# Sp1-Mediated Transcriptional Control of Fibroblast Growth Factor Receptor 4 in Sarcomas of Skeletal Muscle Lineage

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## ABSTRACT

**Fibroblast growth factor receptors (FGFRs) have been implicated in a multitude of differentiating and proliferative actions. FGFR4 is expressed mainly in lung, kidney, pancreas, spleen, and developing muscle. FGFR4 was found to be overexpressed in some human malignancies, where it has been implicated in their pathogenesis. Recently, FGFR4 was found to be overexpressed in pediatric rhabdomyosarcomas, based on cDNA microarray analysis. Using Northern blotting, reverse transcription-polymerase chain reaction, and Western blotting, we classified four human rhabdomyosarcoma-derived cell lines based on their relative expression of FGFR4. We defined a 214 bp (−115/+99) promoter that functioned as a minimal promoter and examined *cis*-DNA elements implicated in the control of expression of the FGFR4 gene in these cells. Overlapping 40- to 50-bp fragments of the minimal promoter were examined by electrophoretic mobility shift assay using nuclear extracts from cell lines with high (HS729-1015) or low (HS729-1016) FGFR4 expression. Fragment C (−65/−26) formed specific complexes with nuclear extracts from both cell lines. Fragment B (−95/−56), however, formed distinct complexes mainly with the high FGFR4-expressing HS729-1015 cells. Both fragments yielded complexes that were competed by an Sp oligonucleotide and supershifted by Sp1 and by Sp3 antibodies. Transfection of Sp1 but not Sp3 efficiently activated FGFR4 promoter activity, an effect that was significantly more pronounced in the HS729-1015 cell line than in the low FGFR4-expressing HS729-1016 cell line. Deletion of each of the two Sp-binding sites in fragments B and C resulted in loss of promoter activity. In particular, deletion of the 5′ Sp-binding site in fragment B was associated with the greatest loss of activity. Sp1 protein expression correlated with FGFR4 expression in cell lines and primary human rhabdomyosarcomas. Furthermore, transfection of Sp1 and methylation inhibition was effective in inducing the endogenous FGFR4 gene in HS729-1015 cells. Our findings point to Sp1 as an important contributor to FGFR4 transcriptional control and elucidate a potential mechanism for the heterogenous expression of FGFR4 in neoplasms derived from the same cell lineage.**

## INTRODUCTION

Myogenic cells proliferate as mononucleated myoblasts before differentiating into postmitotic multinucleated skeletal muscle fibers. This process is governed by signal transduction cascades heavily orchestrated by growth factors. Of these, members of the fibroblast growth factor (FGF) and FGF receptor (FGFR) family have been implicated in sustaining myoblast proliferation and possibly delaying their differentiation (1). FGF-1 and FGF-2 possess well-documented mitogenic activity on skeletal muscle cells; both can activate FGFR1 in proliferating myoblasts (2). Human skeletal muscle-derived malignancies are referred to as rhabdomyosarcoma and are well known to express the FGF-2 gene and protein (3). Moreover, FGF-2 stimulation of myoblasts induces SHP-2 complex formation with fibroblast growth factor receptor substrate (FRS2) and induces Erk activity and Elk-1 transactivation. Overexpression of SHP-2 potentiates the suppressive effects of FGF-2 on muscle-specific gene expression and myogenesis (4), further highlighting the importance of FGFR signaling in myogenic differentiation.

FGF signaling is mediated through four dedicated FGFRs. There are currently four known mammalian FGFR genes encoding a complex family of transmembrane receptor tyrosine kinases (5). Each prototypic receptor is composed of three immunoglobulin-like extracellular domains, a single transmembrane domain, a split tyrosine kinase, and a COOH-terminal tail with multiple autophosphorylation sites (5). Multiple cell-bound or secreted forms of all FGFRs result from alternative transcription initiation, alternative splicing, exon switching, or variable polyadenylation.

Recent evidence has emphasized the importance of FGFR4 in skeletal muscle. There is marked loss of skeletal muscle development in chick embryos resulting from loss of FGFR4 signaling (6). FGFR4 is also expressed in lung, kidney, adrenal gland, pancreas, and spleen (7, 8). The role of FGFR4 in carcinogenesis is poorly understood. FGFR4 mediates membrane ruffling in breast carcinoma cells (9), where it is overexpressed (10), and it has been shown to modulate erythroid cell proliferation (11). A tumor-derived FGFR4 (ptd-FGFR4) isoform recapitulates pituitary tumorigenesis in transgenic mice

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(12). Recently, FGFR4 was found to be overexpressed in pediatric rhabdomyosarcomas (13). Little is known, however, about FGFR4 regulation and the mechanisms leading to FGFR4 overexpression in general or in neoplasms.

In this report, we sought to obtain insight into the regulatory mechanisms governing FGFR4 gene regulation and overexpression. We took advantage of rhabdomyosarcomas as an example of neoplasms derived from the same lineage with variable degrees of FGFR4 expression. We mapped the elements required for minimal promoter activity and identified relevant transcription factors in tumors with low and high FGFR4 expression. Our studies point to a role for the Sp1 transcription factor as an important determinant of FGFR4 regulation.

## MATERIALS AND METHODS

**Cell Culture.** The human rhabdomyosarcoma cell lines examined HS729, HS729-1014, HS729-1015, HS729-1016, and RD3-1019 were generously provided by Dr. J. Squire (University of Toronto, Toronto, Ontario, Canada) as described previously (14). Cells were grown in α-modified Eagle's medium with high glucose supplemented with 20% fetal bovine serum (Sigma, Oakville, Ontario, Canada), 1% glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. For methylation inhibition studies, cells were treated for 16 hours with 5′-azacytidine (Sigma) at a final concentration of 10 μmol/L. Histone deacetylase inhibition was performed using trichostatin A (Sigma) at 100 ng/mL for 24 hours. Primary human embryonal rhabdomyosarcomas were identified on the basis of currently accepted histopathologic criteria.

**Western Blotting Analysis.** Protein concentrations were determined by the Bio-Rad (Hercules, CA) protein assay. Equal amounts of protein (50 μg) from whole cell lysates or nuclear fractions were solubilized in 2× SDS sample buffer, separated on SDS-8% polyacrylamide gels, and transferred to nitrocellulose. Blots were incubated with Sp1 or Sp3 antibody (1:1,000; both from Santa Cruz Biotechnology, Santa Cruz, CA) or with a polyclonal affinity-purified rabbit antiserum directed against the COOH terminus of human FGFR1 (1:1,000; Santa Cruz Biotechnology) and FGFR4 (1:1,000; Santa Cruz Biotechnology) or mouse β-actin (1:500; Sigma).

**Messenger RNA Analysis.** Total RNA was extracted by the Trizol method (Invitrogen, Burlington, Ontario, Canada). One microgram of DNase-treated RNA was used for reverse transcription. Reverse transcription was performed using 2.5 units/mL murine leukemia virus reverse transcriptase, 2.5 mmol/L $MgCl_2$, 1 mmol/L deoxynucleoside triphosphate, 2.5 mmol/L random hexamers, and 1 unit/mL RNase inhibitor. The integrity of RNA from each sample was assessed by amplification of the PGK-1 housekeeping gene as described previously (15). Polymerase chain reaction (PCR) analyses of FGFR4 mRNA were performed with the following primers: the extracellular domain, 5′-CCTGTTGGGGGTCCTGCTGAGTGTG-3′ (sense; corresponding to nucleotides 73 to 93 of the human FGFR4 cDNA) and 5′-AATAGGCACAGTTACCCCCAGCAG-3′ (antisense; corresponding to nucleotides 478 to 490); and the kinase domain, 5′-GGCAGCATCCGCTATAACTACCG-3′ (sense; corresponding to nucleotides 745 to 767) and 5′-CTGACCAAGCCAGCGCCTGTGGC-3′ (antisense; corresponding to nucleotides 1536 to 1557 of the human FGFR4). The identity of all PCR products was verified by sequencing.

For Northern blotting, 20 μg of DNase-treated RNA were electrophoresed on a 1% agarose gel, transferred to a nitrocellulose membrane, fixed with an ultraviolet cross-linker, prehybridized, then hybridized with a 500-bp fragment corresponding to the kinase region of the hFGFR4 cDNA. The blot was washed and exposed for 24 to 72 hours at −70°C. Equal loading of samples was shown by subsequent rehybridization of the same blot for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

**Plasmids.** Promoter analysis of the human FGFR4 gene was performed with the assistance of gene finder.[4] Possible transcription start sites were detected with the TRANSFAC-Promoter 2.0 Prediction Program (gene finder). To generate the −1133/+99 fragment, P1 artificial chromosome (PAC) 32C5 was used for PCR with an upstream primer containing a *Kpn*I restriction site and a downstream primer containing a *Bgl*II or *Hind*III site, permitting subcloning into the corresponding sites of the multiple cloning site of the promoterless firefly luciferase expression vector pGL3 (Promega, Madison, WI) to produce a P(−1133/+99)-Luc construct. Other reporters [P(−855/+99)-Luc, P(−173/+99)-Luc, and P(−115/+99)-Luc] were generated by restriction of the P(−1133/+99)-Luc construct with *Stu*I, *Xho*I, and *Sma*I, respectively. P(−535/+99)-Luc was constructed by PCR using a primer containing an *Fsp*I restriction site. The P(+13/+99)-Luc construct was synthesized (Sigma) and similarly positioned into the pGL3 basic vector. The construct P(−1133/−173)-Luc was generated by deleting the 3′-end of the −1133/+99 construct using *Xho*I with subsequent ligation into the corresponding *Kpn*I and *Xho*I sites in pGL3. The orientation and sequence of all constructs were verified by restriction analysis and nucleotide sequencing.

The pPAC expression vectors encoding Sp1 and Sp3 were kindly provided by Dr. P. Marsden (University of Toronto, Toronto, Ontario, Canada; ref. 16) and cloned into the mammalian pcDNA3.1 vector (Invitrogen). Sequence fidelity was confirmed by direct sequencing. The expression vector pSG5-Ets-1 encoding the chicken Ets-1 isoform was obtained from Dr. A. Bradford (University of Colorado, Denver, CO).

**Transfection and Luciferase Assays.** All plasmid reporters were prepared by column chromatography (Qiagen, Mississauga, Ontario, Canada) for sequencing and transfections. Cells were transfected by the LipofectAMINE method (Invitrogen) according to the manufacturer's protocol. Cells were plated into 12 wells ($3–5 \times 10^4$ cells per well) and transfected the following day with LipofectAMINE (3 μL per well) and DNA (0.5–2 μg per well) as indicated. The total amount of transfected DNA was kept constant by adding empty vector. Transfection efficiency was monitored by simultaneous cotransfection with a β-galactosidase control expression plasmid, CMV-βgal (20 ng per well). Twenty-four hours after transfection, cells were lysed in buffer containing 25 mmol/L glycylglycine, 15 mmol/L $MgSO_4$, 4 mmol/L EGTA, 1% Triton X-100, and 1 mmol/L

[4] http://genome.cbs.dtu.dk/htbin/nph-webface.

dithiothreitol (DTT). Luciferase activity was measured for 20 seconds in a lumat LB 9507 luminometer. β-Galactosidase activity was measured to normalize for variations in transfection efficiency. Promoter activity of each construct was expressed as the ratio of firefly luciferase activity to β-galactosidase activity. Each experiment was performed independently on three separate occasions with triplicate wells in each experiment.

**Preparation of Nuclear Extracts.** Nuclear extracts were prepared by washing cells in 1× PBS and lysis in 100 μL of buffer containing 10 mmol/L HEPES (pH 7.9), 1 mmol/L DTT, 1 mmol/L EDTA, 60 mmol/L KCl, 0.5% Nonidet P-40, and 1 mmol/L phenylmethylsulfonyl fluoride for 5 minutes on ice. The pellet was resuspended in 100 μL of nuclear resuspension buffer [0.25 mmol/L Tris-HCl (pH 7.8), 60 mmol/L KCl, 1 mmol/L DTT, and 1.5 mmol/L phenylmethylsulfonyl fluoride] and lysed with three cycles of freezing and thawing to 37°C. After centrifugation at 13,000 rpm for 10 minutes at 4°C, clear supernatant was collected for further analysis. Protein concentrations were determined by the Bio-Rad protein assay.

**Electrophoretic Mobility Shift Assays.** Oligonucleotides were end-labeled with [α-$^{32}$P]dCTP using the Klenow fragment of DNA polymerase. Overlapping double-stranded oligonucleotide fragments of FGFR4 (Fig. 1) between −115 and +99 were used as probes and for competition in electrophoretic mobility shift assays (EMSAs) as follows: fragment B (−95/−56), 5′-GAAGGAGGGGCGGGCCCGAGCAGGAGGGGGCGGGC-CCGAG-3′; fragment C (−65/−26), 5′-CGGGCCCGAGGGG-CGGGGCGGGACAGGAGGTGGGCCGATC-3′ (sense); fragment D (−35/+4), 5′-TGGGCCGCTCGCGGCACGCCGCCG-TCGCGGGTACATTCCT-3′; fragment E (−5/+24), 5′-GTACA-TTCCTCGCTCCCGGCCGAGGAGCGC-3′; fragment F (+15/+54), 5′-CGAGGAGCGCTCGGGCTGTCTGCGGACCCTGC-CGCGTGCA-3′; and fragment G (+49/+99), 5′-CGTGCAGGG-GTCGCGGCCGGC TGGAGCTGGGAGTGAGGCGGCGGAG-GAGC-3′. Fragment A containing long stretches of Gs [(−115/−86); sense, 5′-GGGTGGGGGGGGGGGCGTGGAAGGAG-GGG-3′] could not be synthesized.

Competitor oligonucleotides containing transcription binding sites were as follows: Sp1, 5′-ATTCGATCG*GGGCGG*GG-CGAGC-3′; Ets-1, 5′-GGGCTGCTT*GAGGAA*GTATAAGA-AT-3′; and Ikaros, 5′-AAGAAG*CGGGAG*TG ACA GG-3′. Other competitors tested included the following: AP-1, 5′-CGCTTGAT-*GAGTCA*GCCGGAA-3′; AP-2, 5′-GATCGAACTGACCG*CCC-GCG*GCCCGT-3′; AP-4, 5′-TTACTCC*CAGC*TCCAGCCGG-3′; cAMP-responsive element binding protein (CREB), 5′-AGAGATTGCC*TGACGTCA*GAGAGCTAG-3′; and Yy-1, 5′-CGCTCCGCG*GCCA*TCTTGGCGGCT-3′. Their complementary strands were synthesized by Sigma. The complementary strands were annealed in annealing buffer [10 mmol/L Tris-Cl (pH 8.0), 50 mmol/L NaCl, and 1 mmol/L EDTA]. Gel shift probes were radiolabeled using Klenow fragment I (Boehringer, Burlington, Ontario, Canada) and purified on a G50 spin column. For EMSA, 100 cpm of $^{32}$P-labeled probe were incubated with 5 μg of nuclear extracts at room temperature for 30 minutes in a binding reaction consisting of 20 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L $MgCl_2$, 2%

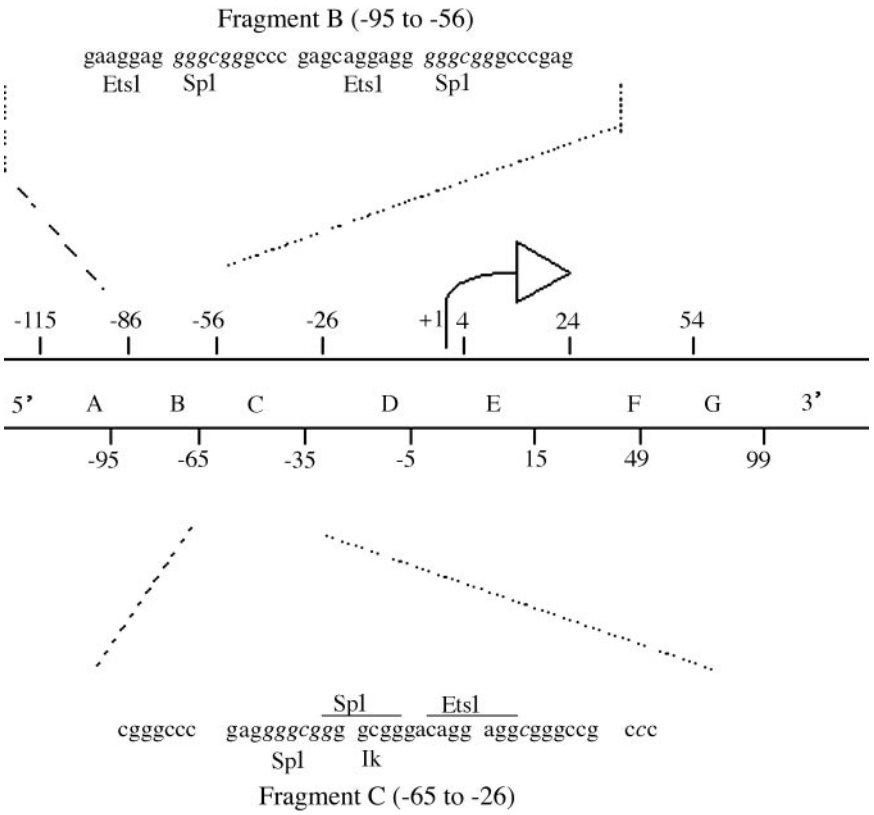


*Fig. 1* Predicted transcription factor binding elements in the human FGFR4 promoter. The putative human FGFR4 promoter was divided into overlapping fragments (*A*−*G*) that were used to further characterize individual binding factors in rhabdomyosarcoma cell lines. Note the presence of two distinct Sp-binding sites in fragments B and C and the flanking Ikaros (*Ik*)- and Ets-binding sites in fragment C.

glycerol, and 1 μg of poly(deoxyinosinic-deoxycytidylic acid) in a final volume of 20 μL. For competition assays, antibody to the relevant transcription factor was added 30 minutes before addition of radiolabeled probe. Antibodies to Sp1, Sp3, Ikaros, and Ap factors were used as described previously (17). Samples were electrophoresed on 4% polyacrylamide nondenatured gels containing 0.5% Tris-borate buffer and 2% glycerol. Gels were dried under vacuum and autoradiographed.

**Sp1-Binding Site Deletions.** The FGFR4 minimal promoter contains four potential Sp-binding sites (Fig. 1). This highly GC-rich region precluded a selective site-directed mutagenesis approach. Instead, we used a combination of restriction digestion and PCR to progressively delete all four Sp-binding sites. The most 5′ site in the P(−115/+99)-Luc construct was deleted using *Apa*I digestion (genomic nucleotide 1055) to generate the P(−84/+99)-Luc reporter. The other three Sp-binding sites were eliminated by PCR using specific mutagenic sense primers as follows: 5′-CCGAGCAGGAGG***GTTCGG***GCCCGAGGG-3′ corresponding to nucleotides 1059 to 1076; 5′- GCGGGCCCA***GGTTCGG***GGCGGGACAGGAG-3′ corresponding to nucleotides 1073 to 1102; and 5′- GCCCGAGG***TTC*G*GTTCGG***GACAGGAGGTG-3′ corresponding to nucleotides 1077 to 1105. The common antisense primer was 5′-GCTCCTCCGCCGCCTCACTC-3′. The generated reporter constructs were referred to as P(−80/+99)-Luc, P(−66/+99)-Luc, and P(−62/+99)-Luc, respectively. The PCR conditions were as follows: denaturation at 95°C for 3 minutes; 30 cycles of 95°C for 40 seconds, 56°C for 45 seconds, and 72°C for 1 minute; and, finally, 72°C for 7 minutes. All inserts were confirmed by restriction digestion and nucleotide sequencing.

**Statistical Analysis.** Data are presented as mean ± SD. Differences were assessed by Student's paired *t* test. Significance level was assigned at $P < 0.05$.

## RESULTS

**Characterization of Fibroblast Growth Factor Receptor 4 Expression in Human Rhabdomyosarcoma Cell Lines.** To investigate protein expression of FGFR4 in human rhabdomyosarcomas, we examined multiple cell lines of human embryonal rhabdomyosarcoma lineage by Western blotting. Using an antibody that specifically recognizes the COOH terminus of human FGFR4, the HS729-1015 cell line revealed strong expression, and one cell line, RD3-1019, exhibited moderate expression of a 110-kDa immunoreactive protein consistent with full-length FGFR4 (Fig. 2*A*). This protein band was abolished when cell lysates were preincubated with FGFR4 blocking peptide (data not shown). In contrast, the HS729 and HS729-1016 cell lines exhibited minimal FGFR4 immunoreactivity. Interestingly, there was an inverse relationship between FGFR4 and FGFR1 expression; the FGFR4-reactive HS729-1015 line was negative for FGFR1, whereas the FGFR4-negative HS729-1016 line exhibited the most FGFR1 reactivity (Fig. 2*A*). RNA examination by reverse transcription-PCR (data not shown) and Northern blotting (Fig. 2*B*) for FGFR4 yielded results similar to those obtained from Western blotting studies. Based on these findings, we selected the HS729-1015 and HS729-1016 cell lines as examples of strong and minimal FGFR4 expression, respectively, and these lines were used for subsequent studies.

**Characterization of the Human Fibroblast Growth Factor Receptor 4 Promoter in Sarcoma Cells.** Interactive elements, such as TATA or CAAT boxes, often regulate the assembly and efficiency of the basic transcriptional machinery. As with other FGFRs, the FGFR4 gene lacks a classic TATA box but includes a 5′ upstream region that is rich in GC residues. The latter contains many consensus motifs that would predict recruitment of RNA polymerase II and initiate gene transcription. We isolated the 5′-upstream region of the human FGFR4 gene from PAC 32C5. The region −1133 to +99 from the transcription start site (18, 19) was placed upstream of the luciferase-reporter vector pGL3-basic. A series of deletion analyses were used to define the minimal promoter and fragments essential for functional activity. To determine potential cell-specific differences, these promoters were transfected into the rhabdomyosarcoma cells and demonstrated to have high activity (20-fold increase) that was maintained within a minimal region of P(−115/+99) in the high FGFR4-expressing HS729-1015 cell line but less activity (only a 10-fold increase) in the low FGFR4-expressing HS729-1016 line (Fig. 3). Serial 5′ deletions

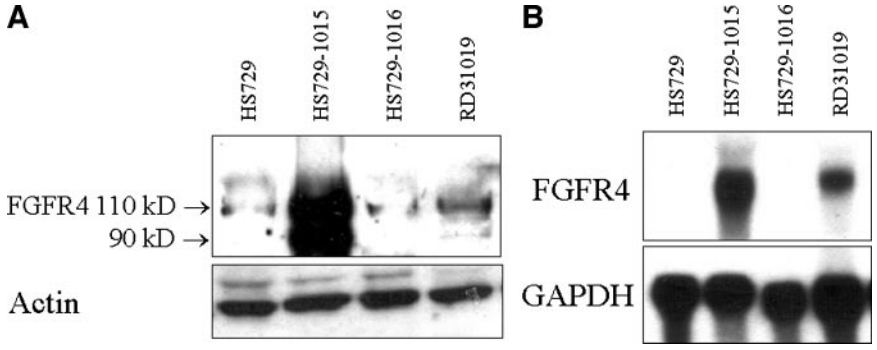


*Fig. 2* Spectrum of FGFR4 expression in human rhabdomyosarcoma cell lines. *A*, Western blotting using an antibody that recognizes the COOH-terminal tail of FGFR4 reveals overexpression of FGFR4 in the HS729-1015 cell line compared with the other cell lines; expression of FGFR4 is intermediate in RD3-1019 cells and low in HS729 and HS729-1016 cells. The β-actin control (*bottom panel*) shows equal protein loading. FGFR1 expression is inversely correlated with FGFR4. *B*, Northern blotting using 20 μg of total RNA confirms the overexpression of FGFR4 mRNA by the HS729-1015 and RD3-1019 cell lines compared with the HS729-1016 and HS729 rhabdomyosarcoma cell lines, whose transcripts are not detectable at this exposure. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) loading control is shown (*bottom panel*).

of the FGFR4 promoter region to +13/+99 markedly abrogated promoter activity, whereas the 3′-deleted construct P(−1133/−173)-Luc showed no significant promoter activity.

**The Fibroblast Growth Factor Receptor 4 Minimal Promoter Contains Multiple Binding Sites for Sp1.** Many transcription factors can be simultaneously involved in the regulation of target genes. To determine which factors are most relevant in regulation of FGFR4 promoter activity in rhabdomyosarcoma cells, we screened potential candidates using EMSA. EMSAs were performed using nuclear extracts from exponentially growing rhabdomyosarcoma cell lines with overlapping 40- to 50-bp oligonucleotide fragments derived from the 214-bp minimal FGFR4 promoter as labeled probes depicted in Fig. 1. Specific DNA-protein complexes were detected when nuclear extracts from the FGFR4-positive HS729-1015 cell line were allowed to interact with fragments B (−95/−56) and C (−65/−26) [Fig. 4*A*]. To further define the relevant binding sites, nuclear extracts from the high FGFR4-expressing HS729-1015 cell line and the low FGFR4-expressing HS729-1016 cell line were allowed to interact with fragments B and C (Fig. 4*B* and *C*). Both cell lines formed complexes with fragment C (Fig. 4*B*). Based on sequence prediction, fragment C contains two Sp-binding sites (Fig. 1). These complexes were competed by an Sp oligonucleotide. The *top* (slower) *migrating band* of the complex was supershifted by Sp1 but not by Sp3 antibody. Conversely, the *bottom* (faster) *migrating component* of the complex was supershifted by the Sp3 antibody by not by anti-Sp1 (Fig. 4*B* and *C*). In contrast to fragment C, which demonstrated binding in both cell lines, fragment B, which also contains two Sp-binding sites (Fig. 1), formed strong complexes with nuclear extracts from the high FGFR4-expressing HS729-1015 cell line but showed minimal interaction with the low FGFR4-expressing HS729-1016 cell line (Fig. 4*C*). Fragment C also contains multiple predicted binding sites for Ap1, Ap2, Ap4, CREB, Yy-1, Ets, and Ikaros. Direct testing of these factors using specific oligonucleotides and supershifting failed to implicate these factors.

**Functional Contribution of Sp1 and Ets in Regulation of Fibroblast Growth Factor Receptor 4 in Sarcoma Cells.** To determine the functional contribution from Sp-type factors in regulating FGFR4 in human rhabdomyosarcomas, we examined the wild-type P(−115/+99)-Luc FGFR4 and the effects of cotransfection with Sp or Ets factors. Fig. 5*A* demonstrates the effects of Sp1 or Sp3 transfection on the FGFR4 minimal promoter activity in FGFR4-positive HS729-1015 (□) and FGFR4-negative HS729-1016 (■) sarcoma cells. Sp1 resulted in a dose-dependent activation of FGFR4 luciferase activity. This effect was more pronounced in the high FGFR4-expressing HS729-1015 cell line than in the low FGFR4-expressing HS729-1016 cell line. In contrast to the effects of Sp1, Sp3 had a minimal inhibitory effect on FGFR4 activity in either cell line. Ets1 transfection in doses ranging from 0.5 to 2 μg resulted in modest peak stimulation (~40%) of FGFR4 reporter activity in both cell lines (data not shown).

To further determine the functional significance of the Sp1 sites in fragments B and C, we progressively deleted these binding elements. Again, the effect of each of the deletions was examined in the low FGFR4- and high FGFR4-expressing cell lines (Fig. 5*B*). Deletion of the first or 5′-most Sp-binding site situated in fragment B resulted in a marked loss of activity. Progressive deletions of the additional three Sp-binding sites resulted in more modest but appreciable reductions in activity

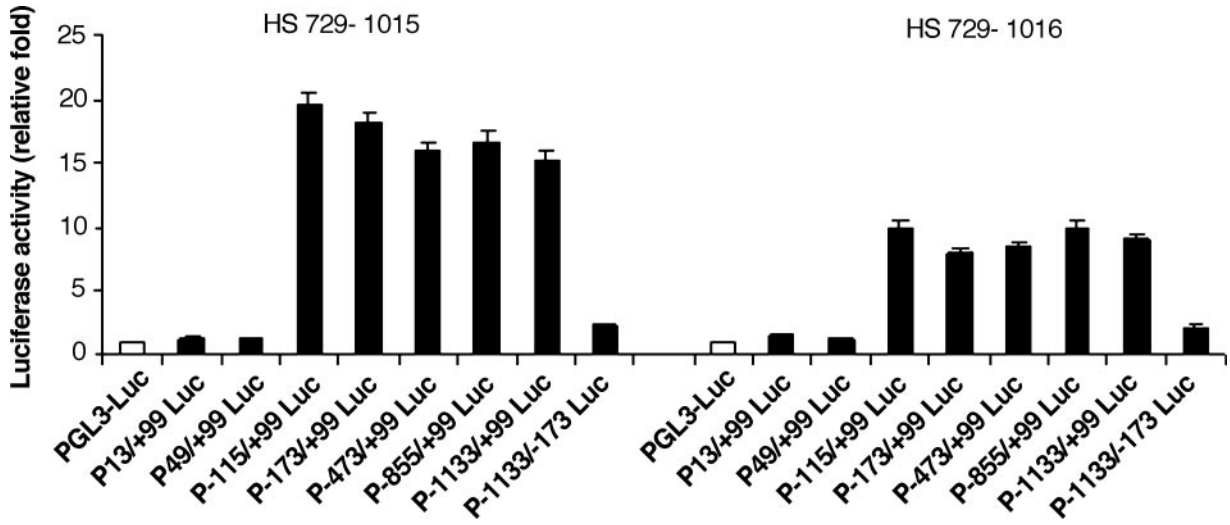


*Fig. 3* Identification of transcriptional activity of the FGFR4 gene in rhabdomyosarcoma cell lines. The effects of sequences spanning the 5′-region of FGFR4 from -1133 to +99 on luciferase reporter activity were assessed in transiently transfected cells of the high FGFR4-expressing HS729-1015 cell line and the low FGFR4-expressing HS729-1016 line. A series of deleted promoter fragments were generated by PCR as described in Materials and Methods, ligated, and subcloned upstream of the promoterless luciferase reporter gene pGL3-basic vector as indicated. Note that serial 5′ deletions of the FGFR4 promoter region to P(+13/+99)-Luc markedly abrogate luciferase activity and that the 3′-deleted construct P(−1133/−173)-Luc shows minimal activity. Most of the activity is retained within the −115/+99-Luc fragment and is significantly higher in the FGFR4-positive cells (HS729-1015) than in the FGFR4-negative cells (HS729-1016). Data are presented as the relative fold increase in mean luciferase activity after adjustment of all calculations for β-galactosidase activity (±SD) compared with control wells of three independent transfections ($P < 0.005$).

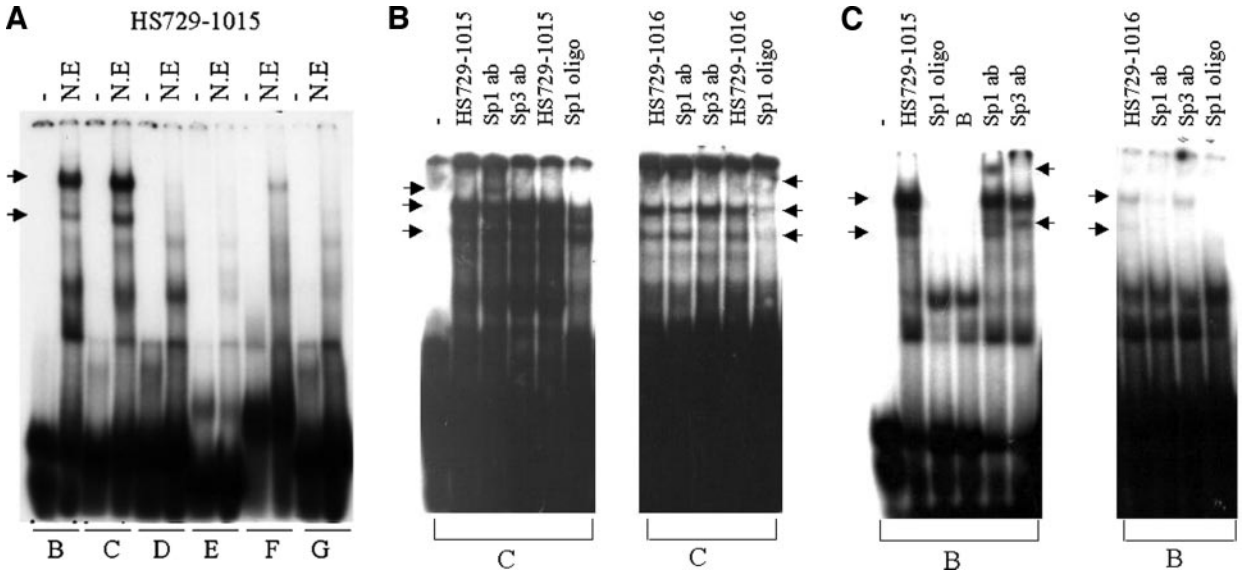


*Fig. 4* Characterization of transcription factor binding elements in rhabdomyosarcoma cells by EMSA. *A*, The minimal promoter of the FGFR4 gene was divided into overlapping fragments (*A*−*G*; see Fig. 1) that were used to assess binding with nuclear extracts from the FGFR4-positive HS729-1015 rhabdomyosarcoma cell line. Only fragments B and C formed distinct DNA-protein complexes with nuclear extracts from these cells. *B* and *C*, Fragments C and B were tested as probes to characterize their binding complexes with nuclear extracts from the FGFR4-positive HS729-1015 and FGFR4-negative H2729-1016 cell lines as indicated. Fragments B and C formed complexes with the FGFR4-positive HS729-1015 cells, where the slower migrating band (*top arrow*) is supershifted with Sp1 antibody, and the faster migrating band is supershifted with an antibody to Sp3 (*bottom arrow*) but not Sp1. The complex is competed by the Sp oligonucleotide. Fragment C formed specific complexes with both cell lines; however, fragment B formed strong complexes with the HS729-1015 cell line but much weaker complexes with the HS729-1016 cell line.

(Fig. 5*B*). Moreover, loss of the 5′ Sp-binding site in fragment B resulted in abrogation of the effect of Sp1 on FGFR4 promoter activity (Fig. 5*C*).

**Overexpression of Sp1 Correlates with and Influences Endogenous Fibroblast Growth Factor Receptor 4 Expression.** To examine whether high FGFR4 expression in rhabdomyosarcoma cell lines correlates with Sp1 expression, we examined the different sarcoma cell lines as well as corresponding primary human embryonal rhabdomyosarcomas for their expression patterns of Sp1 and Sp3 by Western blotting. Nuclear fractions from the cell lines contained a doublet signal of ~105 and 95 kDa recognized by the Sp1-specific antibody in a pattern corresponding to FGFR4 expression (Fig. 6*A*; compare with Fig. 2). In addition, three primary human embryonal rhabdomyosarcoma specimens were also examined, and the tumor that demonstrated the strongest reactivity for FGFR4 also demonstrated relative Sp1 overexpression; this band migrated as a wide band in primary tumor lysates (Fig. 6*B*). Conversely, the cell lines and primary tumors that demonstrated negligible levels of FGFR4 expression revealed minimal Sp1 protein immunoreactivity (Fig. 6*A* and *B*). Consistent with the EMSA and transcriptional data, Sp3 immunoreactivity was negligible in the high FGFR4-expressing HS729-1015 cell line (data not shown).

To determine whether Sp1-mediated transcriptional effects were reflected in endogenous gene changes, HS729-1015 and HS729-1016 cells were transfected with Sp1 and treated with the methylation inhibitor 5-azacytidine. Endogenous FGFR4 was positively influenced by Sp1 in HS729-1015 cells, but Sp1 (Fig. 6*C*) was not sufficient to induce FGFR4 expression in HS729-1016 cells, despite increased Sp1 expression (Fig. 6*C*). Similarly, Sp1 transfection in the presence of histone deacetylase inhibition with trichostatin A resulted in enhanced FGFR4 expression in HS279-1015 cells but not in HS729-1016 cells (data not shown).

## DISCUSSION

In this report, we have characterized the human FGFR4 promoter in transformed human tumor cell lines of skeletal muscle origin (rhabdomyosarcomas) and examined the *cis*-regulatory elements in the proximal region of the FGFR4 promoter that render positive transcriptional activity. We demonstrate that multiple elements mainly in fragments B (−95/−56) and C (−65/−26) of the FGFR4 minimal promoter contain Sp-binding sites. Our findings point to Sp1 as a transcription factor whose overexpression parallels FGFR4 expression in cell lines and primary tumors of skeletal muscle origin. These findings begin to address the mechanisms underlying the differences in FGFR4 expression profiles in neoplasms derived from the myogenic lineage. They also further validate and begin to provide a mechanistic basis for the cDNA microarray finding of FGFR4 overexpression in some pediatric rhabdomyosarcomas (13).

Sp1 has been shown to play a significant role in the regulation of other FGFRs. For example, analysis of the mouse FGFR1 promoter reveals the presence of consensus sequences for binding sites of the transcription factors Sp1, AP1, and AP2 and the absence of TATA and CAAT sequence motifs (20). Transfection of the 5′-regulatory region into NIH3T3 cells defined a minimal promoter within the region defined by −106

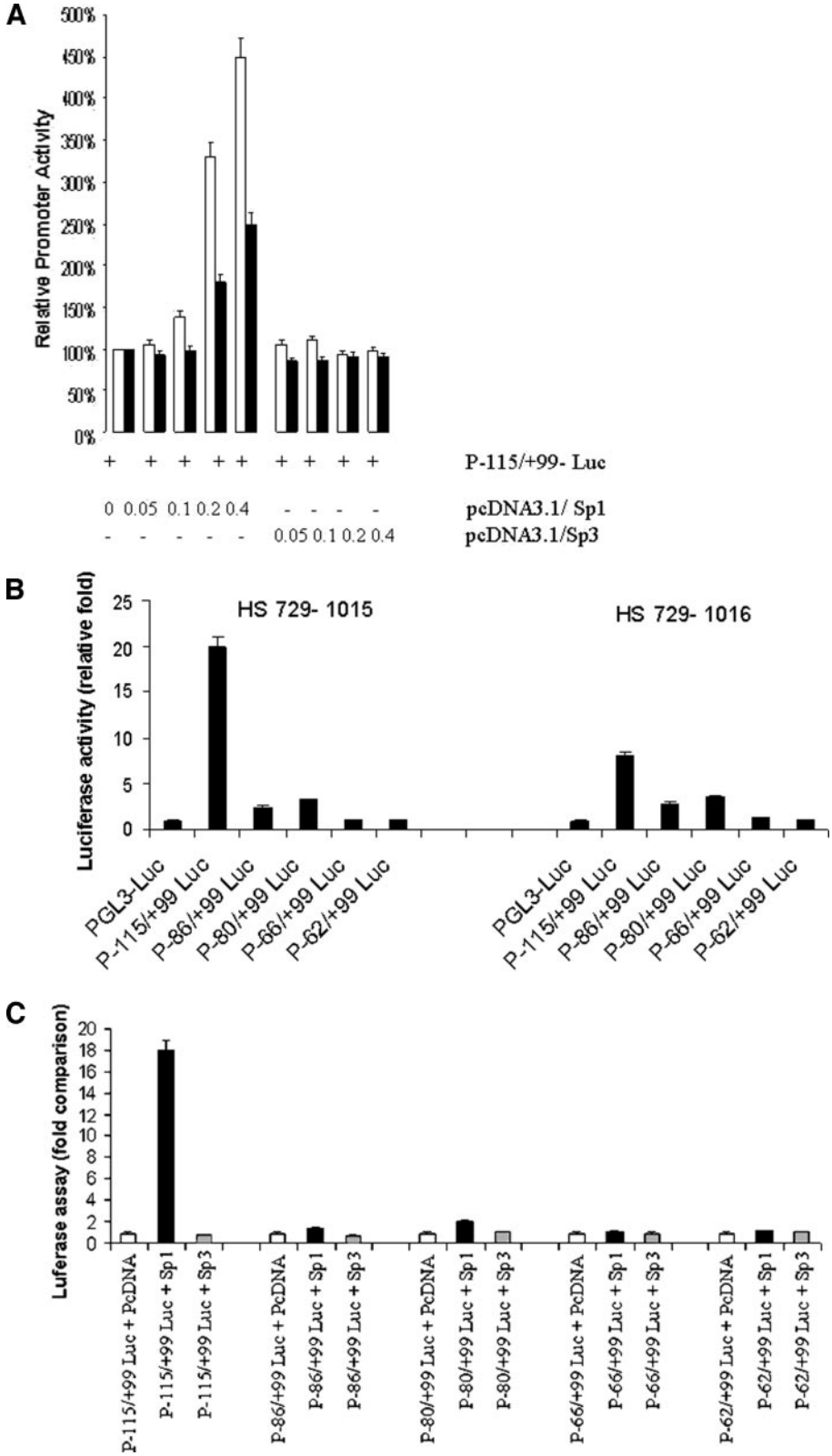


*Fig. 5* Sp1 contributes to FGFR4 transcriptional activity in rhabdomyosarcoma cells. *A*, HS729-1015 (□) and HS729-1016 (■) rhabdomyosarcoma cells were transiently cotransfected with the minimal FGFR4 P(−115/+99)-Luc and pcDNA3.1 expression vectors encoding Sp1 or Sp3 in micrograms of DNA per well. Note the efficient dose-dependent activation of FGFR4 promoter activity by Sp1 (but not by Sp3), an effect that was more pronounced in the FGFR4-positive cell line than in the FGFR4-negative cell line. All transfections included corresponding empty control vectors along with 20 ng of pCMVβgal to normalize for transfection efficiency. Data represent the percentage of induction relative to corresponding empty vector controls. Results are the mean ± SD derived from three independent experiments, with each dose performed in duplicate. *B*, HS729-1015 and HS729-1016 rhabdomyosarcoma cells were transiently cotransfected with wild-type minimal FGFR4 P(−115/+99)-Luc promoter or progressive Sp-binding deletions in fragments B [(−86/+99) (−80/+99)] and C [(−66/+99) (−62/+99)] as indicated. Note the marked loss of promoter activity with loss of the first (5′) Sp-binding site in fragment B. The results are expressed as relative fold induction with mean ± SD derived from three independent experiments, with each construct performed in duplicate. *C*, HS729-1015 cells were transiently cotransfected with the Sp-binding deletion fragments as described in *B*, along with pcDNA 3.1 expression vectors encoding for Sp1 or Sp3 as indicated. The results are expressed as relative fold induction with mean ± SD derived from three independent experiments with each construct performed in duplicate.

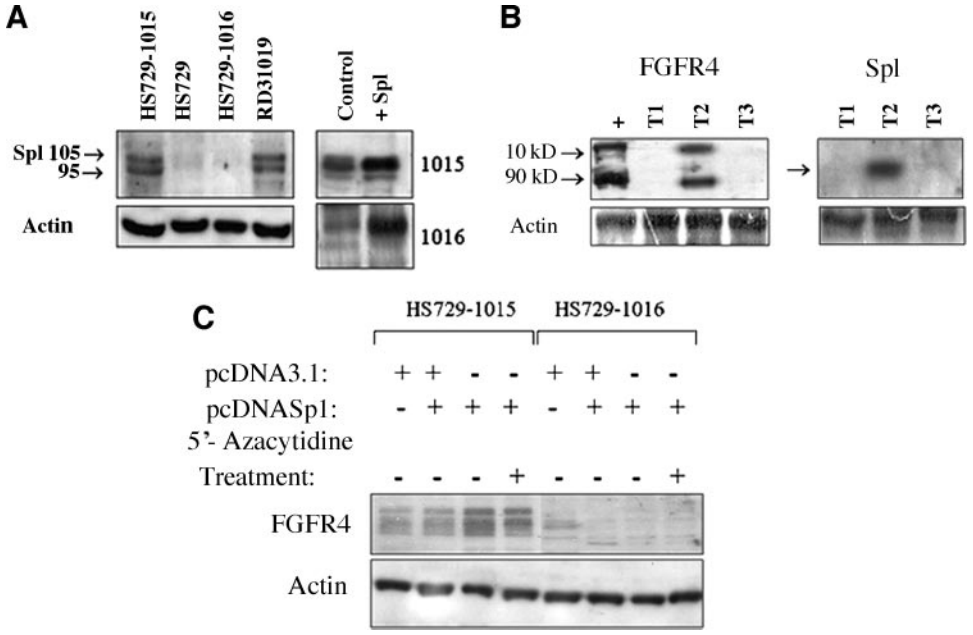


*Fig. 6* FGFR4-expression in rhabdomyosarcoma cells overexpressing Sp1. *A*, Western blotting of nuclear fractions from the indicated rhabdomyosarcoma cell lines reveals overexpression of Sp1 consistent with their pattern of FGFR4 expression (see Fig. 2). The β-actin loading controls are shown (*bottom panel*). *Right*, Western blots for Sp1 on extracts from HS729-1015 cells (*top panel*) or HS729-1016 cells (*bottom panel*) without or with Sp1 transfection are shown. *B*, Lysates of primary human rhabdomyosarcomas reveal concordant relative overexpression of FGFR4 and Sp1 among three primary sarcomas of the same skeletal muscle lineage. Lysate from a human MCF-7 breast cancer positive control for FGFR4 is denoted as +. *C*, HS729-1015 and HS729-1016 rhabdomyosarcoma cells were transfected with pcDNA3.1 expression vectors encoding Sp1 or empty vector to ensure equal amounts of transfected DNA. All cells were treated with the methylation inhibitor 5-azacytidine and subjected to immunoblotting using an antibody that recognizes the COOH terminus of FGFR4 or β-actin as indicated. Preabsorption with purified FGFR4 antigen abolished the 110-/90-kDa reactive band (data not shown).

and +104 of FGFR1 (20). Similarly, deletion analysis of the avian FGFR1 promoter reveals a 78-bp region containing multiple Sp1-binding sites that confers a high level of FGFR1 promoter activity in myoblasts (21). The FGFR2 promoter has also been identified to reside in CpG islands encompassing the 5′-end (22), lacking classical *cis*-regulatory motifs. Sequence analysis of the FGFR3 promoter also reveals multiple transcription binding sites including five classical Sp1 sites (22), all situated within the first 200 bp of the transcription start site. From these studies and our current studies, it may be concluded that for all FGFRs (FGFR1, FGFR2, FGFR3, and FGFR4), as little as 100 bp of sequence 5′ to the transcription initiation site confers significant transcriptional activity.

FGFR promoter studies have provided some insight into the possible mechanisms of FGFR regulation in skeletal muscle. The distal region of the FGFR1 promoter is located >1 kb upstream from the start of transcription and has been shown to positively regulate FGFR1 gene expression in myoblasts (21). This region contains two Sp transcription factor-binding sites, both of which are required for FGFR1 promoter activity in proliferating myoblasts. Although the distal Sp1-binding sites are required for full FGFR1 promoter activity and confer increased transcriptional activity to a minimal promoter, the distal region was not sufficient for promoter activity. The proximal region resides between −69 and −14 of the FGFR1 promoter. Electromobility shift assays revealed that myoblast but not differentiated myotube nuclear proteins specifically bind to these *cis*-elements. Sp1 and Sp3 reactivity was also detected mainly in myoblasts, but not in differentiated myotubes (23), consistent with a role for Sp transcription factors in differentiating myoblasts and the down-regulation of FGFR1 in differentiated muscle fibers. Interestingly, we found no significant repression by Sp3 on FGFR4 regulation, similar to previous findings of Sp3 on FGFR1 activity (23).

Although previous reports have identified the human and murine FGFR4 promoter regions and their transcription start sites (18), these studies did not address characterization of putative transcription factor binding or transcriptional activity. Our studies in other models have identified a cryptic promoter in intron 4 (24), but that promoter is irrelevant to the production of intact FGFR4, and we have found no truncated FGFR4 in rhabdomyosarcomas. A previous report identified an enhancer element in intron 1 that is active in pancreatic carcinoma (25). Our previous analysis of the FGFR4 promoter in endocrine pituitary cells revealed that the 214-bp fragment containing 115 nucleotides upstream of the FGFR4 transcription start site is critical for promoter activity (17). Overlapping 40- to 50-bp fragments of this minimal functional promoter were examined by EMSAs. Specific DNA-protein complexes were noted with two adjacent fragments (fragments B and C). As shown here, these two fragments contain multiple Sp-binding sites. In particular, fragment B contains two Sp sites that formed strong complexes with the high FGFR4-expressing HS729-1015 cell line in comparison with the low FGFR4-expressing HS729-

1016 cell line. Fragment C (−65/−25) also contains multiple predicted binding sites for Ap1, Ap2, Ap4, CREB, Yy-1, Ets, and Ikaros. Direct testing of these factors using specific oligonucleotides and supershifting could not confirm the importance of these factors in the tested sarcomas of skeletal muscle origin. Thus, we further characterized those candidate factors that resulted in different binding and shifting patterns on EMSA screening using nuclear extract from rhabdomyosarcomas with high or low FGFR4 expression. It is noteworthy to emphasize that fragment C also includes a binding site for the zinc finger transcription factor Ikaros that is flanked by two sites for Sp1 and Ets-type factors. Unlike the situation in lymphopoietic and pituitary cells (17), however, we found no evidence for Ikaros expression in rhabdomyosarcoma cells (data not shown). Instead, our data emphasize the importance of fragment B in these cells, where Sp1 acts as a FGFR4 regulator. Because these tumors are thought to reflect a developmental arrest along the myogenic lineage, the patterns of coexpression of Sp1 and FGFR4 may help to further characterize the phenotype of this differentiation arrest in different tumors.

The transcription factor Sp1 is ubiquitously expressed and plays a significant role in the constitutive and induced expression of a variety of mammalian genes and may even contribute to tumorigenesis. In turn, components of cell cycle control elements such as cyclin A have been shown to mediate phosphorylation of Sp1 and enhance the binding activity of Sp1 (26). There is also evidence that p53 transactivation of the p21 cyclin-dependent kinase promoter is dependent on Sp1. Indeed, the DNA-binding domain of Sp1 is required for its physical interaction with p53 (27). Furthermore, whereas Sp1 has been implicated in the positive regulation of vascular endothelial growth factor expression in fibrosarcomas (28), p53-mediated inhibition of vascular endothelial growth factor promoter activity in leiomyosarcomas also appears to require Sp1 (27). Taken together, our current findings on the relevance of Sp1 in targeting FGFR4 expand our understanding of Sp1 as a pivotal factor in important biological processes including cell differentiation and tumorigenesis.

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