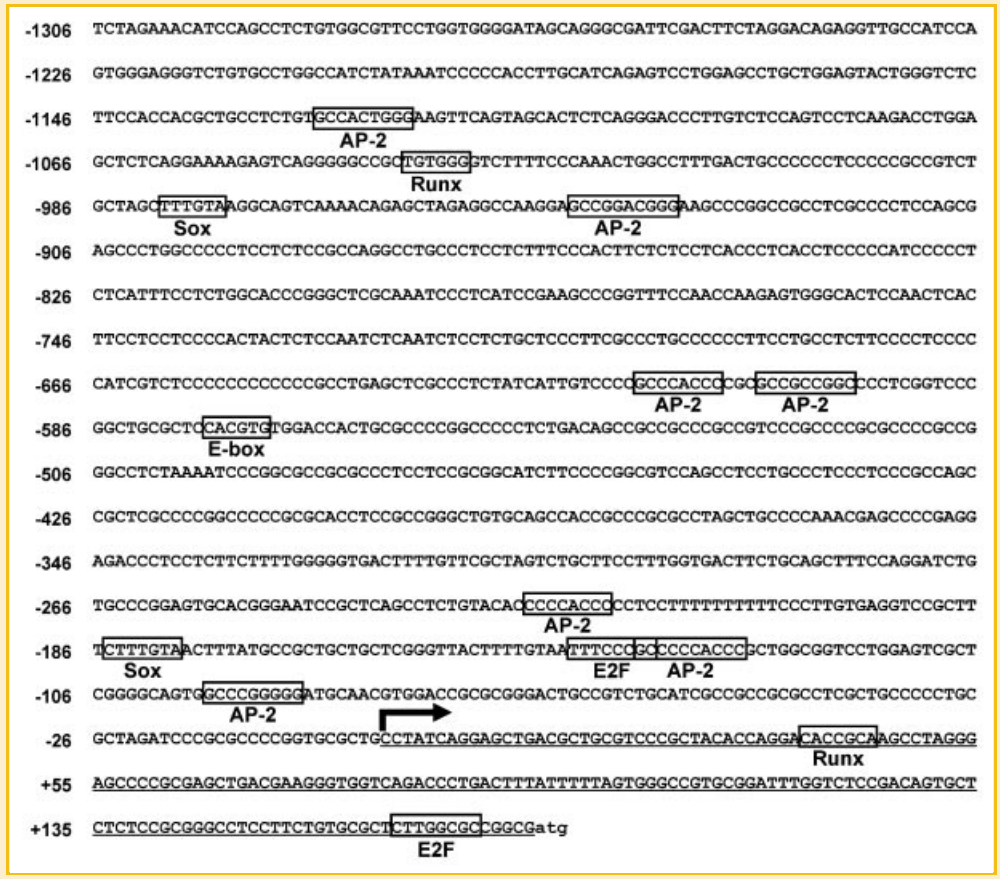

Fig. 2. Nucleotide sequence of the mouse fibrillin-2 promoter region. Nucleotide sequence of a promoter region of mouse fibrillin-2 is illustrated. Transcription factor binding motifs as predicted by TFSERCH are boxed. The putative transcription initiation site was determined by 5′-RACE using mRNA from undifferentiated ATDC5 cells. The transcription initiation site is numbered as +1.

CHARACTERIZATION OF MOUSE FIBRILLIN-2 PROMOTER IN CHONDROCYTES

In an effort to investigate the regulatory mechanism underlying fibrillin-2 expression, the transcription initiation site of the fibrillin-2 gene was determined by 5′-RACE using cDNA prepared from mRNA isolated from undifferentiated ATDC5 cells. Sequence analysis revealed that the transcription initiation site of mouse fibrillin-2 was located at 174 nucleotides upstream of the initiation codon (Fig. 2). Based on this result, we cloned the approximately 1.3-kb 5′-flanking region of the mouse fibrillin-2 gene from the mouse BAC clone. As shown in Figure 2, the fibrillin-2 promoter contains multiple putative recognition motifs for various transcription factors including E2F, Sox, Runx, AP-2, and E-box.

To determine the transcriptionally relevant elements of the fibrillin-2 promoter, a series of deletion constructs between −1306 and −95 of the fibrillin-2 promoter were generated and used for the luciferase reporter assay (Fig. 3A). Basal promoter activities of each fragment of the 5′-upstream region of the fibrillin-2 gene were investigated in ATDC5 cells and mouse embryonic fibroblasts C3H10T1/2. Because C3H10T1/2 cells also possess the ability of differentiation into chondorocytes such as ATDC5 cells, two different cell lines were used for reporter assay. As shown in Figure 3B, the 1.3-kb promoter region F2-PR (−1306/+174) exhibited strong promoter activity compared to the pGL3 empty vector. Promoter activity levels of F2-PR (−986/+174) and F2-PR (−286/+174) were the same as that of F2-PR (−1306/+174). However, a 1.2-kb deletion to position −95 reduced promoter activity to approximately 30% (F2-PR (−95/+174)). As well, only the activity of the shortest sequence in fibrillin-2 promoter was as half as longer region in ATDC5 cells. These results showed that the strong transactivating elements are located between the −95 and −286 bp region of the mouse fibrillin-2 promoter.

UPREGULATION OF FIBRILLIN-2 PROMOTER ACTIVITY BY E2F1

Given the presence of putative transcription factor binding sequences between the −95 and −286 bp region, luciferase reporter assays using F2-PR (−286/+174) and several transcription factors (E2F1, AP-2α, and Runx2) were performed to confirm that specific factors regulate the promoter activity of fibrillin-2. The luciferase activity of F2-PR (−286/+174) was stimulated approximately sevenfold in C3H10T1/2 cells following coexpression with E2F1, while no stimulation was observed with AP-2α or Runx2 (Fig. 4A). On the other hand, the DNA-binding defective E2F1-mutant E132 and transactivation domain-deficient E2F1-mutant E2F1 ΔAD had no effect (Fig. 4B). The putative E2F responsive-like sequence

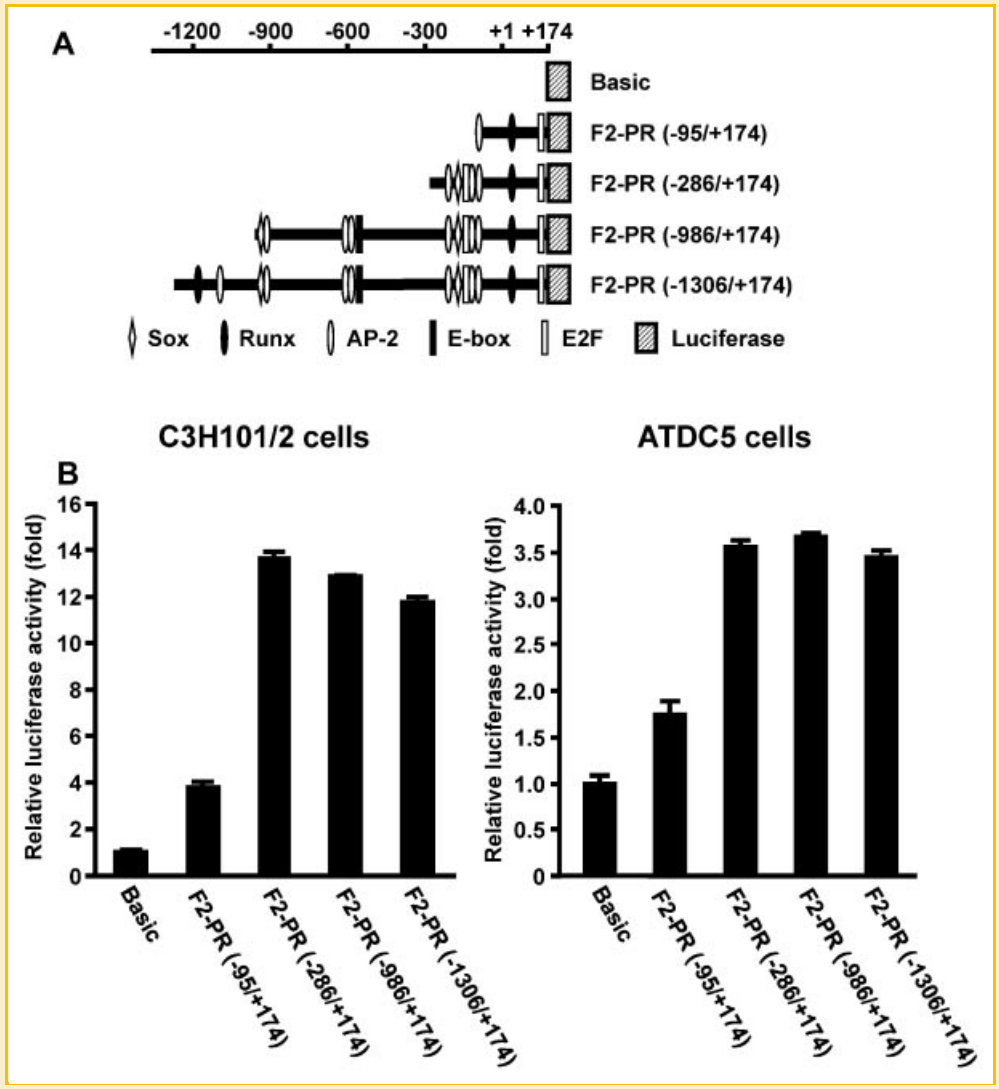

Fig. 3. Deletion mutant analysis of the mouse fibrillin-2 promoter. A: Schematic diagram of the 5′-flanking region of the mouse fibrillin-2 gene promoter. A series of 5′-deletion mutants created from the 5′-flanking sequences were ligated into the luciferase reporter plasmid (pGL3-Basic). B: C3H10T1/2 and ATDC5 cells were transfected with serial deletion mutants of the upstream region of the mouse fibrillin-2 promoter and harvested for the luciferase assay. The luciferase activity was expressed relative to the luciferase activity of Basic (pGL3-Basic), and normalized to the β-galactosidase activity. The results are presented as a mean of the luciferase activity ±SD of at least three independent experiments.

located within the −143/−136 site of the fibrillin-2 promoter was mutated from $^{-143}$tttcccgc^{-136} to $^{-143}$tttcTAgA^{-136} (Fig. 4C). The promoter activity of this mutant was downregulated by greater than 50% in comparison with that of the wild type sequences (Fig. 4D). These data indicated that E2F1 could act as a transcriptional activator of fibrillin-2 gene expression.

BINDING TO THE FIBRILLIN-2 PROMOTER BY E2F1

To identify the binding specificity of E2F transcription factors, EMSA experiments were performed using a probe corresponding to the −151/−126 region of the mouse fibrillin-2 promoter and recombinant bacterially expressed human E2F1 and human DP1 (Fig. 5A). When the fibrillin-2 promoter Wt probe was used, a specific band was observed (Fig. 5B, lane 2). This shifted band completely disappeared following the addition of a molar excess of unlabeled Wt oligonucleotide (self-competition; Fig. 5B, lanes 3 and 4), whereas addition of cold fibrillin-2 promoter Mt had no effect (Fig. 5B, lanes 5 and 6). In competition experiments with nuclear extracts from ATDC5 cells, similar results were obtained (Fig. 5C). Addition of an anti-E2F1 antibody was supershifted the complex, indicating that the E2F1 protein was forming part of the complexes (Fig. 5D). These results provided strong evidence that E2F1 was capable of binding directly to a specific region in the fibrillin-2 promoter.

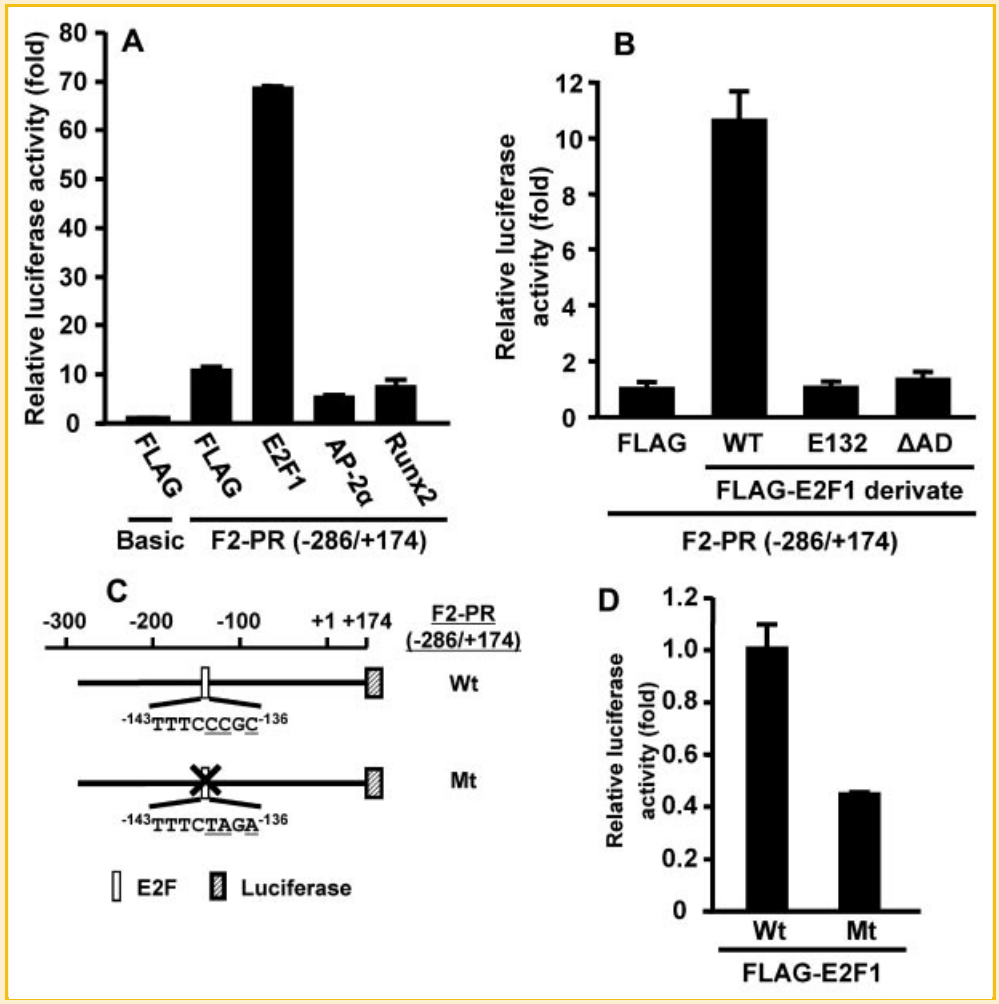

Fig. 4. Activation of the mouse fibrillin-2 promoter by E2F1. A: C3H10T1/2 cells were transiently transfected with pGL3-Basic or pGL3-F2-PR (−286/+174) in combination with pFLAG empty vector, pFLAG-E2F1, pFLAG-AP-2α, or pFLAG-Runx2. Results are shown as relative luciferase activity. The luciferase activity is normalized to the β-galactosidase activity. B: Reporter constructs were transiently cotransfected into C3H10T1/2 cells with pFLAG empty vector, pFLAG-E2F1 WT (WT), pFLAG-E2F1 E132 (E132), or pFLAG-E2F1 ΔAD (ΔAD). The luciferase activity is expressed relative to the luciferase activity of F2-PR (−286/+174) without E2F1 construct, and normalized to the β-galactosidase activity. C: Sequences of the putative E2F DNA-binding site in the fibrillin-2 promoter. This site (−143 to −136) was mutated to the indicated sequences. D: C3H10T1/2 cells were transiently transfected with pGL3-F2-PR (−286/+174) Wt (Wt) or pGL3-F2-PR (−286/+174) Mt (Mt) together with pFLAG-E2F1. After 48 h, the cell lysate was collected and analyzed for luciferase activity. Data represent the means ± SD of four separate determinations.

EFFECT OF E2F FAMILY MEMBERS ON FIBRILLIN-2 EXPRESSION

To investigate the effect of other E2F family members on fibrillin-2 expression, F2-PR (−286/+174) was cotransfected with E2F1, E2F2, E2F3, or E2F4 into C3H10T1/2 cells. As shown in Figure 6A, the activity of the gene promoter increased modestly following E2F2 or E2F3 cotransfection, while coexpression of transcriptional repressor E2F4 had no significant effect. Furthermore, we examined the expression patterns of E2F family members using RT-PCR analysis in an effort to demonstrate a correlation between the E2F family and fibrillin-2 gene expression during chondrogenesis. As shown in Figure 7A, ATDC5 cells constitutively expressed E2F1 mRNA except for the decrease of E2F1 expression at a late stage. E2F2 and E2F3 mRNA levels increased slightly during early chondrogenic differentiation and then finally diminished. In contrast, E2F4 transcripts were not detected in undifferentiated cells, but increased markedly during chondrogenic differentiation. Moreover, the expression changes of E2F1 and E2F4 protein were coincident with that of each mRNA during differentiation. It is highly likely that the expression of fibrillin-2 is upregulated by transactivators (E2F1, E2F2, and E2F3) during the early phase of chondrogenesis and subsequently downregulated as a result of rapid increases in transrepressor (E2F4). This hypothesis was supported by our finding that forced expression of E2F4 inhibited the E2F1-mediated increase in fibrillin-2 promoter activity in a dose-dependent manner (Fig. 6B).

DISCUSSION

Fibrillin-rich microfibrils are specialized ECM assemblies that impart connective tissue with mechanical stability and elastic

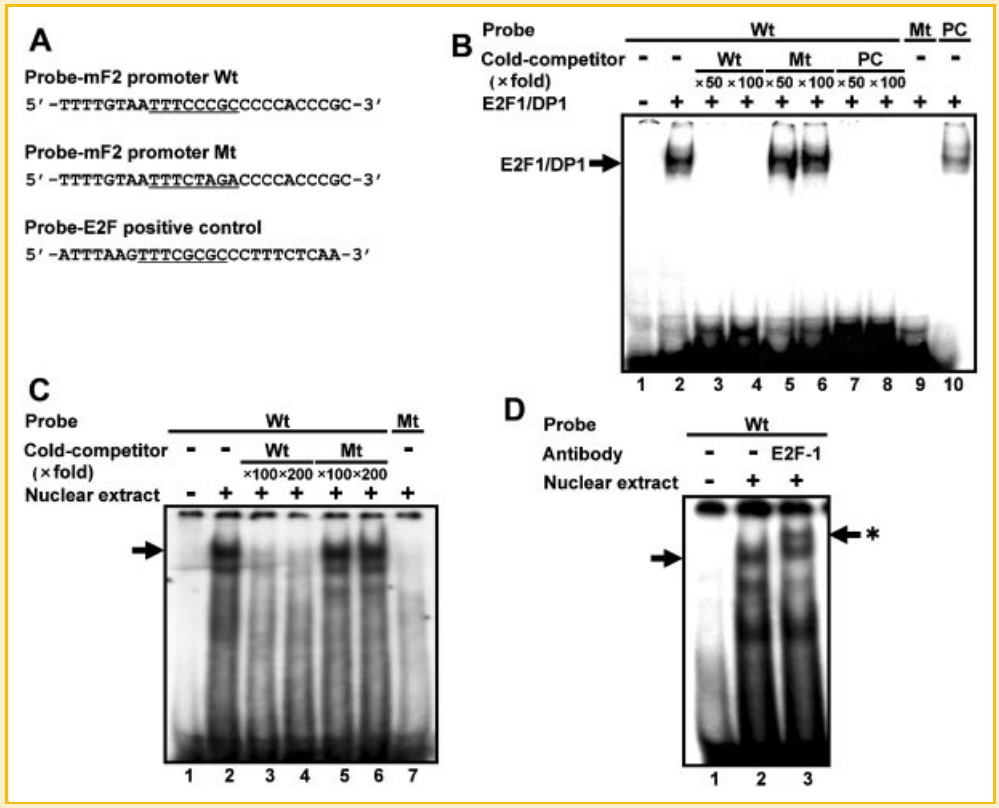

Fig. 5. Analysis of the interaction of the E2F1 with the putative E2F binding site (−143/−136) by gel mobility-shift assays. A: Sequence of oligonucleotides used in the electrophoretic mobility-shift analysis. Mutated sites are shown in upper case letters. Wt, wild type; Mt, mutant type. B: E2F1/DP1 complex was incubated with ^{32}P-labeled oligonucleotide probes (probe Wt and Mt) in the absence or presence of a 50- or 100-fold molar excess of cold competitor. The DNA–protein complexes were analyzed by electrophoresis through 4.5% polyacrylamide gels. PC, positive control. C: Nuclear extracts from ATDC5 cells was used by cold-competitor analysis. Arrow indicates E2F-probe complex. D: ^{32}P-labeled Wt probe was incubated with nuclear extracts in absence or presence of anti-E2F1 antibody. Arrow and asterisk indicate E2F-probe complex and supershifted E2F1-probe complex, respectively.

properties, and participate in the regulation of organ formation, growth and homeostasis [Ramirez and Dietz, 2007]. Unlike the case with fibrillin-1 and other ECM-related proteins, the physiological role of fibrillin-2 remains unknown. Previous findings have indicated that the differential expression of fibrillin genes reflects diversified microfibril function during development [Carta et al., 2006]. This study represents the first demonstration of E2F-mediated regulation of mouse fibrillin-2 expression during chondrocyte differentiation. As the putative E2F binding sequence is located at $^{-350}$TTTTACGC^{-343} and $^{-13}$TTCGGCGC^{-6} upstream from the initiation codon of the human fibrillin-2 gene, which is found on chromosome 5q23-31, as is the case with mouse fibrillin-2, E2F transcription factors may act similarly on fibrillin-2 gene expression in humans and mouse. The molecular basis for CCA, a heritable disorder of connective tissue, is now known to be associated with mutations in the human *fibrillin-2* gene [Putnam et al., 1995]. Classic phenotypic manifestations of CCA include several skeletal abnormalities associated primarily with overgrowth of long bones. Furthermore, skeletal abnormalities of *fibrillin-2* null mice lend credence to the hypothesis that fibrillin-2 plays an essential role in skeletal development [Arteaga-Solis et al., 2001]. As previously described for cartilaginous tissue [Zhang et al., 1995], RT-PCR analysis confirmed that fibrillin-2 was also expressed in mouse chondrogenic ATDC5 cells. The highest mRNA levels for fibrillin-2 were detected during the early stage of chondrocyte differentiation in ATDC5 cells, consistent with a previous study that found maximum expression of fibrillin-2 prior to cartilage development [Zhang et al., 1995]. In contrast, there was no significant change in fibrillin-1 expression during differentiation. These results indicated that transcriptional regulation of each member of the fibrillin family in chondrocytes might be regulated by a distinct manner, and that fibrillin-2 gene expression was more sensitive with respect to chondrogenesis compared with fibrillin-1. Therefore, we focused our attention on the transcriptional regulatory mechanism of fibrillin-2 during chondrogenesis. The E2F transcription factor is known to play a pivotal role in mediating gene expression during cell proliferation [Stevens and La Thangue, 2003]. One of the functional roles of E2F relates to cell differentiation [Fajas et al., 2002; McClellan and Slack, 2007]. Overexpression of E2F transactivators disturbed chondrocyte maturation [Scheijen et al., 2003], indicating the involvement of E2F family proteins in chondrocyte differentiation. Thus, we speculated that E2F plays a role in the regulation of fibrillin-2 expression in ATDC5 cells. When E2F1 was coexpressed with E2F4, the promoter activity of fibrillin-2 decreased in a dose-dependent manner in comparison with only E2F1 overexpression. E2F1 is known as a transactivator, whereas E2F4

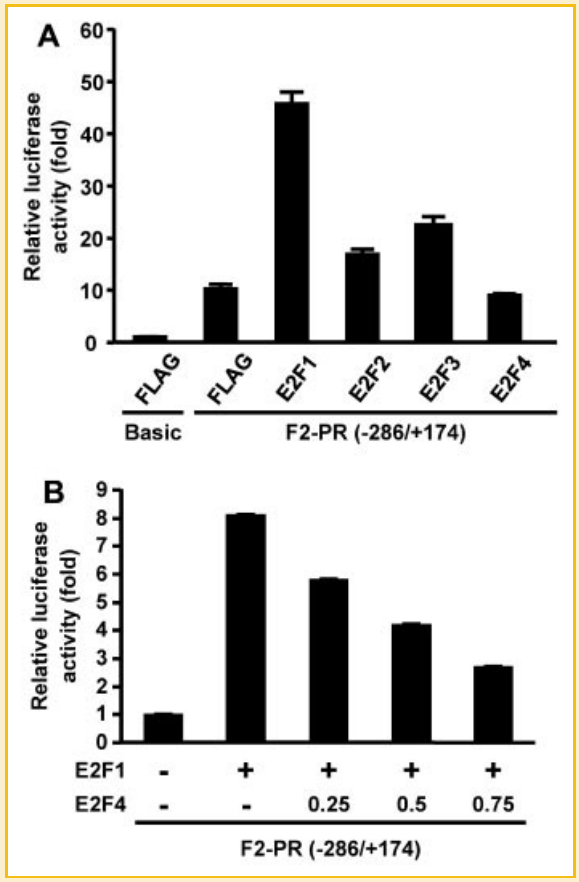

Fig. 6. Regulation of the mouse fibrillin-2 promoter by the E2F family. A: C3H10T1/2 cells were transiently transfected with pGL3-Basic or pGL3-F2-PR (−286/+174) in combination with pFLAG empty vector, pFLAG-E2F1, pFLAG-E2F2, pFLAG-E2F3, or pFLAG-E2F4. Results are shown as relative luciferase activity after 48 h. The luciferase activity is normalized to the β-galactosidase activity. B: The reporter plasmid pGL3-F2-PR (−286/+174) was transiently cotransfected into C3H10T1/2 cells in combination with pFLAG empty vector, pFLAG-E2F1, and/or pFLAG-E2F4. After 48 h, results were obtained and are shown as relative luciferase activity.

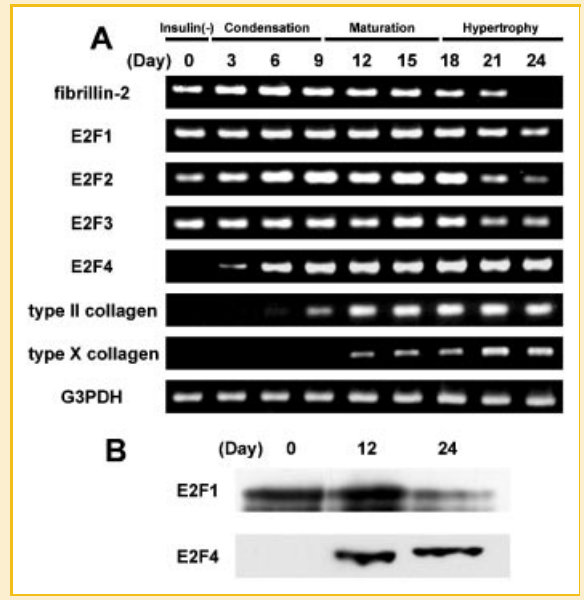

Fig. 7. Expression of fibrillin-2 and E2F family member genes during chondrogenesis of ATDC5 cells. A: ATDC5 cells were induced to differentiate by treatment with insulin (10 μg/ml) for 0–24 days and total RNA was harvested at different times as indicated. The expression levels of fibrillin-2 and E2F transcription factors were analyzed by RT-PCR. B: Western blot analysis of E2F1 and E2F4 protein in differentiating ATDC5 cells.

can repress E2F-responsive genes [Stevens and La Thangue, 2003]. We examined the correlation of gene expression between fibrillin-2 and E2F family proteins during chondrogenesis of ATDC5 cells (Fig. 7). While the E2F transactivators were highly expressed during early differentiation and were downregulated during the terminal stages of differentiation, E2F4 mRNA levels continued to increase with the progression of chondrogenesis. Taken together, we elucidated that E2F family members were regulators of fibrillin-2 gene expression during chondrocyte differentiation.

The two fibrillins belong to a small family of structurally related glycoproteins that includes the latent TGF-β binding proteins (LTBPs) [Handford et al., 2000; Kielty et al., 2002; Charbonneau et al., 2004; Ramirez et al., 2004; Hubmacher et al., 2006]. LTBPs and fibrillins are components of microfibrils, and both protein types are known to interact directly with members of the TGF-β superfamily [Isogai et al., 2003; Gregory et al., 2005; Sengle et al., 2008]. For example, interactions between LTBP-1 and TGF-β or between fibrillins and BMP-7 are well known. Additionally, latent TGF-β/LTBP complexes also interact with fibrillins [Isogai et al., 2003]. These interactions are required to properly regulate TGF-β signaling, since mutations in TGF-β, LTBPs or fibrillins cause dysregulation of TGF-β signaling [Neptune et al., 2003; Rifkin, 2005; Habashi et al., 2006; Cohn et al., 2007]. On the other hand, TGF-β was shown to promote chondrocyte proliferation but inhibit chondrocyte differentiation [Ferguson et al., 2000; Pateder et al., 2000; Beier et al., 2001; Ionescu et al., 2003; Dong et al., 2005]. It has been reported that TGF-β regulates cyclin D1 expression through the activation of activating transcription factor-2 or β-catenin signaling [Ionescu et al., 2003; Li et al., 2006]. Cyclin D1 binds to Cdk4 and Cdk6 to form a pRB kinase [Kozar and Sicinski, 2005]. Following phosphorylation, pRB loses its repressive activity for the E2F transcription factor [Sherr and Roberts, 1999, 2004]. It is possible that the TGF-β signal pathway upregulates the transcriptional activity of E2F molecules in proliferating chondrocytes. Importantly, our data demonstrated that maximum fibrillin-2 mRNA levels were observed during chondrocyte proliferation of ATDC5 cells. Therefore, TGF-β signals may control fibrillin-2 gene expression through the regulation of E2F activity.

In conclusion, our data demonstrated that the transcriptional regulation for fibrillin-2 was related to E2F family members in terms of chondrogenic differentiation of ATDC5 cells. It is likely that fibrillin-1 and fibrillin-2 have distinct roles in chondrogenesis. The physiological function of fibrillin-2 in the regulation of chondrogenesis remains to be determined. The expression patterns of the two fibrillins differed during chondrogenesis. Both fibrillins may operate as part of the same function in early developing bone and may compensate each other in terms of their fundamental roles in

skeletal development. Accordingly, our discovery contributes towards our understanding of the role of fibrillin molecules in bone development and disease.

ACKNOWLEDGMENTS

The authors thank Ms. Michie Kimura, Mrs. Kaori Hayashi, Mr. Yutaka Shiraishi and Mr. Suguru Fukui for their technical assistance.

REFERENCES

Arteaga-Solis E, Gayraud B, Lee SY, Shum L, Sakai L, Ramirez F. 2001. Regulation of limb patterning by extracellular microfibrils. J Cell Biol 154:275–281.

Beier F, Ali Z, Mok D, Taylor AC, Leask T, Albanese C, Pestell RG, LuValle P. 2001. TGFbeta and PTHrP control chondrocyte proliferation by activating cyclin D1 expression. Mol Biol Cell 12:3852–3863.

Carta L, Pereira L, Arteaga-Solis E, Lee-Arteaga SY, Lenart B, Starcher B, Merkel CA, Sukoyan M, Kerkis A, Hazeki N, Keene DR, Sakai LY, Ramirez F. 2006. Fibrillins 1 and 2 perform partially overlapping functions during aortic development. J Biol Chem 281:8016–8023.

Charbonneau NL, Ono RN, Corson GM, Keene DR, Sakai LY. 2004. Fine tuning of growth factor signals depends on fibrillin microfibril networks. Birth Defects Res C Embryo Today 72:37–50.

Cohn RD, van Erp C, Habashi JP, Soleimani AA, Klein EC, Lisi MT, Gamradt M, ap Rhys CM, Holm TM, Loeys BL, Ramirez F, Judge DP, Ward CW, Dietz HC. 2007. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. Nat Med 13:204–210.

Dong Y, Drissi H, Chen M, Chen D, Zuscik MJ, Schwarz EM, O'Keefe RJ. 2005. Wnt-mediated regulation of chondrocyte maturation: Modulation by TGF-beta. J Cell Biochem 95:1057–1068.

Fajas L, Landsberg RL, Huss-Garcia Y, Sardet C, Lees JA, Auwerx J. 2002. E2Fs regulate adipocyte differentiation. Dev Cell 3:39–49.

Ferguson CM, Schwarz EM, Reynolds PR, Puzas JE, Rosier RN, O'Keefe RJ. 2000. Smad2 and 3 mediate transforming growth factor-beta1-induced inhibition of chondrocyte maturation. Endocrinology 141:4728–4735.

Gregory KE, Ono RN, Charbonneau NL, Kuo CL, Keene DR, Bächinger HP, Sakai LY. 2005. The prodomain of BMP-7 targets the BMP-7 complex to the extracellular matrix. J Biol Chem 280:27970–27980.

Habashi JP, Judge DP, Holm TM, Cohn RD, Loeys BL, Cooper TK, Myers L, Klein EC, Liu G, Calvi C, Podowski M, Neptune ER, Halushka MK, Bedja D, Gabrielson K, Rifkin DB, Carta L, Ramirez F, Huso DL, Dietz HC. 2006. Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. Science 312:117–121.

Handford PA, Downing AK, Reinhardt DP, Sakai LY. 2000. Fibrillin: From domain structure to supramolecular assembly. Matrix Biol 19:457–470.

Hubmacher D, Tiedemann K, Reinhardt DP. 2006. Fibrillins: From biogenesis of microfibrils to signaling functions. Curr Top Dev Biol 75:93–123.

Ionescu AM, Schwarz EM, Zuscik MJ, Drissi H, Puzas JE, Rosier RN, O'Keefe RJ. 2003. ATF-2 cooperates with Smad3 to mediate TGF-beta effects on chondrocyte maturation. Exp Cell Res 288:198–207.

Isogai Z, Ono RN, Ushiro S, Keene DR, Chen Y, Mazzieri R, Charbonneau NL, Reinhardt DP, Rifkin DB, Sakai LY. 2003. Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. J Biol Chem 278:2750–2757.

Judge DP, Dietz HC. 2005. Marfan's syndrome. Lancet 366:1965–1976.

Kielty CM, Sherratt MJ, Shuttleworth CA. 2002. Elastic fibres. J Cell Sci 115:2817–2828.

Koide S, Yoshida I, Tsuji A, Matsuda Y. 2003. The expression of proprotein convertase PACE4 is highly regulated by Hash-2 in placenta: Possible role of placenta-specific basic helix-loop-helix transcription factor, human achaete-scute homologue-2. J Biochem 134:433–440.

Kozar K, Sicinski P. 2005. Cell cycle progression without cyclin D-CDK4 and cyclin D-CDK6 complexes. Cell Cycle 4:388–391.

Kwon EJ, Oh EJ, Kim YS, Hirose F, Ohno K, Nishida Y, Matsukage A, Yamaguchi M, Yoo MA. 2001. E2F-dependent transcription of the raf proto-oncogene during Drosophila development. Nucleic Acids Res 29:1808–1814.

Li TF, Chen D, Wu Q, Chen M, Sheu TJ, Schwarz EM, Drissi H, Zuscik M, O'Keefe RJ. 2006. Transforming growth factor-beta stimulates cyclin D1 expression through activation of beta-catenin signaling in chondrocytes. J Biol Chem 281:21296–21304.

McClellan KA, Slack RS. 2007. Specific in vivo roles for E2Fs in differentiation and development. Cell Cycle 6:2917–2927.

Montero JA, Hurlé JM. 2007. Deconstructing digit chondrogenesis. Bioessays 29:725–737.

Neptune ER, Frischmeyer PA, Arking DE, Myers L, Bunton TE, Gayraud B, Ramirez F, Sakai LY, Dietz HC. 2003. Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. Nat Genet 33:407–411.

Pateder DB, Rosier RN, Schwarz EM, Reynolds PR, Puzas JE, D'Souza M, O'Keefe RJ. 2000. PTHrP expression in chondrocytes, regulation by TGF-beta, and interactions between epiphyseal and growth plate chondrocytes. Exp Cell Res 256:555–562.

Putnam EA, Zhang H, Ramirez F, Milewicz DM. 1995. Fibrillin-2 (FBN2) mutations result in the Marfan-like disorder, congenital contractural arachnodactyly. Nat Genet 11:456–458.

Quondamatteo F, Reinhardt DP, Charbonneau NL, Pophal G, Sakai LY, Herken R. 2002. Fibrillin-1 and fibrillin-2 in human embryonic and early fetal development. Matrix Biol 21:637–646.

Ramirez F, Dietz HC. 2007. Fibrillin-rich microfibrils: Structural determinants of morphogenetic and homeostatic events. J Cell Physiol 213:326–330.

Ramirez F, Sakai LY, Dietz HC, Rifkin DB. 2004. Fibrillin microfibrils: Multipurpose extracellular networks in organismal physiology. Physiol Genomics 19:151–154.

Rifkin DB. 2005. Latent transforming growth factor-beta (TGF-beta) binding proteins: Orchestrators of TGF-beta availability. J Biol Chem 280:7409–7412.

Scheijen B, Bronk M, van der Meer T, Bernards R. 2003. Constitutive E2F1 overexpression delays endochondral bone formation by inhibiting chondrocyte differentiation. Mol Cell Biol 23:3656–3668.

Sengle G, Charbonneau NL, Ono RN, Sasaki T, Alvarez J, Keene DR, Bächinger HP, Sakai LY. 2008. Targeting of bone morphogenetic protein growth factor complexes to fibrillin. J Biol Chem 283:13874–13888.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512.

Sherr CJ, Roberts JM. 2004. Living with or without cyclins and cyclin-dependent kinases. Genes Dev 18:2699–2711.

Smith DB, Johnson KS. 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 67:31–40.

Stevens C, La Thangue NB. 2003. E2F and cell cycle control: A double-edged sword. Arch Biochem Biophys 412:157–169.

Viljoen D. 1994. Congenital contractural arachnodactyly (Beals syndrome). J Med Genet 31:640–643.

Yuasa K, Suzue K, Nagahama M, Matsuda Y, Tsuji A. 2007. Transcriptional regulation of subtilisin-like proprotein convertase PACE4 by E2F: Possible role of E2F-mediated upregulation of PACE4 in tumor progression. Gene 402:103–110.

Zhang H, Hu W, Ramirez F. 1995. Developmental expression of fibrillin genes suggests heterogeneity of extracellular microfibrils. J Cell Biol 129:1165–1176.