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The role of Sp1 in BMP2-up-regulated Erk2 gene expression☆

Xiaoyun Xing,[a] Paul R. Manske,[a] Yen-Yao Li,[b] and Jueren Lou[a,*]

[a] *Department of Orthopaedic Surgery, Barnes-Jewish Hospital at Washington University, St. Louis, MO 63110, USA*
[b] *Department of Orthopaedic Surgery, Chang-Gung Memorial Hospital, Taiwan*

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Abstract

Extracellular signal-regulated kinase (Erk) is an important component in many cellular processes, including cell differentiation and proliferation. We previously showed that Erk is involved in BMP2-induced osteoblastic differentiation in mesenchymal progenitor cells and Erk protein level is up-regulated under BMP2 inducement. In this study, the molecular mechanism which mediates the regulation of Erk2 gene expression by BMP2 was investigated. Northern blot analysis showed that increased Erk2 protein level under BMP2 inducement comes from BMP2-up-regulated Erk2 mRNA expression. Transient transfection of C3H10T1/2 cells with a series of constructs of mouse Erk2 promoter demonstrated that a sequence residing between nucleotides −148 and −42 of Erk2 promoter is one of the BMP2-responsive elements. Electrophoresis mobility shift assays indicated that BMP2 treatment on C3H10T1/2 cells increases the binding of cell nuclear extracts to the −148/−42 fragment, and the BMP2-enhanced binding bands are Sp1 transcription factors. A series of competitive gel shift assays and the supershift assays by mapping oligos S1–S5 on −148/−42 identified that S1 and S5 contain Sp1 binding sites, which are located, respectively, in −147/−139 and −51/−46. Transfection studies showed that the addition of the Sp1 binding inhibitor mithramycin or mutation of the Sp1 site residing at −147/−139 abolishes the up-regulation of Erk2 promoter activity induced by BMP2. All these results indicate that Sp1-mediated transcription is one of the mechanisms, which is responsible for BMP2-induced up-regulation of Erk2 expression.

Keywords: BMP2; Erk2; Erk2 promoter; Sp1; Signal transduction; Transcription

Bone morphogenetic proteins (BMPs), a member of the transforming growth factor-β superfamily, are multiple functional molecules, which have important functions in embryogenesis and organogenesis [1,2], as well as in bone formation [3]. BMPs initiate a signaling cascade through the ligand-induced hetero-oligomerization of type I and II serine/threonine kinase receptors [4–7]. Smad proteins are well demonstrated as the mediators of the signal transduction for BMP receptors [8–10]. Nuclear cofactors, extra- and intracellular negative autofeedback loops, and the cross talk between the Smad pathway and other signaling pathways are also thought to regulate Smads to accomplish the diverse functions of BMPs [11]. In addition to Smad proteins, accumulating evidence has shown that an alternative pathway such as mitogen-activated protein kinases (MAPK) is involved in BMP signal transduction. It was reported that TGF-β-activated kinase (TAK1), a member of the MAPK kinase kinase (MAPKKK) family, is activated by BMP4 and TGF-β [12,13]. TAK1 was also reported to be involved in the cardiogenic BMP signaling pathway [14]. p38 MAP kinase pathway was demonstrated to take part in BMP2-induced neuronal differentiation in PC12 cells [15] and BMP4-stimulated osteocalcin synthesis in osteoblasts [16]. TAK1 and p38 pathways also mediate BMP2-induced apoptosis in MH60 cells [17]. p44/p42 MAP kinase (Erk) was shown to participate in the up-regulation of α(I) collagen gene both by TGF-β and BMP2 in rat osteoblastic cells [18].

Among BMPs, BMP2 is well defined to induce bone formation both in vivo and in vitro. When implanted subcutaneously or intramuscularly in animals, BMP2

☆ *Abbreviations:* BMP, bone morphogenetic protein; TGF, transforming growth factor; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; Mad, mother against dpp; TAK, TGF-β-activated kinase; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay.

* Corresponding author. Fax: 1-314-289-6521.
E-mail address: louj@msnotes.wustl.edu (J. Lou).

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induces ectopic bone formation [19,20]. In vitro, BMP2 promotes the differentiation of mesenchymal cells into the osteoblastic lineage [21–23]. In addition, BMP2 can stimulate chemotactic migration of osteoblasts, which play an important physiological role in bone healing and bone remodeling [24]. Smad signaling pathway has been shown to be involved in BMP2-induced osteoblast differentiation [25,26]. In addition, extracellular signal-regulated kinase (Erk), the member of MAPK cascade which is crucial for cell proliferation and differentiation [27], has also been reported to be involved in BMP2-induced osteoblast differentiation of mesenchymal cells by our laboratory and other groups [28,29]. Although BMP2 regulating osteoblast growth and differentiation, and Erk involvement in osteoblastic functions have been defined, the signal transduction mechanism between BMP2 and Erk remains unclear.

In this study, we confirmed that the up-regulation of Erk2 by BMP2 occurs at the transcriptional level. We identified two functional Sp1 sites (−147/−139 and −51/−46) within nucleotides −148/−42 of the Erk2 promoter and found that only the Sp1 site residing at −147/−139 is involved in the transcriptional up-regulation of Erk2 by BMP2 in C3H10T1/2 cells.

Materials and methods

Materials. Mouse mesenchymal progenitor cell line C3H10T1/2 was purchased from American Type Culture Collection (Rockville, MD). pGL3-basic plasmid and luciferase enzyme assay system were from Promega (Madison, WI). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and pCH110 vector were from Amersham Pharmacia Biotech (Piscataway, NJ). LipofectAMINE Plus reagent was from Invitrogen (Carlsbad, CA). β-Galactosidase enzyme assay system was from Tropix (Bedford, MA). All chemicals for SDS–PAGE and protein assays were from Bio-Rad (Hercules, CA). Sp-1 and Egr consensus oligonucleotides, antibodies against Sp1 and Egr2 were from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY). All restriction and modifying enzymes were from Boehringer–Mannheim (Indianapolis, IN). Other chemical reagents were from Sigma (St. Louis, MO).

Cell culture. C3H10T1/2 cells were grown in DMEM containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 in air. For BMP2 inducement, C3H10T1/2 cells were grown to 90% confluence. The cells were then maintained in serum starvation with DMEM without serum and antibiotics for 18 h. Recombinant BMP2 protein at 500 ng/ml was then added to the cells. Induced cells were harvested on a time course for further experiments. Uninduced cells were harvested on the same time course as BMP2-treated cells to serve as controls.

Northern blot analysis. Cells were treated with or without BMP2 as described above. At time points of 24, 48, and 72 h after treatment, cells were harvested and the total cellular RNA was extracted by the guanidine method. RNA was then subjected to electrophoresis in 1.0% formaldehyde–agarose gel. After electrophoresis, the gel was transferred to hybond-XL nylon membrane, followed by UV light fixation. The membrane was prehybridized in 50% formamide, 5× Denhardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 5× SSC at 42 °C for 1.5 h. Rat Erk2 DNA probe was labeled with [α-^{32}P]dCTP by Klenow fragment using the random-prime labeling technique. Hybridization was performed at 42 °C for 16 h with ^{32}P-labeled Erk2 probe in the same solution as prehybridization, except for a different concentration of Denhardt' (2.5×) and supplement with 6.5% dextran sulfate. The membrane was washed for 2 × 15 min in 6× SSC/0.4% SDS at room temperature, 2 × 15 min in 1× SSC/0.4% SDS at 37 °C, and 2 × 30 min in 0.1× SSC/0.1% SDS at 65 °C. The membrane was then exposed by autoradiography and quantitated by NIH image analysis. Applied total RNA was routinely standardized with ethidium bromide-stained rRNA to ensure equal loading.

Erk2 promoter luciferase reporter constructs. A series of 5′-deleted Erk2 promoter reporter constructs in pCAT-Basic were provided by Dr. Kunio Takishima (National Defense Medical College, Japan). We subcloned them into a PGL3-Basic plasmid to create a series of luciferase reporter constructs, which spanned from −2425, −1235, −1156, −781, −542, −366, −219, −174, −148, −42, and +20 to +223 from the transcription start site. The point mutations were made on pGL3(−148) to create constructs Mu-1 and Mu-5 as described below in detail. All constructs were confirmed by dideoxy sequencing.

Cell transfection and luciferase assay. C3H10T1/2 cells were maintained in complete DMEM. Reporter constructs described above were transfected into cells using lipofectAMINE Plus reagent according to the protocol recommended by the manufacturer. Briefly, cells at concentration of 3×10^4/well were seeded on 48-well plates one day before transfection. As much as 0.2 μg/well reporter construct was cotransfected with 0.1 μg/well pCH110 β-Gal plasmid, which served as the internal control to normalize transfection efficiencies. FBS was added to transfection medium 3 h later to a final concentration of 10%. After 18 h of incubation, cells were replaced with fresh complete medium and incubated for an additional 24 h. Serum starvation and BMP2 inducement were followed on cells as described above. Cells were lysed after 48 h of BMP2 inducement and luciferase and β-galactosidase activities were measured using Optocomp II Luminometer (MGM Instruments, Hamden, CT). For the Sp1 function inhibition assay, pGL3(−148)-transfected cells were treated with 100 nM mithramycin for 18 h during serum starvation followed by BMP2 inducement. Mithramycin untreated cells served as control.

Nuclear extract preparation and gel mobility shift assay. C3H10T1/2 cells were cultured until confluence. Serum starvation and BMP2 inducement were performed on cells as described above. Nuclear extracts were prepared [30] on the following time course: cells were washed twice with phosphate-buffered saline (PBS) and lysed in ice-cold hypotonic lysis buffer containing 10 mM Hepes–KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.6% Nonidet P-40 (NP-40) for 10 min to allow swelling. After vortexing for 15 s, the nuclei were spun down and resuspended in nuclear extraction buffer (20 mM Hepes–KOH, pH 7.9, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 0.12 TIU/ml aprotinin) for 1 h at 4 °C. Nuclei lysates were spun. The supernatants (nuclear extracts) were aliquoted and stored at −80 °C. Protein concentration of nuclear extract was determined using Bio-Rad protein assay kit. For gel shift, double-stranded oligos were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Purified probe (5×10^4 cpm) was mixed with 5 μg of nuclear extracts in a total volume of 20 μl containing 1× binding buffer (10 mM Hepes–KOH, pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM DTT) and 2 μg poly(dI–dC). Mixtures were incubated for 20 min on ice and subjected to electrophoresis at 4 °C using a 4% non-denaturing polyacrylamide gel in 1× Tris–glycine buffer (pH 8.3). The gel was dried and visualized after autoradiography. For competition and supershift experiments, either a 100-fold molar excess of unlabeled competitors or indicated antibodies were incubated with nuclear extracts for 45 min prior to the addition of the probe.

Mapping of transcriptional factor binding sites. A 115 bp fragment of Erk2 promoter (−152 to −38) containing potential transcriptional factor binding sites was created by standard polymerase chain reaction (PCR). Five segments of oligos, which covered −148 to −42 of Erk2

promoter, were synthesized [S1 (−152 to −126), S2 (−132 to −104), S3 (−110 to −82), S4 (−88 to −60), and S5 (−66 to −38)]. These segments overlapped 7 bp with their adjacent segments. These oligos were used as probes in the gel shift experiment to map transcription factor binding sites within the −148/−142 fragment of Erk2 promoter.

Site-directed mutagenesis. Point mutations were introduced in the context of pGL3(−148) using a sequential PCR mutagenesis method [31]. Oligo primers used to mutate the putative Sp1 binding site within S1 were sense (−126 to −152) (5′-GCACTGCGGCTGCGGttGAGGGA CGTC-3′) and anti-sense (−152 to −126) (5′-GACGTCCCTCaaCCGC AGCCGCAGTGC-3′), with the lowercase letters indicating the mutation sites. The sense primer and primer 1 (a fragment from pGL3-basic vector, 1–27) (5′-GCGGGTACCGAGCTCTTACGCGTGCTA-3′) containing a *Kpn*I site were used to make PCR product 1 and the antisense primer and primer 2 (+223 to +205 of Erk2 promoter) (5′-AGAGT CGACGTTGGCTGCACAGCCGCC-3′) containing a *Sal*I site were used to make PCR product 2 by standard PCR. The two PCR products were combined and amplified by primers 1 and 2. The final PCR product was digested by *Kpn*I and *Sal*I, subcloned into the corresponding *Kpn*1 and *Sal*I sites of pGL3-basic, named Mu-1. To make Mu-5, the same method as above was applied, except oligos of sense (−38 to −66) (5′-GG AGTCAAttGCGTGCGTGTTGATTGGCT-3′) and anti-sense (−66 to −38) (5′-AGCCAATCAACACGCACGCaaTTGACTCC-3′) were used to mutate the putative Sp1 binding site within S5. pGL3(−148) was used as a template in PCR mutation. All mutants were confirmed by DNA sequencing.

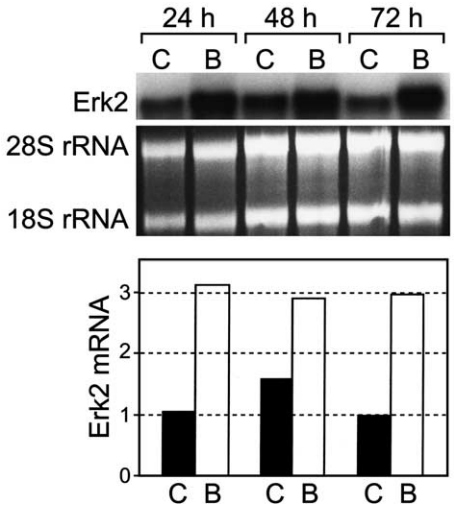

Fig. 1. Effect of BMP2 on Erk2 expression in mouse C3H10T1/2 cells. Cells were treated with BMP2 (B) at 500 ng/ml for 24, 48, and 72 h. Control cells (C) received no BMP2. Total RNA (10 μg/lane) samples from treated and untreated cells were analyzed by Northern blot analysis with a ^{32}P-labeled rat Erk2 DNA probe. Erk2 mRNA levels were normalized with ethidium bromide-stained rRNA.

Results

BMP2 increased Erk2 mRNA expression in mouse mesenchymal progenitor cells

To determine whether BMP2 influences C3H10T1/2 cells at the transcription level, RNA was extracted from a series of cell samples harvested according to the time course with or without BMP2 inducement and evaluated by Northern blot analysis. The results demonstrated that BMP2 treatment induced cell overexpression of Erk2 mRNA. At 24 h following inducement, Erk2 mRNA level of BMP2-treated cells increased 3-fold compared to the untreated cell samples. The up-regulation of Erk2 mRNA overexpression was maintained during the entire time course up to 72 h following BMP2 inducement (Fig. 1). Our previous results [28] showed that Erk2 protein level is elevated in BMP2-induced C3H10T1/2 cells. Northern blot results from current study demonstrate that the elevation of Erk2 protein level by BMP2 inducement is from the up-regulation at the transcription level.

BMP2 enhanced the activity of the Erk2 promoter

To further confirm BMP2-induced Erk2 overexpression at the transcription level, we generated a reporter construct in which −2425 to +223 bp of Erk2 promoter was fused to a luciferase gene. This construct was transfected into C3H10T1/2 cells and the production of luciferase was monitored as a measure of promoter activity. By normalization with cotransfected β-galactosidase activity for transfection efficiency, we observed a 1.5-fold higher promoter activity after 48 h-exposure to BMP2 (Fig. 2). To define the BMP2-responsive elements in the Erk2 promoter, a series of sequential truncations in the 5′-flanking region of the Erk2 promoter were fused to the luciferase reporter gene. The promoter activities of these constructs were measured following transfection into C3H10T1/2 cells. The results showed that truncations as far as −148 bp from the transcription initiation site did not affect promoter responsiveness to BMP2, although promoter activity was varied in each deletion. All these constructs presented 1.5–2.0-fold higher promoter activities after 48 h of BMP2 exposure. However, truncation to −42 bp upstream from the transcription start site attenuated promoter activity. Moreover, the activity-attenuated constructs were not responsive to BMP2 inducement (Fig. 2). These data further confirmed that the Erk2 promoter activity was induced by BMP2. The results also suggested that there was a BMP2-responsive sequence in the Erk2 promoter and it was possibly located between −42 and −148 bp of the Erk2 promoter.

BMP2 increased binding of nuclear protein to the −148/−42 bp fragment of Erk2 promoter and the BMP2-enhanced binding bands were Sp1 transcription factors

Since BMP2 induction increased the promoter activity of −148 to −42 bp fragment in the Erk2 promoter, it was highly likely that enhancement of transcriptional factors binding to this region should occur. To examine this hypothesis, nuclear extracts from BMP2-treated cells or untreated cells were used to perform an electrophoresis mobility shift assay (EMSA) with ^{32}P-labeled double strand oligo −152 to −38 bp of Erk2

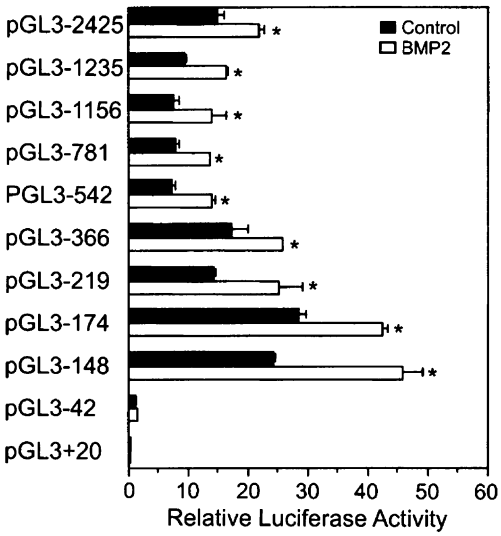

Fig. 2. Effect of BMP2 on Erk2 promoter activity in C3H10T1/2 cells and a 106 bp (−148/−42) BMP2-responsive element. C3H10T1/2 cells were transfected with a series of 5′-truncated mouse Erk2 promoter constructs fused with luciferase (LUC) reporter gene. After 18 h of starvation, cells were treated with either BMP2 (500 ng/ml) or control medium for 48 h. LUC activity was measured and normalized with the activity of co-transfected β-galactosidase. Values represent means ± SD of determinations from at least four independent transfections. Groups marked by an asterisk are significantly different from the control group, $p \leqslant 0.001$.

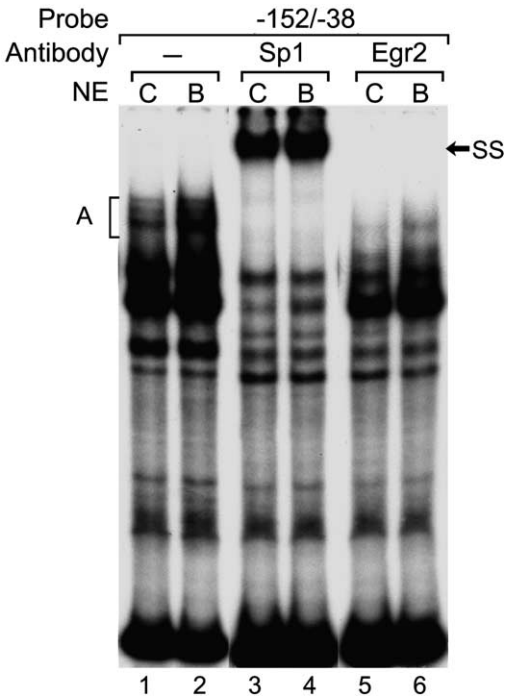

Fig. 3. BMP2 increases the binding of nuclear protein to the probe that spans −148 to −42 of mouse Erk2 promoter and BMP2-enhanced binding bands are Sp1 transcription factors. Cells were treated with control medium (C) or BMP2 (B) for 48 h. Nuclear extracts (NE) were isolated and incubated with ^{32}P-labeled oligo −152/−38 (lanes 1 and 2) or with Sp1 antibody (lanes 3 and 4) and Egr2 antibody (lanes 5 and 6) for supershift assays before addition of radiolabeled −152/−38. The region marked SS designates supershifted bands.

promoter. As the results indicate in Fig. 3 (lanes 1and 2), multiple binding bands were detected. Band A migrated slowly and presented a smear feature. Careful examination demonstrated that there were three bands close to each other in the smear. This group of bands was responsive significantly to BMP2 exposure. Other fast migrating bands did not show any significant change under BMP2 inducement. The results of this experiment demonstrated that certain nuclear transcription factors did bind to the −148/−42 fragment of the Erk2 promoter and BMP2 inducement could enhance such binding. Computer analysis indicated that there were potential Sp1 binding sites within the −148/−42 fragment, which were located in (−147 to −139) and (−51 to −46).

To confirm Sp1 binding sites in the −148/−42 fragment of the Erk2 promoter, as well as to examine whether the Sp1 transcription factor specifically bound to the Sp1 sites in the fragment, a series of competitive gel shift assays and supershift assays were conducted. Results of competitive assays demonstrated that a 100-fold excess unlabeled oligo containing the Sp1 consensus sequence abolished band A completely in both control and BMP2-treated cells, whereas the mutated Sp1 consensus sequence and consensus Egr oligo had no such effect (data not shown). The results of supershift assays (Fig. 3) showed that the anti-Sp1 antibody supershifted band A completely (lanes 3 and 4). As the control, anti-Egr2 antibody did not show such supershift (lanes 5 and 6). These results demonstrated that the slowly migrating binding bands on the −148/−42 fragment which were responsive to BMP2 were Sp1 transcription factors.

Mapping of Sp1 transcriptional factor binding sites in the −148/−42 fragment of Erk2 promoter

After confirming BMP2 dependent increase of Sp1 binding on the −148/−42 fragment of the Erk2 promoter, the location of the Sp1 binding sites in this fragment was further investigated. Five synthesized oligos, which covered the −148/−42 fragment and had 7 bp overlapping with adjacent oligos, were used as probes to map the Sp1 binding sites (Fig. 4A). Gel shift assays using these oligos and nuclear extracts from C3H10T1/2 demonstrated that only oligos S1 and S5 gave rise to slow migration binding bands (bands a, b, and c) (Fig. 4B, lanes 2, 3 and 14, 15), which were similar to the pattern in Fig. 3 (lanes 1 and 2). Moreover, these slow migration bands in oligos S1 and S5 were enhanced under BMP2 inducement. Oligos S2, S3, and S4 did not show such slow migration binding bands in the same assay. This result suggested that BMP2-responsive Sp1 sites in the −148/−42 fragment, which were responsible for creating slow migration binding bands with cell nuclear extracts, were likely located in oligos S1 and S5.

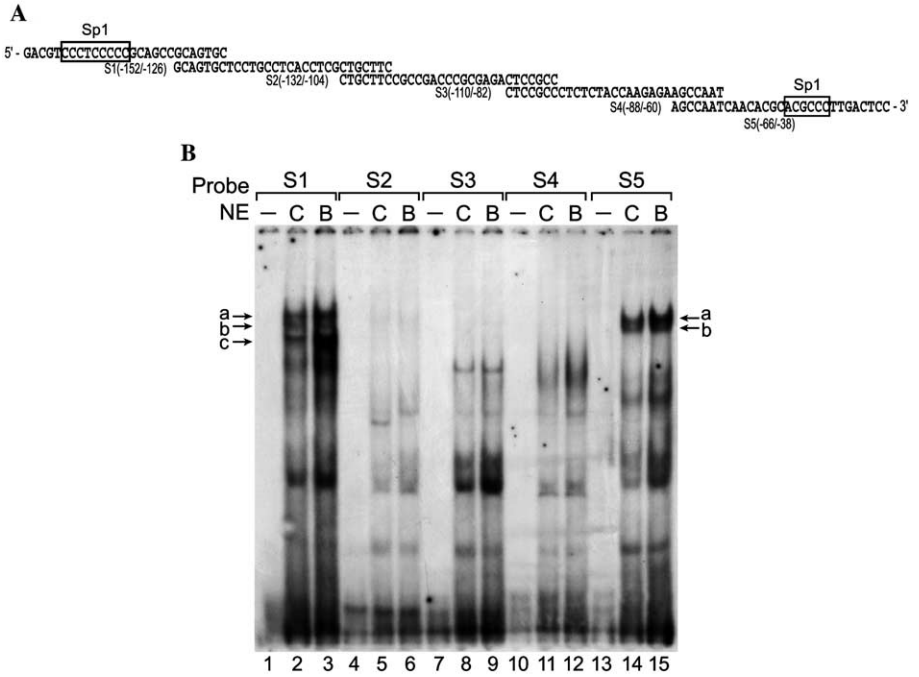

Fig. 4. Mapping of transcriptional factor binding sites in −148/−42 fragment. (A) Five segments (S1–S5) used in gel shift assays were synthesized, which covered nucleotides −148 to −42 and each overlapped 7 bp with its adjacent oligo. The putative Sp1 binding sites were shown as solid boxes. (B) Gel shift assays using radiolabeled oligos S1–S5 only (lanes 1, 4, 7, 10, and 13, respectively). Nuclear extracts (NE) from control cells (C) or BMP2 (B)-treated cells (lanes 2, 5, 8, 11, and 14 or lanes 3, 6, 9, 12, and 15) were reacted with radiolabeled oligos S1–S5.

To further determine whether Sp1 binding sites contributed to such binding in oligos S1 and S5, a series of competitive gel shift assays and supershift assays were conducted. Results of Fig. 5B showed that the slowly migrating bands a and b that S1 probe generated (lanes 1 and 2) were abolished by unlabeled wild-type S1 (lanes 7 and 8) and Sp1 consensus sequence (lanes 3 and 4), whereas the mutant oligos mSp1 and mS1 (Fig. 5A) failed to compete for such binding (lanes 5, 6 and 9, 10) nor did consensus Egr oligo (lanes 11 and 12). Similar results were shown in oligo S5 assay (Fig. 5C): S5 binding with the nuclear extracts was interfered by unlabeled Sp1 consensus sequence and oligo S5 itself (lanes 7, 8 and 3, 4). However, neither mutant mSp1 and mS5 (Fig. 5A) nor consensus Egr oligo affected these bindings (lanes 5, 6; 9, 10; and 11, 12). Also, anti-Sp1 antibody supershifted bands a and b in S1 and S5 probed assays (Figs. 5B and C, lanes 13 and 14). No supershift bands were observed in S2–S4 probed supershift assays (data not shown).

These results indicated that Sp1 binding sites in the −148/−42 fragment, which responded to BMP2 inducement and gave rise to slowly migrating bands, were located in the sequence of oligo S1 (−147 to −139) and oligo S5 (−51 to −46).

Sp1 was involved in BMP2-induced Erk2 expression

After identifying the two Sp1 binding sites in the BMP2-responsive elements in the −148/−42 fragment of the Erk2 promoter, the participation of Sp1 in BMP2-up-regulated Erk2 gene expression needed to be confirmed. Mithramycin, which binds to GC pairs of the DNA and interferes with Sp1 binding, was used in the transfection study. Results showed that the effect of BMP2 on pGL3(−148) of the Erk2 promoter was abolished in mithramycin-treated cells as compared with non-mithramycin-treated cells (data not shown). Furthermore, we investigated whether both the Sp1 sites in the −148/−42 fragment mediated BMP2-induced up-regulation of Erk2 transcription in C3H10T1/2 cells. The point mutation of the Sp1 binding site on the S1 or the S5 sequence was introduced into pGL3(−148) (Fig. 6A). Transfection studies showed that BMP2 treatment increased the activity of the wild-type promoter pGL3(−148) by 86.6%, whereas Mu-1 abolished the stimulation of BMP2 completely. Mu-5 did not affect BMP2 stimulation (Fig. 6B). This result confirmed that the Sp1 site residing at −147/−139 in the −148/−42 fragment of the Erk2 promoter was essential for BMP2-induced up-regulation of Erk2 transcription.

Discussion

In this study, we demonstrated that BMP2 enhances Erk2 gene expression at the transcriptional level and that transcription factor Sp1 mediates the BMP2-up-regulated Erk2 transcription. Our experiments showed

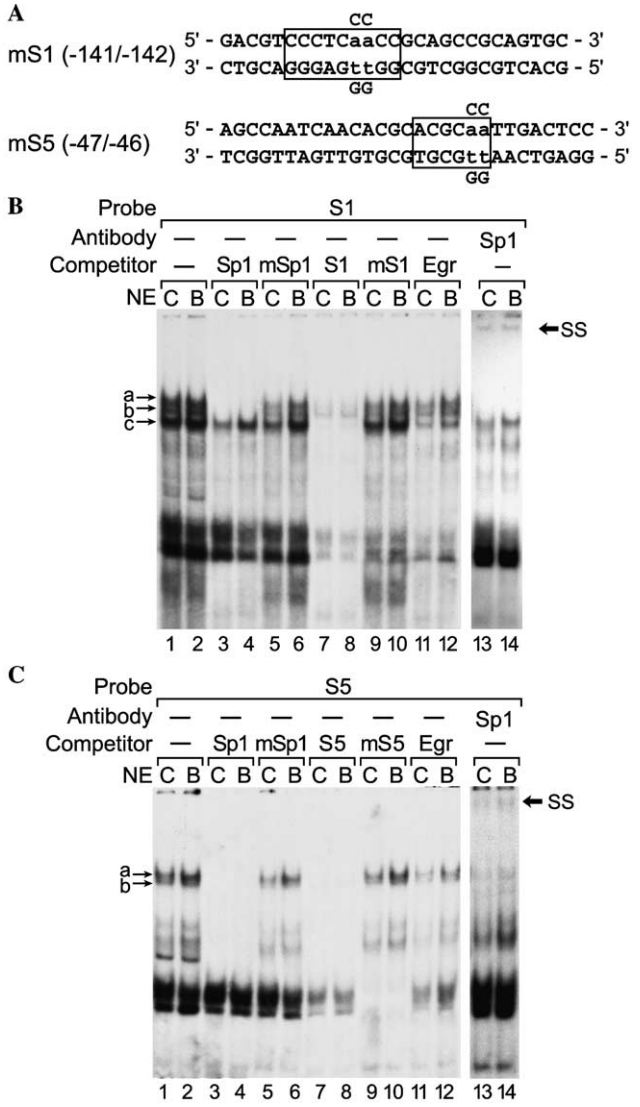

Fig. 5. Competition of Sp1 transcription factor binding sites and Sp1 supershift in oligos S1 and S5 of Erk2 promoter. (A) Sequences of wild-type (uppercase) and mutant (lowercase) S1 and S5. The putative Sp1 binding sites in both oligos are boxed. (B) Gel shift assays using ^{32}P-labeled oligo S1 were competed with 100-fold excess unlabeled Sp1 consensus oligo (5′-ATTCGATCGGGGCGGGGCGAGC-3′) (lanes 3 and 4), mutated Sp1 consensus oligo (5′-ATTCGATCGGttCGGGGCGA GC-3′) (lanes 5 and 6), wild-type oligo S1 (lanes 7 and 8), mutant mS1 (lanes 9 and 10), and Egr consensus oligo (5′-GGATCCAGCGGG GGCGAGCGGGGGCGA-3′) (lanes 11 and 12). (C) Gel shift assays were performed with C3H10T1/2 nuclear extracts and oligo S5 probe. The reactions were competed with excess of unlabeled Sp1 consensus oligo (lanes 3 and 4), mutant Sp1 consensus oligo (lanes 5 and 6), wild-type oligo S5 (lanes 7 and 8), mutant mS5 (lanes 9 and 10), and Egr consensus oligo (lanes 11 and 12). Nuclear extracts prepared from untreated control cells or BMP2-treated cells are indicated as C or B, respectively. For supershift, nuclear extracts (NE) were incubated with Sp1 antibody (Figs. 5B and C, lanes 13 and 14) before addition of radiolabeled S1 or S5. The region marked SS designates supershifted bands.

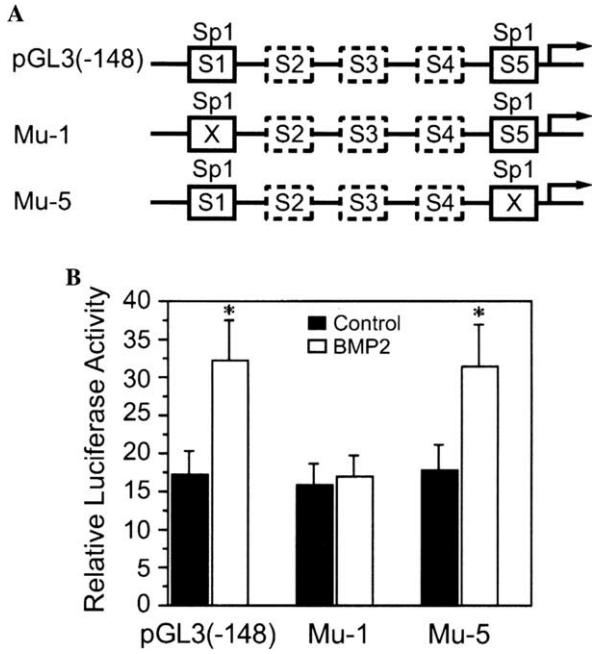

Fig. 6. Effects of mutation in Sp1 sites on BMP2-up-regulated Erk2 promoter activity. (A) pGL3(−148) constructs containing mutations in different Sp1 binding sites. Each contains a point mutation, which abrogated binding in gel shift assays (Fig. 5). (B) Cells were cotransfected with wild-type or mutant (Mu-1 or Mu-5) promoter constructs and PCH110 β-Gal plasmid. After treatment with control medium or BMP2, luciferase activities were measured and normalized to the level of β-Gal. Values represent means ± SD of determinations from at least six independent transfections. Groups marked by an asterisk are significantly different from the control group, $p \leqslant 0.001$.

that the increase of Erk2 protein level in BMP2-induced cells results from the transcriptional up-regulation of Erk2. By analyzing the promoter activity of a series of deletion constructs of the 5′-flanking region of the Erk2 promoter, we found that the −148/−42 fragment of the Erk2 promoter is one of the BMP2-responsive elements. Using gel shift assays, we found that BMP2 enhances the binding of nuclear extracts to −148/−42 and the enhanced binding bands on −148/−42 are Sp1 transcription factors. Mapping study further demonstrated the presence of two BMP2-responsive Sp1 sites in the −148/−42 fragment, which reside in −147/−139 and −51/−46. Transfection assays showed that the addition of the Sp1 binding inhibitor mithramycin or point mutation of the Sp1 site residing at −147/−139 completely abolished the effect of BMP2 on the up-regulation of Erk2 promoter activity. These data indicate that Sp1 can be one of the mediators in BMP2-up-regulated Erk2 gene expression.

Sp1 is a ubiquitous transcriptional factor containing three zinc fingers, Cys_2–His_2, which binds the homologous GC-boxes and related motifs present in many promoters including the mouse Erk2 promoter [32]. Sp1 is involved in the expression of many different genes in a constitutive or an inducible manner [33–35]. The other three Sp1-related transcription factors Sp2, Sp3, and Sp4 were also cloned [33]. All four members have similar domain structures, but Sp1, Sp3, and Sp4 are more

closely related to each other than to Sp2 [33]. Sp3 has been shown to act as a transcriptional activator or as a repressor of Sp1-mediated activation [36,37]. Sp4 is a transcriptional activator, predominantly found in the brain [38]. It is known that Sp1 and Sp3 recognize the same consensus sequence [38]. We assumed that these two proteins compete for the same binding sites on the BMP2-responsive element −148/−42 of the Erk2 promoter. Supershift assays using the anti-Sp3 antibody and the probe −152/−38 fragment of the Erk2 promoter were performed in our laboratory. The results demonstrated that supershift bands were present in both control and BMP2-treated groups (data not shown). This result indicated that Sp3 also binds to the Erk2 promoter. Co-relations between Sp1 and Sp3 in BMP2-induced Erk2 promoter activity are unclear.

In this study, we have found that multiple binding bands occur between Sp1 factor and the related probes. These results indicate that transcription factor Sp1 in C3H10T1/2 cells nuclei exits in different forms. It may combine other nuclei factors or it has multiple post-translational modifications [39]. In addition, different spliced forms and different sizes of Sp1 mRNA have been reported [40,41]. All these differences will lead to changes in the sizes of Sp1–DNA binding complexes, which result in different migrations in electrophoresis. A similar phenomenon was also found in other papers [42,43].

In this study, we also found that although there are two Sp1 sites in the BMP2-responsive element −148/−42 of Erk2 promoter, only Sp1 site at −147/−139 is involved in BMP2-up-regulation of Erk2 promoter activity. The Sp1 site at −51/−46 does not take part in regulating Erk2 expression. There is an interpretation that variations in sequence of the regions flanking Sp1 binding domains may lead to altered DNA conformation and, hence, function [44]. Similar findings have been reported for other promoters, such as integrin β^5, p21, $p15^{INK4B}$, and α(I) procollagen [34,44–46].

A physical and/or functional interaction between Sp1 and other transcription factors have been documented [47–49] and Smad proteins, which are important in BMP2-induced osteoblast differentiation, have also been reported to interact with Sp1 in TGF-β signaling [44,50,51]. Such interactions have not been observed during the signal transduction between BMP2 and Erk, although we have identified the role of Sp1 in BMP2-up-regulated Erk2 gene expression.

MAPK plays an important role in cell proliferation and differentiation [27]. However, its role in BMP signaling is not clear and these two pathways appear to coordinate or oppose in different experimental systems [18,52]. Our previous data demonstrated that Erk/MAPK is involved in BMP2-induced osteoblast differentiation via an increase of Erk protein expression [28]. In this study, we confirmed that the increase of Erk2 protein level in BMP2-induced cells is from BMP2-up-regulated mRNA expression and transcription factor Sp1 is involved in BMP2-up-regulation of Erk2 gene expression.

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References

[1] B.L. Hogan, Bone morphogenetic proteins: multifunctional regulators of vertebrate development, Genes Dev. 10 (1996) 1580–1594.
[2] D.M. Kingsley, The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms, Genes Dev. 8 (1994) 133–146.
[3] T. Sakou, Bone morphogenetic proteins: from basic studies to clinical approaches, Bone 22 (1998) 591–603.
[4] P. ten Dijke, H. Yamashita, T.K. Sampath, A.H. Reddi, M. Estevez, D.L. Riddle, H. Ichijo, C.H. Heldin, K. Miyazono, Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4, J. Biol. Chem. 269 (1994) 16985–16988.
[5] B.L. Rosenzweig, T. Imamura, T. Okadome, G.N. Cox, H. Yamashita, P. ten Dijke, C.H. Heldin, K. Miyazono, Cloning and characterization of a human type II receptor for bone morphogenetic proteins, Proc. Natl. Acad. Sci. USA 92 (1995) 7632–7636.
[6] F. Liu, F. Ventura, J. Doody, J. Massague, Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs, Mol. Cell. Biol. 15 (1995) 3479–3486.
[7] T. Nohno, T. Ishikawa, K. Hosokawa, S. Noji, D.H. Wolsing, J.S. Rosenbaum, Identification of a human type II receptor for bone morphogenetic protein-4 that forms differential heteromeric complexes with bone morphogenetic protein type I receptors, J. Biol. Chem. 270 (1995) 22522–22526.
[8] F. Liu, A. Hata, J.C. Baker, J. Doody, J. Carcamo, R.M. Harland, J. Massague, A human Mad protein acting as a BMP-regulated transcriptional activator, Nature 381 (1996) 620–623.
[9] G.H. Thomsen, Xenopus mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor, Development 122 (1996) 2359–2366.
[10] M. Kretzschmar, J. Massague, Smads: mediators and regulators of TGF-β signaling, Curr. Opin. Genet. Dev. 8 (1998) 103–111.
[11] A. von Bubnoff, K.W. Cho, Intracellular BMP signaling regulation in vertebrates: pathway or network? Dev. Biol. 239 (2001) 1–14.
[12] K. Yamaguchi, K. Shirakabe, H. Shibuya, K. Irie, I. Oishi, N. Ueno, T. Taniguchi, E. Nishida, K. Matsumoto, Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction, Science 270 (1995) 2008–2011.
[13] H. Shibuya, H. Iwata, N. Masuyama, Y. Gotoh, K. Yamaguchi, K. Irie, K. Matsumoto, E. Nishida, N. Ueno, Role of TAK1 and TAB1 in BMP signaling in early Xenopus development, EMBO J. 17 (1998) 1019–1028.
[14] K. Monzen, I. Shiojima, Y. Hiroi, S. Kudoh, T. Oka, E. Takimoto, D. Hayashi, T. Hosoda, A. Habara-Ohkubo, T. Nakaoka, T. Fujita, Y. Yazaki, I. Komuro, Bone morphogenetic

proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4, Mol. Cell. Biol. 19 (1999) 7096–7105.
[15] S. Iwasaki, M. Iguchi, K. Watanabe, R. Hoshino, M. Tsujimoto, M. Kohno, Specific activation of the p38 mitogen-activated protein kinase signaling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2, J. Biol. Chem. 274 (1999) 26503–26510.
[16] O. Kozawa, D. Hatakeyama, T. Uematsu, Divergent regulation by p44/p42 MAP kinase and p38 MAP kinase of bone morphogenetic protein-4-stimulated osteocalcin synthesis in osteoblasts, J. Cell. Biochem. 84 (2002) 583–589.
[17] N. Kimura, R. Matsuo, H. Shibuya, K. Nakashima, T. Taga, BMP2-induced apoptosis is mediated by activation of the TAK1–p38 kinase pathway that is negatively regulated by Smad6, J. Biol. Chem. 275 (2000) 17647–17652.
[18] S. Palcy, D. Goltzman, Protein kinase signalling pathways involved in the up-regulation of the rat α1(I) collagen gene by transforming growth factor β1 and bone morphogenetic protein 2 in osteoblastic cells, Biochem. J. 343 (1999) 21–27.
[19] M.R. Urist, Bone morphogenetic protein: the molecularization of skeletal system development, J. Bone Miner. Res. 12 (1997) 343–346.
[20] J.M. Wozney, V. Rosen, Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair, Clin. Orthop. 346 (1998) 26–37.
[21] J. Lou, F. Xu, K. Merkel, P. Manske, Gene therapy: adenovirus-mediated human bone morphogenetic protein-2 gene transfer induces mesenchymal progenitor cell proliferation and differentiation in vitro and bone formation in vivo, J. Orthop. Res. 17 (1999) 43–50.
[22] F. Lecanda, L.V. Avioli, S.-L. Cheng, Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2, J. Cell. Biochem. 67 (1997) 386–396.
[23] T. Katagiri, A. Yamaguchi, M. Komaki, E. Abe, N. Takahashi, T. Ikeda, V. Rosen, J.M. Wozney, A. Fujisawa-Sehara, T. Suda, Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage, J. Cell Biol. 127 (1994) 1755–1766.
[24] M. Lind, E.F. Eriksen, C. Bunger, Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells, Bone 18 (1996) 53–57.
[25] R. Nishimura, Y. Kato, D. Chen, S.E. Harris, G.R. Mundy, T. Yoneda, Smad5 and DPC4 are key molecules in mediating BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal precursor cell line C2C12, J. Biol. Chem. 273 (1998) 1872–1879.
[26] N. Yamamoto, S. Akiyama, T. Katagiri, M. Namiki, T. Kurokawa, T. Suda, Smad1 and Smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts, Biochem. Biophys. Res. Commun. 238 (1997) 574–580.
[27] M.H. Cobb, MAP kinase pathways, Prog. Biophys. Mol. Biol. 71 (1999) 479–500.
[28] J. Lou, Y. Tu, S. Li, P.R. Manske, Involvement of ERK in BMP-2 induced osteoblastic differentiation of mesenchymal progenitor cell line C3H10T1/2, Biochem. Biophys. Res. Commun. 268 (2000) 757–762.
[29] S. Gallea, F. Lallemand, A. Atfi, G. Rawadi, V. Ramez, S. Spinella-Jaegle, S. Kawai, C. Faucheu, L. Huet, R. Baron, S. Roman-Roman, Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells, Bone 28 (2001) 491–498.
[30] E. Schreiber, P. Matthias, M.M. Muller, W. Schaffner, Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells, Nucleic Acids Res. 17 (1989) 6419.
[31] J.G. Jiang, B. Gao, R. Zarnegar, The concerted regulatory functions of the transcription factors nuclear factor-1 and upstream stimulatory factor on a composite element in the promoter of the hepatocyte growth factor gene, Oncogene 19 (2000) 2786–2790.
[32] G. Suske, The Sp-family of transcription factors, Gene 238 (1999) 291–300.
[33] L. Lania, B. Majello, P. De Luca, Transcriptional regulation by the Sp family proteins, Int. J. Biochem. Cell Biol. 29 (1997) 1313–1323.
[34] S.A. Jimenez, J. Varga, A. Olsen, L. Li, A. Diaz, J. Herhal, J. Koch, Functional analysis of human α1(I) procollagen gene promoter. Differential activity in collagen-producing and -non-producing cells and response to transforming growth factor β1, J. Biol. Chem. 269 (1994) 12684–12691.
[35] A. Moustakas, D. Kardassis, Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members, Proc. Natl. Acad. Sci. USA 95 (1998) 6733–6738.
[36] L. Zhao, L.S. Chang, The human POLD1 gene. Identification of an upstream activator sequence, activation by Sp1 and Sp3, and cell cycle regulation, J. Biol. Chem. 272 (1997) 4869–4882.
[37] G. Hagen, S. Muller, M. Beato, G. Suske, Sp1-mediated transcriptional activation is repressed by Sp3, EMBO J. 13 (1994) 3843–3851.
[38] G. Hagen, S. Muller, M. Beato, G. Suske, Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes, Nucleic Acids Res. 20 (1992) 5519–5525.
[39] A.R. Black, J.D. Black, J. Azizkhan-Clifford, Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer, J. Cell. Physiol. 188 (2001) 143–160.
[40] T. Takahara, S.I. Kanazu, S. Yanagisawa, H. Akanuma, Heterogeneous Sp1 mRNAs in human HepG2 cells include a product of homotypic trans-splicing, J. Biol. Chem. 275 (2000) 38067–38072.
[41] S.P. Persengiev, P.J. Raval, S. Rabinovitch, C.F. Millette, D.L. Kilpatrick, Transcription factor Sp1 is expressed by three different developmentally regulated messenger ribonucleic acids in mouse spermatogenic cells, Endocrinology 137 (1996) 638–646.
[42] J.H. Yu, G. Schwartzbauer, A. Kazlman, R.K. Menon, Role of the Sp family of transcription factors in the ontogeny of growth hormone receptor gene expression, J. Biol. Chem. 274 (1999) 34327–34336.
[43] H. Fukuda, T. Noguchi, N. Iritani, Transcriptional regulation of insulin receptor gene promoter in rat hepatocytes, Biochem. Biophys. Res. Commun. 280 (2001) 1274–1278.
[44] C.F. Lai, X. Feng, R. Nishimura, S.L. Teitelbaum, L.V. Avioli, F.P. Ross, S.L. Cheng, Transforming growth factor-β up-regulates the β5 integrin subunit expression via Sp1 and Smad signaling, J. Biol. Chem. 275 (2000) 36400–36406.
[45] M.B. Datto, Y. Yu, X.F. Wang, Functional analysis of the transforming growth factor β responsive elements in the WAF1/Cip1/p21 promoter, J. Biol. Chem. 270 (1995) 28623–28628.
[46] J.M. Li, M.A. Nichols, S. Chandrasekharan, Y. Xiong, S.F. Wang, Transforming growth factor β activates the promoter of cyclin-dependent kinase inhibitor p15INK4B through an Sp1 consensus site, J. Biol. Chem. 270 (1995) 26750–26753.
[47] A. Gualberto, A.S. Baldwin, p53 and Sp1 interact and cooperate in the tumor necrosis factor-induced transcriptional activation of the HIV-1 long terminal repeat, J. Biol. Chem. 270 (1995) 19680–19683.
[48] S.Y. Lin, A.R. Black, D. Kostic, S. Pajovic, C.N. Hoover, J.C. Azizkhan, Cell cycle-regulated association of E2F1 and Sp1 is

related to their functional interaction, Mol. Cell. Biol. 16 (1996) 1668–1675.

[49] D. Kardassis, E. Falvey, P. Tsantili, M. Hadzopoulou-Cladaras, V. Zannis, Direct physical interactions between HNF-4 and Sp1 mediate synergistic transactivation of the apolipoprotein CIII promoter, Biochemistry 41 (2002) 1217–1228.

[50] X.H. Feng, X. Lin, R. Derynck, Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-β, EMBO J. 19 (2000) 5178–5193.

[51] P.K. Datta, M.C. Blake, H.L. Moses, Regulation of plasminogen activator inhibitor-1 expression by transforming growth factor-β-induced physical and functional interactions between Smads and Sp1, J. Biol. Chem. 275 (2000) 40014–40019.

[52] G. Ghosh Choudhury, D.C. Jin, Y. Kim, A. Celeste, N. Ghosh-Choudhury, H.E. Abboud, Bone morphogenetic protein-2 inhibits MAPK-dependent Elk-1 transactivation and DNA synthesis induced by EGF in mesangial cells, Biochem. Biophys. Res. Commun. 258 (1999) 490–496.