Expression and Regulation of the Osteoarthritis-associated Protein Asporin*

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Asporin (ASPN) is a small leucine-rich proteoglycan that is involved in pathological processes of osteoarthritis. Previously, we showed that asporin can inhibit transforming growth factor-β1 (TGF-β1)-mediated expression of cartilage matrix genes and chondrogenesis in vitro (Kizawa, H., Kou, I., Iida, A., Sudo, A., Miyamoto, Y., Fukuda, A., Mabuchi, A., Kotani, A., Kawakami, A., Yamamoto, S., Uchida, A., Nakamura, K., Notoya, K., Nakamura, Y., and Ikegawa, S. (2005) Nat. Genet. 37, 138–144). However, details about regulation of asporin itself are not yet known. Here, we examined ASPN expression in skeletal tissue and potential regulation of ASPN by TGF-β. In situ hybridization revealed the presence of ASPN mRNA in the perichondrium/periosteum of long bones, but its absence in articular cartilage and growth plates. Immunohistochemical analysis also showed ASPN protein expression predominantly in the perichondrium/periosteum. TGF-β1 induced endogenous ASPN mRNA expression over time in vitro, and this induction was suppressed by the TGF-β type I receptor kinase inhibitor SB431542. Inhibition of Smad3 significantly reduced TGF-β1-induced ASPN expression, whereas overexpression of Smad3 augmented the induction. Characterization of the human ASPN promoter region revealed a region from −126 to −82 that is sufficient for full promoter activity; however, TGF-β1 failed to increase activity through the ASPN promoter. Our findings indicate that TGF-β1 induces ASPN through Smad3 but that this induction is indirect.

Asporin (ASPN)² belongs to the class I small leucine-rich proteoglycan (SLRP) family (1, 2) and contains a unique asparagine acid repeat polymorphism in its N-terminal region. Recently, we found that the Asp repeat polymorphism in ASPN is a multifunctional cytokine involved in many growth processes, including cartilage formation (5). TGF-β signaling is initiated by interaction of the TGF-β ligand with two transmembrane serine/threonine kinase receptors (types I and II) (6–8). These receptors activate downstream signaling through the Smad pathway (9, 10). In this pathway, Smad2 or Smad3 forms heterodimer complexes with Smad4 and then binds directly or via other DNA-binding proteins to TGF-β-responsive promoters, stimulating or repressing gene transcription (9, 11).

Expression of two other members of the class I SLRP protein family, biglycan (BGN) and decorin (DCN), is regulated by TGF-β in vitro (12–15). BGN expression is stimulated by TGF-β, whereas DCN expression is repressed. It is thus reasonable to hypothesize that ASPN expression can be influenced by TGF-β. In this study, we demonstrate that ASPN expression is up-regulated by TGF-β1 and that the Smad pathway is crucial for this induction. In addition, the −126 to −82 in the ASPN promoter shows strong basal promoter activity in articular chondrocytes, but no TGF-β-responsive regions reside within −1.3 kb of the ASPN promoter region. These results suggest that ASPN expression is induced by TGF-β1 through Smad3 in an indirect manner.

**EXPERIMENTAL PROCEDURES**

**RNA in Situ Hybridization**—A mouse asporin cDNA (GenBank¹ accession number NM_025711) spanning nucleotides 187–457 was subcloned into the pGEM-T vector (Promega Corp., Madison, WI) for generation of sense and antisense RNA probes. RNA probes were prepared using the digoxigenin RNA labeling mixture (Roche Applied Science, Mannheim, Germany). Paraffin-embedded slides and sections of C57BL/6 mouse knee joint were obtained from Genostaff (Tokyo, Japan). The mouse knee joint was dissected, fixed with tissue fixative (Genostaff), and then embedded in paraffin using the manufacturer’s proprietary procedures. Tissue sections (4 μm) were dewaxed and hybridized as described (16), with some modifications. We carried out hybridization with digoxigenin-labeled RNA probes at 60 °C for 16 h. The bound label was detected using BM purple alkaline phosphatase substrate (Roche Applied Science), and the sections were counterstained with Kernechtrot stain solution (Mutoh Chemical Co., Tokyo), dehydrated, and then mounted with malinol (Mutoh Chemical Co.).

**Immunohistochemistry**—Deparaffinized sections were incubated in 3% hydrogen peroxide in methanol for 15 min at room

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2 The abbreviations used are: ASPN, asporin; SLRP, small leucine-rich proteoglycan; TGF-β1, transforming growth factor-β1; BGN, biglycan; DCN, decorin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; NHAC, normal human articular chondrocyte; ALK, activin receptor-like kinase; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; dpc, day(s) post-coitus.
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temperature to block endogenous peroxidase activity. The sections were washed with Tris-buffered saline, and excess protein was blocked with protein block (code X0909, Dakopatts, Copenhagen, Denmark). Next, the sections were incubated overnight with an anti-ASPN polyclonal antibody (2229-B01) at a concentration of 1 μg IgG/ml at 4 °C. Normal rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as the control primary antibody. After washing, tissues were blocked with the biotin-blocking system (code X0590, Dakopatts), and then incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (code E0432, Dakopatts). After washing, the sections were exposed to horseradish peroxidase-conjugated streptavidin (Nichirei Biosciences, Tokyo, Japan), and signals were detected with dimethylaminoazovenezene/H2O2.

Antibody—A polyclonal antibody against mouse ASPN was prepared to immunizing a rabbit with recombinant mature mouse ASPN. Its titer was detected by enzyme-linked immunosassays on microtiter plates. The anti-ASPN antibody (2229-B01) was separated by protein A affinity chromatography. This polyclonal antibody cross-reacts with human ASPN.

Cell Lines and Cell culture—OUMS-27 cells (17) were purchased from the Osaka Animal Cell Bank of the Institute for Fermentation (Osaka, Japan) and cultured as described previously (18). HCS-2/8 cells (provided by Dr. Masaharu Takigawa) (19) were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/high glucose supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (FBS). Normal human articular chondrocyte (NHAC) cells from the knee joint were purchased from Cambrex (East Rutherford, NJ). To obtain monolayer cultures, NHAC cells were maintained in a standard chondrocyte growth medium (Cambrex). Differentiated articular chondrocytes were maintained in three-dimensional cultures using alginate beads (Cambrex) according to the manufacturer’s instructions. Unless noted otherwise, we used primary cells following redifferentiation in three-dimensional culture for experiments.

Induction of ASPN by TGF-β—NHAC cells were cultured in 12-well plates at a density of 1 × 10^5 cells/well in DMEM/nutrient mixture F-12 containing 10% FBS. When cells reached confluence, the culture medium was replaced with DMEM/nutrient mixture F-12 containing 0.2% FBS. After 12 h, cells were treated with TGF-β (10 ng/ml) (R&D Systems, Wiesbaden, Germany) for the indicated time periods. The induction of ASPN by TGF-β was also examined in OUMS-27 and HCS-2/8 cells using DMEM instead of DMEM/nutrient mixture F-12.

Inhibitor Treatment—SB431542 (a potent and selective inhibitor of ALK4, ALK5, and ALK7) and two macromolecular synthesis inhibitors (cycloheximide (CHX) and actinomycin D) were purchased from Sigma (Deisenhofen, Germany). To examine the effects of SB431542, NHAC cells were cultured in 12-well plates at a density of 1 × 10^5 cells/well in DMEM/nutrient mixture F-12 containing 10% FBS. The culture medium was replaced with DMEM/nutrient mixture F-12 containing 0.2% FBS when cells reached confluence. After 12 h, cells were treated with TGF-β1 (10 ng/ml) for 24 h in the absence or presence of SB431542 (10 μM). SB431542 was added to cells 1 h prior to treatment with TGF-β1. The effects of SB431542 on TGF-β1-induced ASPN expression in OUMS-27 and HCS-2/8 cells were also examined using DMEM instead of DMEM/nutrient mixture F-12. To examine the effects of CHX and actinomycin D, NHAC cells were treated with TGF-β1 (10 ng/ml) for 24 h in the absence or presence of CHX (5 and 25 μg/ml) or actinomycin D (1 μg/ml). CHX and actinomycin D were added to cells 0.5 h prior to TGF-β1 treatment. ASPN mRNA levels were quantified using real-time PCR. The expression of SMAD7 and GAPDH was examined as a positive and a negative control, respectively.

Transient Transfection—HCS-2/8 cells were cultured for 24 h with DMEM containing 10% FBS in 12-well plates at a density of 1 × 10^5 cells/well. The expression plasmid pCMV6-Smad3 and the control empty vector were purchased from OriGene Technologies (Rockville, MD). Cells were transfected using TransIT-LT1 (Mirus Bio Corp., Madison) according to the manufacturer’s instructions. 24 h following transfection, cells were treated with TGF-β1 (10 ng/ml) and incubated for another 24 h. Total mRNA from harvested cells was subjected to real-time PCR.

Small Interfering RNA (siRNA)—Target sequences for Smad2 and Smad3 siRNAs (Smad2, 5'-UCUUUGUGCAGAGCCTCATT-3' and 5'-UUGGGGGCUCGCAAAAGATT-3' and Smad3, 5'-ACCUUCCCCGAUCCGAUTT-3' and 5'-AUCGGAUUGGGGAUGGUTT-3') were selected using the siRNA design support system on the TaKaRa Bio web site. These siRNAs and control scrambled siRNA were purchased from Takara Bio Inc. (Shiga, Japan). HCS-2/8 cells were cultured for 24 h with DMEM containing 10% FBS in 12-well plates at a density of 1 × 10^5 cells/well. Cells were transfected with 25 nm siRNA using TransIT-TKO (Mirus Bio Corp.) according to the manufacturer’s instructions. 24 h after transfection, cells were treated with TGF-β1 (10 ng/ml) for 24 h, and total mRNA from harvested cells was subjected to real-time PCR.

RNA Isolation and Real-time Quantitative PCR Assay—Total RNAs were isolated using the SV total RNA isolation system (Promega Corp.). Equal amounts of total RNA were reverse-transcribed into cDNA using the TaqMan core reagent kit (Applied Biosystems, Foster City, CA). 2 μl of each reverse transcription reaction was used as a template for SYBR Green real-time PCR (Qiagen Inc., Hilden, Germany). The following primers were used for amplification: ASPN, 5'-CTGGGCTTAGAAACACAAA-3' and 5'-TTCATCTTT-5-GCCACTGTGG-3'; and GAPDH, 5'-ACCACAGTCCAT-GCCATCAC-3' and 5'-TCACACACCGTTGCTGTAG-3'. SYBR Green PCR and real-time fluorescence detection were performed using the ABI PRISM 7700 sequence detection system. The PCR conditions were as follows: 94 °C for 15 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. To adjust for differences in cDNA synthesis, relative values for ASPN mRNA concentration were normalized to GAPDH mRNA levels.

Western Blotting—Cells were washed twice with phosphate-buffered saline and lysed in mammalian protein extraction reagent (M-PER, Pierce) with a protease inhibitor mixture (Roche Applied Science). The proteins in the cell lysate were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The primary antibody to ASPN (2229-B01) was used at 1:1000 dilution, and then horseradish peroxi-
dase-conjugated anti-rabbit IgG (GE Healthcare) was used at 1:5000 dilution. The membranes were stripped with a Restore Western blot stripping buffer (Pierce) and reprobed with an anti-actin antibody (1:2000 dilution; Santa Cruz Biotechnology, Inc.) or an anti-GAPDH antibody (1:2000 dilution; Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution; Upstate, Temecula, CA).

Luciferase Reporter Assay—ASPN-luciferase fusion plasmids were constructed in the promoterless vector pGL3-Basic (Promega Corp.). All ASPN fragments were amplified by PCR using a primer pair containing MluI- or XhoI-cleaved restriction sites at the 5′-ends. The 45-bp (−126 to −82) and 29-bp (−110 to −82) fragments were synthesized as double-stranded oligonucleotides containing MluI-cleaved sites at both ends (Invitrogen). All plasmids were verified by DNA sequencing. For detection of luciferase activity, HCS-2/8 cells were seeded in 24-well plates at a density of 5 × 10^4 cells/well in DMEM containing 10% FBS. After 24 h, the medium was replaced with DMEM containing 0.2% FBS. After 12 h, cells were transiently transfected using TransIT-LT1 reagent with appropriate ASPN promoter plasmids and incubated for 24 h in the absence or presence of TGF-β1 (10 ng/ml). Luciferase analysis in NHAC cells was performed using DMEM/nutrient mixture F-12 instead of DMEM. In these experiments, the pGL3-Basic vector was used as a negative control and the pRL-SV40 vector (Promega Corp.) as an internal control for normalization. The SBE4-luciferase plasmid (where SBE is Smad-binding element) (20) was used as a positive control to confirm the effect of TGF-β1 under our experimental conditions. Luciferase activity was measured using the PG-DUAL-SP reporter assay system (Toyo Ink Co., Tokyo). All assays were performed at least two times in duplicate.

RESULTS

Expression of Asporin in Adult Mouse—In previous work, we showed that ASPN expression is increased in articular cartilage from osteoarthritis patients using real-time PCR (3) and immunohistochemistry.3 To investigate the expression pattern of Aspn in limbs, we performed RNA in situ hybridization using mouse embryos at 15.5 days post-coitus (dpc). At this stage, Aspn mRNA was present around the maxilla, mandible, and vertebrae, consistent with previous findings (2). Detailed analysis of the limbs revealed a pronounced signal in the perichondrium and periosteum of the long bones, but not in the epiphysial cartilage (Fig. 1, A and B). Next, we investigated Aspn expression in the knee joints of 8-week postnatal mice. No substantial expression was detected in the articular cartilage and growth plate (Fig. 1, C and D), whereas the adjacent perichondrion showed expression similar to that of the 15.5-dpc embryo (Fig. 1E) (2).

Immunohistochemical analysis revealed ASPN expression patterns similar to those of Aspn mRNA. In 15.5-dpc mouse embryos, ASPN staining was seen in fibroblasts surrounding the subcutaneous tissue and vertebrae (Fig. 2, B and C). Weak ASPN staining was also found in fibroblasts surrounding the

3 M. Nakajima, H. Kizawa, M. Saitoh, I. Kou, K. Miyazono, and S. Iregawa, unpublished data.
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FIGURE 3. TGF-β isoforms similarly induce ASPN mRNA. TGF-β1, -β2, and -β3 (10 ng/ml) induced ASPN expression in NHAC, HCS-2/8, and OUMS-27 cells. When cells reached confluence, the medium containing 10% FBS was replaced with medium containing 0% FBS. After 12 h, cells were treated with TGF-β (10 ng/ml) for 24 h. ASPN mRNA expression values were normalized to GAPDH mRNA levels and are given relative to those of the TGF-β1-unstimulated control. Results are shown for the mean ± S.E. of at least two independent experiments for each cell line. **, p < 0.01 (Student’s t test).

FIGURE 4. TGF-β1 stimulates ASPN mRNA expression up to 24 h following treatment. NHAC (black bars), HCS-2/8 (gray bars), and OUMS-27 (black bars) cells were grown in normal growth medium containing 10% FBS, incubated in medium with 0.2% FBS for 12 h, and then stimulated with TGF-β1 (10 ng/ml) for the appropriate times up to 24 h. ASPN mRNA expression values were normalized to GAPDH mRNA levels and are given relative to those of the TGF-β1-unstimulated control. Results are shown for the mean ± S.E. of at least two independent experiments for each cell line. *, p < 0.05; **, p < 0.01 (Student’s t test).

FIGURE 5. Induction of ASPN mRNA by TGF-β1 occurs through the TGF-β type I receptor. The TGF-β type I receptor kinase inhibitor SB431542 (10 μM) was added to NHAC (white bars), HCS-2/8 (gray bars), and OUMS-27 (black bars) cells 1 h prior to treatment with TGF-β1 (10 ng/ml, 24 h). SB431542 completely suppressed TGF-β1-induced ASPN expression. ASPN mRNA expression values were normalized to GAPDH mRNA levels and are given relative to those of the TGF-β1-unstimulated control. Results are shown for the mean ± S.E. of at least three independent experiments for each cell line. ***, p < 0.01 (Student’s t test).

sphenoid bone and thoracic cavity (Fig. 2A). In contrast, the knee joints of 8-week postnatal mice showed ASPN expression predominantly in the periosteum, fascia, and tendon, with no apparent expression in growth plate cartilage (Fig. 2, D–F).

TGF-β Induces ASPN Expression—TGF-β can regulate SLRP gene expression either positively or negatively (21–25). To examine the potential effects of TGF-β on ASPN expression, we first investigated ASPN mRNA levels in response to TGF-β isoforms (β1, β2, and β3). Each isoform similarly induced ASPN expression in all chondrocytic cells examined (Fig. 3). We next examined the temporal profile of ASPN expression in response to TGF-β1, the isoform most abundantly expressed in articular cartilage and NHAC cells. ASPN expression was induced within 3–6 h following TGF-β1 (10 ng/ml) stimulation and increased with time up to 24 h. These findings were replicated in HCS-2/8 and OUMS-27 cells (Fig. 4), suggesting that TGF-β1 induction of ASPN is a common feature of chondrocytes. Thus, TGF-β1 positively regulates ASPN in cartilage. However, in TGF-β1-unstimulated NHAC cells, ASPN expression was induced after 24 h of culture.

ALK5 Kinase Activity Is Required for TGF-β1-induced ASPN Expression—SB431542 selectively inhibits the ALK5 group of TGF-β/bone morphogenetic protein type I receptors (26). This inhibitor completely suppressed TGF-β1-induced ASPN expression in NHAC, HCS-2/8, and OUMS-27 cells (Fig. 5) as a smad7 (data not shown). These results indicate that TGF-β1 induces ASPN expression through ALK5 activity (TGF-β type I receptor).

Smad3 Mediates TGF-β1-induced ASPN Expression—Intracellular TGF-β signaling is initiated by activation of the receptor-regulated Smad proteins Smad2 and Smad3 via ALK5. To determine whether TGF-β1 activates the Smad pathway in HCS-2/8 cells, we analyzed extracts from cells treated with TGF-β1 (10 ng/ml) by immunoblotting for phosphorylated and total Smad2 and Smad3. Smad2 and Smad3 were present in both control and TGF-β1-treated cells, and phosphorylation of both proteins increased within 10 min of TGF-β1 treatment (data not shown). These results indicate that HCS-2/8 cells can respond to TGF-β1 through a Smad pathway.

To clarify the role of Smad2 and Smad3 in TGF-β1-induced ASPN induction, we investigated the effects of endogenous Smad2 and Smad3 after TGF-β1 stimulation using knockdown experiments. It is interesting that only Smad3 siRNA repressed TGF-β1-induced ASPN expression effectively. Furthermore, simultaneous knockdown of Smad2 and Smad3 produced results similar to those of knockdown of Smad3 alone (Fig. 6, A and B). ASPN expression was again repressed by Smad3 siRNA at the protein level (Fig. 6C), and Smad3 overexpression increased TGF-β1-induced ASPN expression at both the mRNA and protein levels (Fig. 7). Taken together, these results
indicate that TGF-β1-induced ASPN expression occurs through the Smad pathway and involves Smad3 in particular.

**TGF-β Induction of ASPN mRNA Requires de Novo Protein Synthesis**—To evaluate whether the TGF-β/Smad signal regulates ASPN expression directly, we examined the effect of CHX on ASPN induction in NHAC cells. Pretreatment with CHX 0.5 h prior to addition of TGF-β1 effectively blocked ASPN induction (Fig. 8, A and C). Pretreatment with actinomycin D 0.5 h prior to addition of TGF-β1 also effectively blocked ASPN induction (Fig. 8, B and C). These results suggested that de novo protein synthesis is required in this process.

**Analysis of the ASPN Promoter**—To analyze the promoter region of human ASPN, we first characterized the transcription start site by rapid amplification of cDNA 5’-ends using RNA extracts from chondrocytic cells. Sequence analysis confirmed the previously reported start site (2) in almost all clones examined. Next, we measured the activity of the putative promoter region in NHAC and HCS-2/8 cells by luciferase reporter analysis using various ASPN-luciferase fusion plasmids. In both cell lines, deletion of sequences between −826 and −140 had no impact on promoter activity, whereas a construct further truncated to −104 abolished nearly all activity (Fig. 9, A and B). These results suggest that a strong positive element resides between −140 and −104. Further investigation of this region narrowed full promoter activity to the sequence between −126 and −82. It is interesting that this sequence contains a homopyrimidine segment (−124 to −104) that has been reported previously to possess enhancer activity in decorin (27). ASPN promoter analysis using non-chondrocytic cell lines showed the same results (data not shown). These observations indicate that this homopyrimidine region represents a strong, non-cell-specific positive element that is crucial to ASPN promoter activity.

**Responsiveness of the ASPN Promoter to TGF-β1**—To investigate whether TGF-β induction of ASPN expression results from an increase in transcriptional activity from the ASPN promoter, we performed transient transfection assays using various ASPN-luciferase fusion plasmids. We saw no difference in the response to TGF-β stimulation in HCS-2/8 cells. In NHAC cells, TGF-β caused a significant decrease in luciferase activity (Fig. 9B) as BGN in MG-63 cells (22). In HCS-2/8 cells, TGF-β1 increased transcription of TGF-β/Smad response elements by 2-fold under the identical conditions (data not shown). This finding stands in contrast with the 5-fold induction of endogenous ASPN expression in response to TGF-β1. Therefore, the promoter sequences contained in the genomic clone cannot account for the increased ASPN expression following TGF-β1 stimulation.

**DISCUSSION**

We have found that Aspn is expressed in the perichondria of the maxilla, mandible, vertebrae, and long bones in mice. BGN shows similar expression patterns, appearing in the perichondria of the vertebrae, ribs, and long bones of the
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Inhibition of TGF-β induction of ASPN expression by CHX and actinomycin D, respectively, in NHAC cells. Cells were treated with TGF-β1 (10 ng/ml) for 24 h in the absence or presence of the indicated concentrations of the protein synthesis inhibitor CHX or 1 μg/ml actinomycin D. CHX and actinomycin D were each added to cells 0.5 h prior to TGF-β1 treatment. ASPN mRNA levels were quantified by reverse transcription-PCR. Results are shown for the mean ± S.E. of two independent experiments. *, p < 0.05; **, p < 0.01 (Student’s t test). C, effects of actinomycin D and CHX on ASPN expression in the presence of TGF-β1 (10 ng/ml, 24 h). ASPN and GAPDH were analyzed using anti-ASPN (2229-B01) and anti-GAPDH antibodies, respectively. NC, negative control.

TGF-β1 promotes extracellular matrix accumulation primarily by inducing the synthesis of matrix proteins such as collagens, fibronectin, and proteoglycans. ASPN expression is increased by TGF-β in fibroblasts (24), osteosarcoma cells (31), and human articular chondrocytes (32). DCN expression is reduced by TGF-β in human skin fibroblasts (23) and articular chondrocytes (32). In addition, TGF-β-induced ASPN expression is regulated through the Smad pathway, mediated by both Smad2 and Smad3 (33). Here, we have demonstrated that ASPN expression is significantly enhanced by TGF-β1 and that the Smad pathway mediates the effects of TGF-β on ASPN expression in cartilage. Inhibition of Smad3, but not Smad2, reduces TGF-β1-induced ASPN expression, and overexpression of Smad3 augments TGF-β1 effects on ASPN in chondrocytic cells. TGF-β-induced BGN expression in PANC-1 cells relies on both Smad2 and Smad3 (33). Here, we have shown that Smad3, but not Smad2, is necessary for TGF-β1-induced ASPN expression in cartilage. This observation is consistent with a previous report that Smad3, and not Smad2, contributes significantly to cartilage degeneration in osteoarthritis (34). Therefore, the role of ASPN in maintaining articular cartilage may regulate endochondral bone formation through TGF-β1/Smad3 signaling.

ASPN binds directly to TGF-β1 and inhibits Smad signaling in chondrocytes (3). Therefore, ASPN and TGF-β/Smad appear to form a functional feedback loop. However, ASPN is
responsive to TGF-β1. Some constructs containing extra regions in addition to the response element contains no obvious Smad response element. In contrast to two chondrocytic cell lines, significant ASPN induction was identified in TGF-β1-un-treated NHAC cells at 24 h of culture (Fig. 4). Endogenous TGF-β1 in the cells may be a cause for the ASPN induction. We found an increase in endogenous TGF-β1 expression in NHAC cells at the mRNA level, although we could not confirm it at the protein level (data not shown). Alternatively, the different cell response in ASPN induction may be due to cell-specific signal mediator(s). Our results suggest that ASPN is the indirect target of Smad3, and hence, its expression could be influenced by other mediators. In this context, a recent study reported that up-regulation of BGN by TGF-β needs intermittent transcriptional induction of GADD45β (38). GADD45β is a target gene of TGF-β that depends on Smad3, but not on Smad2 (39), and TGF-β-mediated ASPN expression depends on Smad3. Therefore, GADD45β may be a potential mediator of TGF-β-induced ASPN expression. Further study is required to establish whether a TGF-β/Smad pathway induces ASPN in chondrocytes in vivo and to determine the precise role of Smad3 in ASPN mRNA expression.

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REFERENCES

1. Lorenzo, P., Aspberg, A., Onnerford, P., Bayliss, M. T., Neame, P. J., and Heinegard, D. (2001) J. Biol. Chem. 276, 12201–12211

2. Henry, S. P., Takanosu, M., Boyd, T. C., Mayne, P. M., Eberspaecher, H., Zhou, W., de Crombrugghe, B., Hook, M., and Mayne, R. (2001) J. Biol. Chem. 276, 12212–12221

3. Kizawa, H., Kou, I., Iida, A., Sudo, A., Miyamoto, Y., Fukuda, A., Mabuchi, A., Kotani, A., Kawakami, A., Yamamoto, S., Uchida, A., Nakamura, K., Notoya, K., Nakamura, Y., and Ikeyama, S. (2005) Nat. Genet. 37, 138–144

4. Jiang, Q., Shi, D., Yl, L., Ikegawa, S., Wang, Y., Nakamura, T., Qiao, D., Liu, C., and Dai, J. (2006) J. Hum. Genet. 51, 1068–1072

5. Seyedin, S. M., Thompson, A. Y., Bentz, H., Rosen, D. M., McPherson, J. M., Conti, A., Siegel, N. R., Galluppi, G. R., and Piez, K. A. (1986) J. Biol. Chem. 261, 5693–5695

6. Ebner, R., Chen, R. H., Shum, L., Lawler, S., ZIoncheck, T. F., Lee, A., Lopez, A. R., and Derynck, R. (1993) Science 260, 1344–1348

7. Wrana, L. J., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Nature 370, 341–347

8. Wrana, L. J., Attisano, L., Carcamo, J., Zentella, A., Moody, J., Laiho, M., Wang, X. F., and Massague, J. (1992) Cell 71, 1003–1014

9. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 390, 465–471

10. Nakao, A., Afrahhite, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., and ten Dijke, P. (1997) Nature 389, 631–635

11. Massagüé, J., and Wotton, D. (2000) EMBO J. 19, 1745–1754

12. Ioizzo, R. V. (1997) Crit. Rev. Biochem. Mol. Biol. 32, 141–174

13. Ioizzo, R. V., and Murdoch, A. D. (1996) FASEB J. 10, 598–614

14. Demoor-Fossard, M., Galera, P., Santra, M., Ioizzo, R. V., Pujol, J. P., and Redini, F. (2001) J. Biol. Chem. 276, 36983–36992

15. Hocking, A. M., Shimohama, T., and McQuillan, D. J. (1998) Matrix Biol. 17, 1–19

16. Hoshino, M., Sone, M., Fukata, M., Kuroda, S., Kaibuchi, K., Nabeshima, Y., and Hama, C. (1999) J. Biol. Chem. 274, 17837–17844

17. Kunisada, T., Miyazaki, M., Mihara, K., Gao, C., Kawai, A., Inoue, H., and Namba, M. (1998) Int. J. Cancer. 77, 854–859

18. Kou, I., and Ikegawa, S. (2004) J. Biol. Chem. 279, 50942–50948

19. Kobayashi, H., Hirashima, Y., Sun, G. W., Fujie, M., Nishida, T., Takigawa, M., and Terao, T. (2000) J. Biol. Chem. 275, 21185–21191

20. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) Mol. Cell 1, 611–617

21. Mauviel, A., Santra, M., Chen, Y. Q., Uitto, J., and Ioizzo, R. V. (1995) J. Biol. Chem. 270, 11692–11700

22. Ungefroren, H., and Krull, N. B. (1996) J. Biol. Chem. 271, 15787–15795

23. Kahari, V. M., Larjava, H., and Uitto, J. (1991) J. Biol. Chem. 266, 10608–10615

24. Romaris, M., Heredia, A., Molist, A., and Bassols, A. (1991) Biochim. Biophys. Acta 1093, 229–233

25. Westergren-Thorsson, G., Antonsson, P., Malmstrom, A., Heinegard, D., and Oldberg, A. (1991) Matrix 11, 177–183

26. Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., and Hill, C. S. (2002) Mol. Pharmacol. 62, 65–74

27. Santra, M., Danielski, K. G., and Ioizzo, R. V. (1994) J. Biol. Chem. 269, 579–587

28. Wilda, M., Bachner, D., Just, W., Geerkens, K., Kraus, P., Vogel, W., and Hameister, H. (2000) J. Bone Miner. Res. 15, 2187–2196

29. Scholzen, T., Solursh, M., Suzuki, S., Reiter, R., Morgan, J. L., Buchberg, A. M., Siracusa, L. D., and Ioizzo, R. V. (1994) J. Biol. Chem. 269, 28270–28281

30. Danielson, K. G., Barbiall, H., Holmes, D. F., Graham, H., Kadler, K. E., and Ioizzo, R. V. (1997) J. Cell Biol. 136, 729–743

31. Breuer, B., Schmidt, G., and Kresse, H. (1990) Biochem. J. 269, 551–554

32. Roughley, P. J., Melching, L. I., and Recklies, A. D. (1994) Matrix Biol. 14, 51–59

33. Chen, W. B., Lenschow, W., Tiede, K., Fischer, J. W., Kalthoff, H., and Ungefroren, H. (2001) J. Biol. Chem. 277, 36118–36128

34. Yang, X., Chen, L., Xu, X., Li, C., Huang, C., and Deng, C. X. (2001) J. Cell Biol. 153, 35–46

35. Moore, P. S., Sipos, B., Orlandini, S., Sorio, C., Real, F. X., Lemoine, N. R., Gress, T., Bassi, C., Kloppeg, G., Kalthoff, H., Ungefroren, H., Lohr, M., and Scarpa, A. (2001) Virchows Arch. 439, 798–802

36. Weber, C. K., Sommer, G., Fensterer, H., Weimer, M., Gan-sauge, F., Leber, G., Adam, L., and Gress, T. M. (2001) Gastroenterology 121, 657–667

37. Heegaard, A. M., Xia, Z., Young, M. F., and Nielsen, K. L. (2004) J. Cell. Biochem. 93, 463–475

38. Ungefroren, H., Groth, S., Ruhnke, M., Kalthoff, H., and Fantrich, F. (2005) J. Biol. Chem. 280, 2644–2652

39. Major, M. B., and Jones, D. A. (2004) J. Biol. Chem. 279, 5278–5287