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Vascular Smooth Muscle Cell-Specific Regulation of Cyclin-Dependent Kinase Inhibitor $p21^{WAF1/Cip1}$ Transcription by Sp1 Is Mediated via Distinct *cis*-Acting Positive and Negative Regulatory Elements in the Proximal $p21^{WAF1/Cip1}$ Promoter

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Abstract Smooth muscle cells (SMC) play a central role in common vascular pathologies such as atherosclerosis and restenosis. Understanding the molecular regulation of SMC proliferation at a transcriptional level may provide important clues for the targeted control of vascular hyperplasia. We recently reported the capacity of the transcription factor Sp1 to down-regulate $p21^{WAF1/Cip1}$ production thereby reducing $p21^{WAF1/Cip1}$–cyclin D1–Cdk4 complex formation and inhibiting vascular SMC proliferation (Kavurma and Khachigian [2003] J. Biol. Chem. 278, 32537–32543). We have now localized the Sp1-response elements in the $p21^{WAF1/Cip1}$ promoter responsible for $p21^{WAF1/Cip1}$ repression in WKY12-22 SMCs. The proximal region of the $p21^{WAF1/Cip1}$ promoter contains five distinct Sp1-binding elements that we have termed A, B, C, D, and E. Electrophoretic mobility shift analysis revealed that SMC nuclear Sp1 interacts with all five Sp1-binding sites, and each of these sites is critical for Sp1 repression of the $p21^{WAF1/Cip1}$ promoter, since mutation in any one element ablates repression, and in some cases results in activation. In contrast, only elements C, D, and E are bound by Sp1 in endothelial cells. Sp1 overexpression activates the $p21^{WAF1/Cip1}$ promoter in this cell type. Furthermore, mutation in any of these five elements is not sufficient to prevent activation of the $p21^{WAF1/Cip1}$ promoter by Sp1 in endothelial cells. Surprisingly, double mutations of elements C and E facilitates superactivation by Sp1 in both cell types, whereas triple mutations of C, D, and E inactivate the promoter. These findings demonstrate cell type-specific regulation of $p21^{WAF1/Cip1}$ transcription by Sp1 via distinct *cis*-acting positive and negative regulatory elements in the proximal $p21^{WAF1/Cip1}$ promoter. J. Cell. Biochem. 93: 904–916, 2004.

Key words: Sp1; $p21^{WAF1/Cip1}$; *cis*-acting elements; cell-type specific; promoter; repression

Sp1, first described to bind and activate the simian virus 40 (SV40) early promoter [Dynan and Tjian, 1983] belongs to the Sp family of transcription factors which are characterized by a highly-conserved DNA-binding domain consisting of three Krüppel-like C_2H_2 zinc fingers. Sp1 can positively [Rafty and Khachigian, 1998; Gartel et al., 2000; Kavurma et al., 2001; Rance et al., 2003] or negatively influence gene expression [Tang et al., 1999; Song et al., 2001; Zaid et al., 2001] by direct interactions with DNA or via interactions with other transcription factors.

The cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ controls multiple cellular processes including apoptosis, senescence, differentiation, and the regulation of cell cycle events [Dotto, 2000]. During mitogenic influences, $p21^{WAF1/Cip1}$ acts as an assembly factor, recruiting D-type cyclins and Cdk4/6 to promote cell cycle progression [Sherr and Roberts, 1999]. $p21^{WAF1/Cip1}$ can also negatively regulate cell cycle progression by inhibiting the activity of cyclin E–Cdk2 complexes [Sherr and Roberts,

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1999] or proliferating cell nuclear antigen (PCNA) [Kelman, 1997]. The stoichiometry of p21$^{WAF1/Cip1}$ within the cellular system appears to control such processes [LaBaer et al., 1997; Cheng et al., 1999; Kavurma and Khachigian, 2003].

p21$^{WAF1/Cip1}$ levels and expression can be influenced by a variety of agents such as FGF [Johnson et al., 1998; Kivinen and Laiho, 1999], EGF [Thomas et al., 1999], TGF-beta; [Kivinen and Laiho, 1999; Yoo et al., 1999; Pardali et al., 2000], IL-6 [Bellido et al., 1998], PMA [Shanmugam et al., 2001; Lai et al., 2002], and DNA damaging agents [Martinez et al., 2002]. The expression of p21$^{WAF1/Cip1}$ can also be modulated by transcription factors that bind to *cis*-regulatory elements located within the promoter [reviewed by Gartel and Tyner, 1999]. p21$^{WAF1/Cip1}$ is positively controlled by the STAT family of transcription factors [Chin et al., 1996; Matsumura et al., 1997; Coqueret and Gascan, 2000], Smad proteins [Moustakas and Kardassis, 1998; Pardali et al., 2000], p300 [Snowden et al., 2000; Zhang et al., 2001], Ets-1 [Zhang et al., 2001], vitamin D receptor [Liu et al., 1996], and Sp1 [Moustakas and Kardassis, 1998; Gartel et al., 2000; Han et al., 2001]. Negative regulation of p21$^{WAF1/Cip1}$ transcription has also been observed and involves the transcription factors c-Myc [Gartel et al., 2001], Runx2 [Westendorf et al., 2002], and c-Jun [Wang et al., 2000].

We recently demonstrated that Sp1 down-regulates p21$^{WAF1/Cip1}$ expression reducing the assembly of the p21$^{WAF1/Cip1}$–cyclin D–Cdk4 complex and Rb phosphorylation, thereby inhibiting vascular SMC proliferation [Kavurma and Khachigian, 2003]. Here we demonstrate that negative regulation of p21$^{WAF1/Cip1}$ transcription in WKY12-22 SMCs depends upon the integrity of each of the five separate *cis*-acting elements in the proximal region of the p21$^{WAF1/Cip1}$ promoter which bind Sp1. Disruption in any of these sites abrogates the interaction with Sp1 and converts these negative regulatory elements to positive regulatory elements in a cell type-specific manner.

MATERIALS AND METHODS

Plasmid Constructs

A 1,975 bp fragment of the p21$^{WAF1/Cip1}$ promoter was generated using the Erase-a-base system (Promega Corporation, Annandale, Australia) originally from 0-Luc (a gift from Dr. Wafik El-Diery, Howard Hughes Medical Institute, University of Pennsylvania) and cloned into pGL3-basic (3*6, Fig. 1A). CMV-Sp1 was obtained from Robert Tjian (Howard Hughes Medical Institute University of California), pEBGNLS and pEBGSp1 were obtained from Gerald Thiel (Institute For Genetics, University of Cologne), and pCEP-WAF1 was obtained from Bert Vogelstein (John Hopkins Oncology Centre). Mutations of Sp1 sites in 3*6 (Fig. 1A) were generated using the QuikChange Site-directed Mutagenesis Kit (Stratagene, Mount Waverley, Australia).

Cell Culture and Transfections

WKY12-22 SMCs were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Waymouth's medium pH 7.4 (Invitrogen, Mount Waverley, Australia). Primary bovine aortic endothelial cells (used between passages 3 and 9) were maintained in Dulbeccos' modified medium (Invitrogen). The medium was supplemented with 10% fetal bovine serum, penicillin 10 U/ml, streptomycin 10 μg/ml, and 1 mM L-glutamine (Invitrogen). Transient transfections were performed at 50–60% confluence with FuGENE6 transfection agent (Roche Molecular Biochemicals, Castle Hill, Australia) and indicated plasmid constructs for 24 h. For assays involving Luciferase, PRL-TK was co-transfected as an internal control. Cells were harvested and luciferase activity was quantified 24 h later using the Dual Luciferase Assay System (Promega). Luciferase activity was normalized with data generated from pRL-TK.

BrdU Incorporation Studies

Cell proliferation following Sp1 overexpression was determined using the Cell Proliferation ELISA system version 2 (Amersham Pharmacia Biotech, Castle Hill, Australia). Briefly, 5,000 WKY12-22 SMC and endothelial cells were plated into a 96 well-titre plate and transfected with CMV-Sp1 or pcDNA3. BrdU (10 μM) was added to cells for 4 h prior to harvest (24 h following transfection). Cells were fixed and incubated with anti-BrdU for 90 min. The plate was washed several times with PBS and immune complexes were detected by subsequent substrate reaction and OD_{450}.

Electrophoretic Mobility Shift Assay (EMSA)

WKY12-22 SMCs and endothelial cells were harvested for nuclear extract (NE) as previously

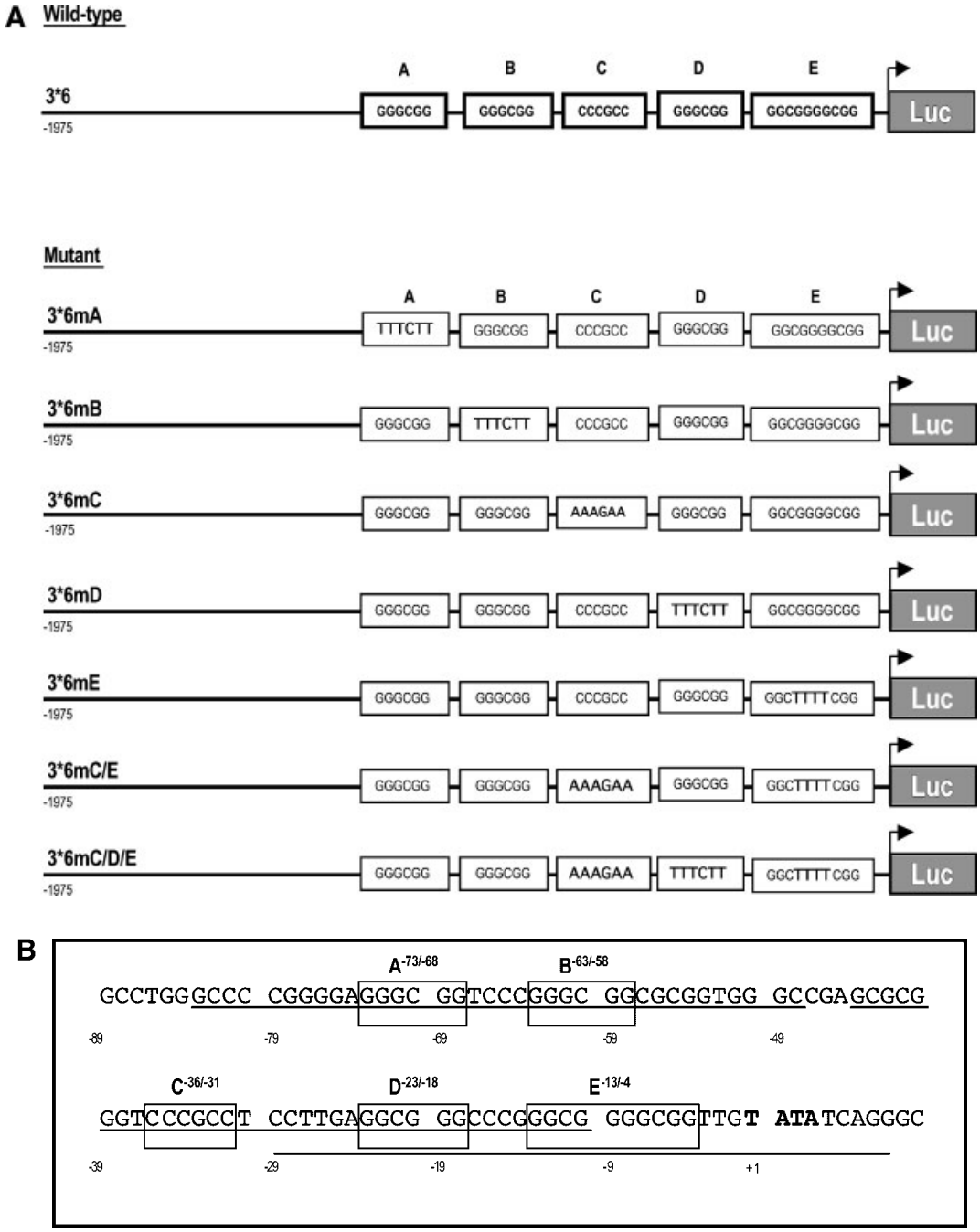

Fig. 1. Schematic depicting 3*6, a luciferase reporter vector driven by 1975 bp of the $p21^{WAF1/Cip1}$ promoter. **A**: Transverse mutations of individual Sp1 elements (A, B, C, D, and E) in 3*6. Mutant sequences are in bold. **B**: Sequence of the proximal $p21^{WAF1/Cip1}$ promoter. Sp1 binding elements are shown in boxes. Sequences of probes used for EMSA are underlined. Sequence positions of Sp1 elements are relative to the TATA.

described [Kavurma et al., 2001; Kavurma and Khachigian, 2003]. Briefly, NEs were incubated on ice for 20 min with 10 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 μg of salmon sperm DNA, 5% sucrose, 3 μg of poly(dI-dC), 1 mM PMSF, and indicated ^{32}P-labeled double-stranded oligonucleotides (Fig. 1B) in a total volume of 20 μl. In our experience, these conditions are optimal for Sp1 binding. In supershift assays, NEs were incubated with an anti-rabbit polyclonal Sp1 antibody (Santa Cruz Biotechnology, Santa

Cruz, CA) that recognizes Sp1 of mouse to human origin, for 15 min on ice, prior to addition of the probe. Samples were resolved by 6% non-denaturing polyacrylamide gel electrophoresis, gels were dried and visualized by autoradiography.

Western Immunoblot Analysis

WKY12-22 SMC and endothelial cells were grown to 60% confluence. Cells were transfected with 20 μg of CMV-Sp1 or pcDNA3 for 24 h using FUGENE6. Cells were harvested for NE. Proteins were resolved by 8% SDS–polyacrylamide gel electrophoresis, transferred onto Immobilon-P transfer membranes (Millipore, North Ryde, Australia), and membranes were blocked overnight in PBS containing 5% skim milk and 0.05% Tween 20. Sp1 was detected using a rabbit polyclonal antibody (1:1,500) purchased from Accurate Chemical Scientific.

RESULTS AND DISCUSSION

Sp1 Represses p21$^{WAF/Cip1}$ Promoter Activity and WKY12-22 Cell Proliferation, but Increases p21$^{WAF/Cip1}$ Promoter Activity and Growth in Endothelial Cells

We previously demonstrated that the zinc finger transcription factor Sp1 inhibits vascular SMC proliferation yet stimulates endothelial cell proliferation in a p21$^{WAF1/Cip1}$-dependent manner using a variety of approaches [Kavurma and Khachigian, 2003]. Sp1 can repress p21$^{WAF1/Cip1}$ promoter activity and gene expression in SMCs, a finding not observed in endothelial cells [Kavurma and Khachigian, 2003], nor indeed, in multiple other cell types including HepG2 [Moustakas and Kardassis, 1998], HaCaT keratinocytes [Pardali et al., 2000], Caco-2 colon adenocarcinomas [Gartel et al., 2000], metastatic prostate adenocarcinomas [Shan et al., 2000], HeLa cells [Xiao et al., 2000], and PC12 cells [Yan and Ziff, 1997].

The proximal region of the p21$^{WAF1/Cip1}$ promoter contains five distinct sites for Sp1 [Koutsodontis et al., 2002]. We have termed these sites:

A ($^{-73}$-GGGCGG^{-68}), B ($^{-63}$-GGGCGG^{-58}), C ($^{-36}$-CCCGCC^{-31}), D ($^{-23}$-GGGCGG^{-18}), and E ($^{-13}$-GGCGGGGCGG^{-4}, in which two Sp1 elements overlap) (Fig. 1B).

In this study, we hypothesized that cell-specific Sp1 repression of p21$^{WAF1/Cip1}$ transcription is mediated by the differential utilization of these elements by Sp1. We overexpressed Sp1 in a transient transfection setting with a Firefly-luciferase reporter vector driven by 1,975 bp of the p21$^{WAF1/Cip1}$ promoter (Fig. 1A). Sp1, as expected, repressed p21$^{WAF1/Cip1}$ promoter activity in SMCs, whereas it activated the promoter in endothelial cells (Fig. 2A). Sp1 was clearly overexpressed in both cell lines compared to pcDNA3 transfectants (Fig. 2B). Moreover, overexpression of Sp1 reduced BrdU incorporation into DNA in SMCs (Fig. 2C, left panel), yet stimulated the synthesis of DNA in endothelial cells (Fig. 2C, right panel).

WKY12-22 SMC Endogenous Sp1 Binds all Recognition Elements in the p21$^{WAF1/Cip1}$ Promoter

We next determined the capacity of the proximal region of the p21$^{WAF1/Cip1}$ promoter to interact with endogenous Sp1 in NEs from WKY12-22 SMCs. Electrophoretic mobility shift and supershift analysis using ^{32}P-labelled double-stranded oligonucleotides p21$^{WAF1/Cip1}$[−83/−48], p21$^{WAF1/Cip1}$[−44/−10], and p21$^{WAF1/Cip1}$-[−29/+9] (Fig. 1B) revealed that Sp1 bound to each of these promoter fragments in a sequence-specific manner (Fig. 3A–C). Two Sp1–DNA complexes were supershiftable with antibodies to Sp1 using p21$^{WAF1/Cip1}$ [−44/−10] as probe (Fig. 3B). Interestingly, only one Sp1–DNA complex was observed using SMC NE, p21$^{WAF1/Cip1}$ [−83/−48], and p21$^{WAF1/Cip1}$ [−29/+9], even though these probes each contain two Sp1 sites, suggesting cooperative binding. Mutation of Sp1 elements A, B, and C completely abrogated Sp1 binding (Fig. 3A,B); mutation of sites D and E caused a dramatic reduction in Sp1 complex formation (Fig. 3B,C). These findings demonstrate that in WKY12-22 SMCs, endogenous nuclear Sp1 interacts with elements A–E in the proximal p21$^{WAF1/Cip1}$ promoter.

Repression of the p21$^{WAF1/Cip1}$ Promoter Activity by Sp1 in WKY12-22 SMCs Involves not one but Multiple Sp1 Recognition Elements

We next determined the functional consequence of the interaction of Sp1 with each of its binding elements in the p21$^{WAF1/Cip1}$ promoter. Mutations in each element were serially introduced into the p21$^{WAF1/Cip1}$ promoter construct 3*6 (Fig. 1A) and transient transfection analysis was performed using WKY12-22 SMCs.

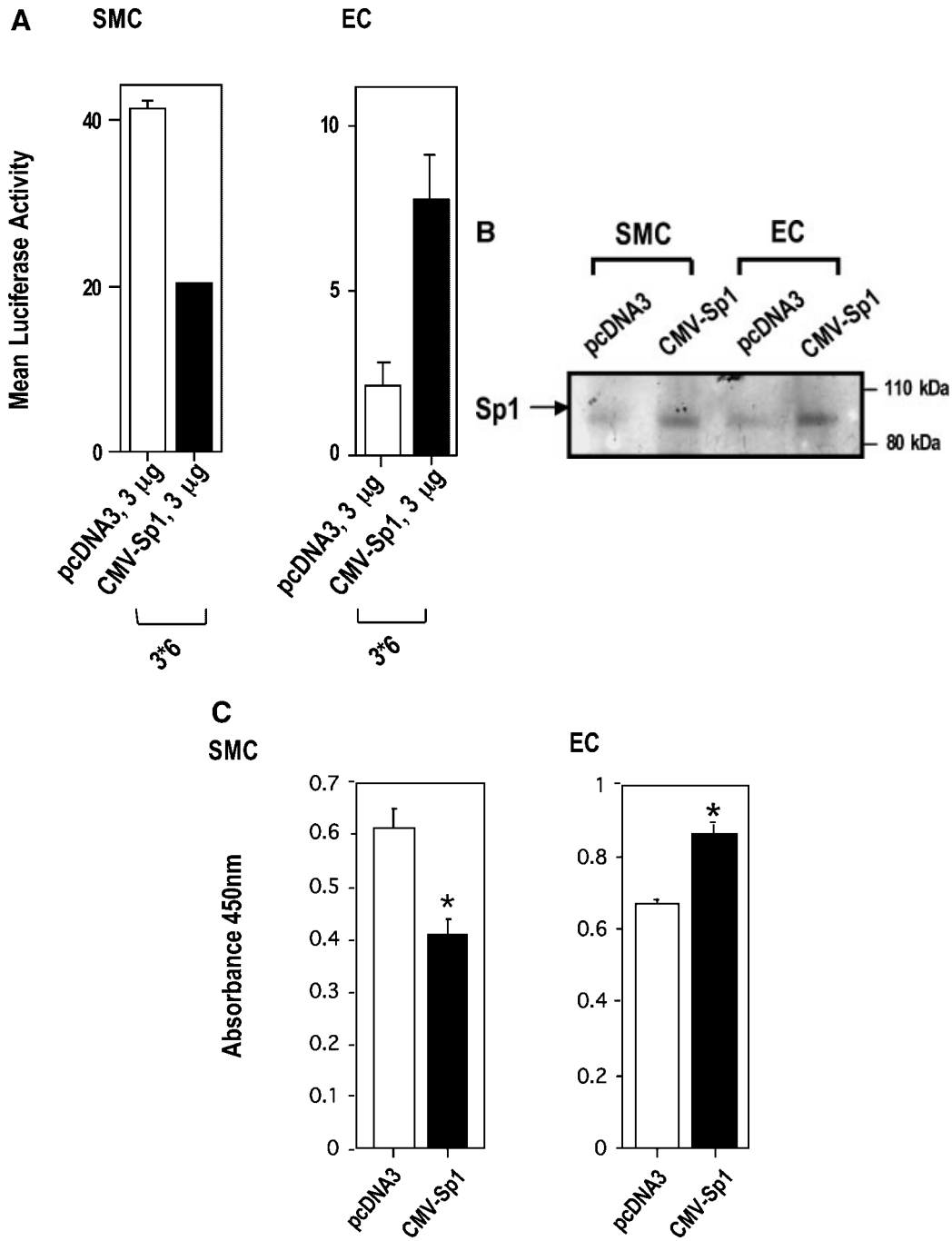

Fig. 2. Sp1 repressess $p21^{WAF1/Cip1}$ promoter activity in WKY12-22 SMCs, but activates in endothelial cells. **A**: Sp1 overexpression represses the $p21^{WAF1/Cip1}$ promoter construct 3*6 in SMCs (**right panel**). Sp1 overexpression leads to $p21^{WAF1/Cip1}$ promoter activation in endothelial cells (EC, **left panel**). Unless indicated, 5 µg of 3*6 was co-transfected with 3 µg of CMV-Sp1 or pcDNA3. Firefly luciferase activity was normalized to Renilla activity. Error bars represent standard errors of the mean. The data is representative of two independent determinations. **B**: CMV-driven Sp1 is expressed in WKY12-22 SMC and endothelial cells. Western immunoblot for Sp1 using nuclear extracts (NEs) from SMC and endothelial cells. Twenty micrograms of CMV-Sp1 or pcDNA3 were transfected 24 h prior to harvest. **C**: Sp1 overexpression inhibits DNA synthesis in WKY12-22 SMCs (**left panel**) and stimulates DNA synthesis in endothelial cells (**right panel**). BrdU incorporation is reduced in WKY12-22 SMCs and increased in endothelial cells following Sp1 overexpression compared to pcDNA3. BrdU incorporation was assessed by ELISA 24 h post transfection as described in the experimental procedures. Asterisk denotes statistical significance $P < 0.05$ (Student's *t*-test).

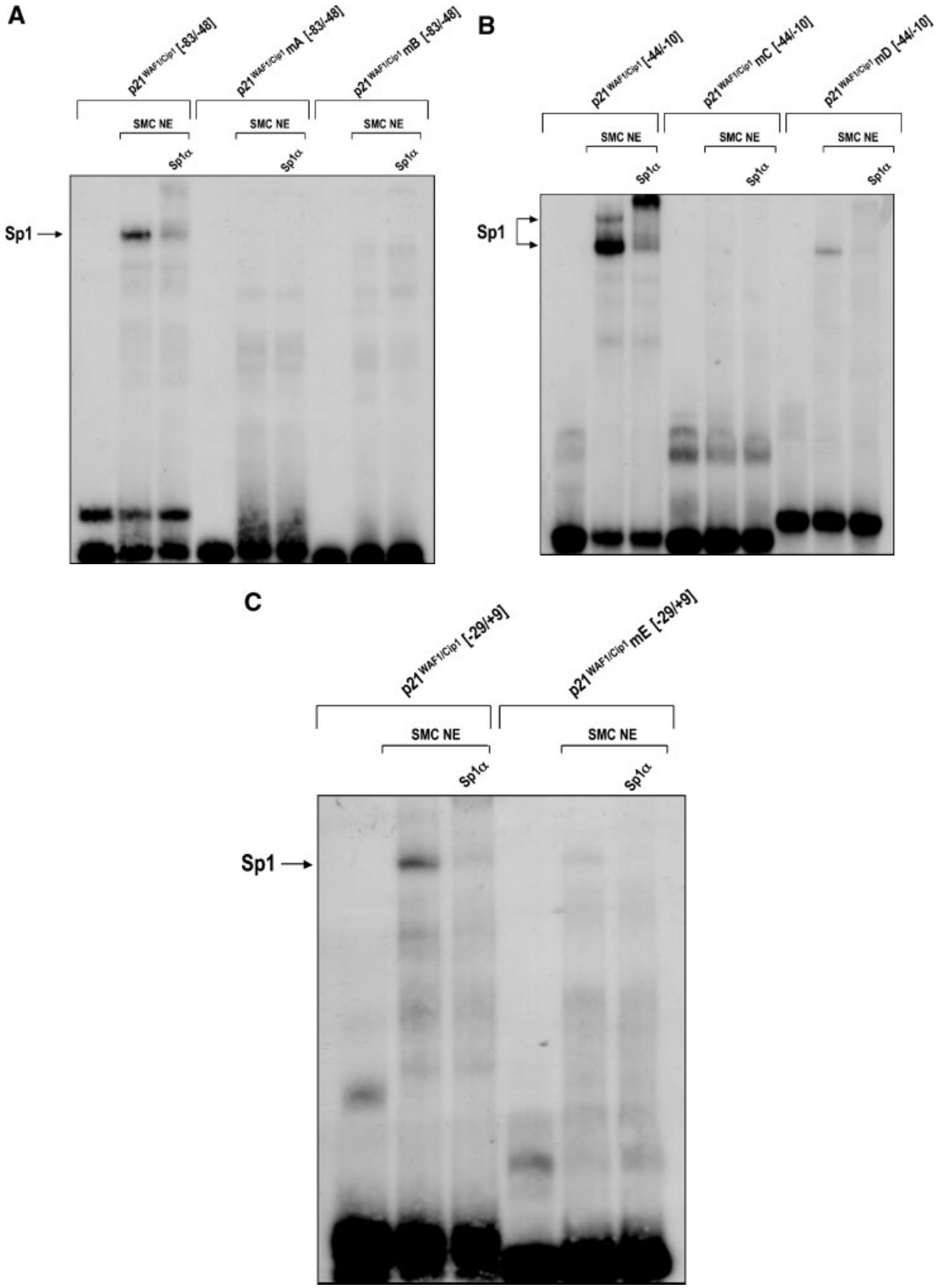
A
p21WAF1/Cip1 [-83/-48]
p21WAF1/Cip1 mA [-83/-48]
p21WAF1/Cip1 mB [-83/-48]
SMC NE
Sp1α
Sp1
B
p21WAF1/Cip1 [-44/-10]
p21WAF1/Cip1 mC [-44/-10]
p21WAF1/Cip1 mD [-44/-10]
C
p21WAF1/Cip1 [-29/+9]
p21WAF1/Cip1 mE [-29/+9]

Fig. 3.

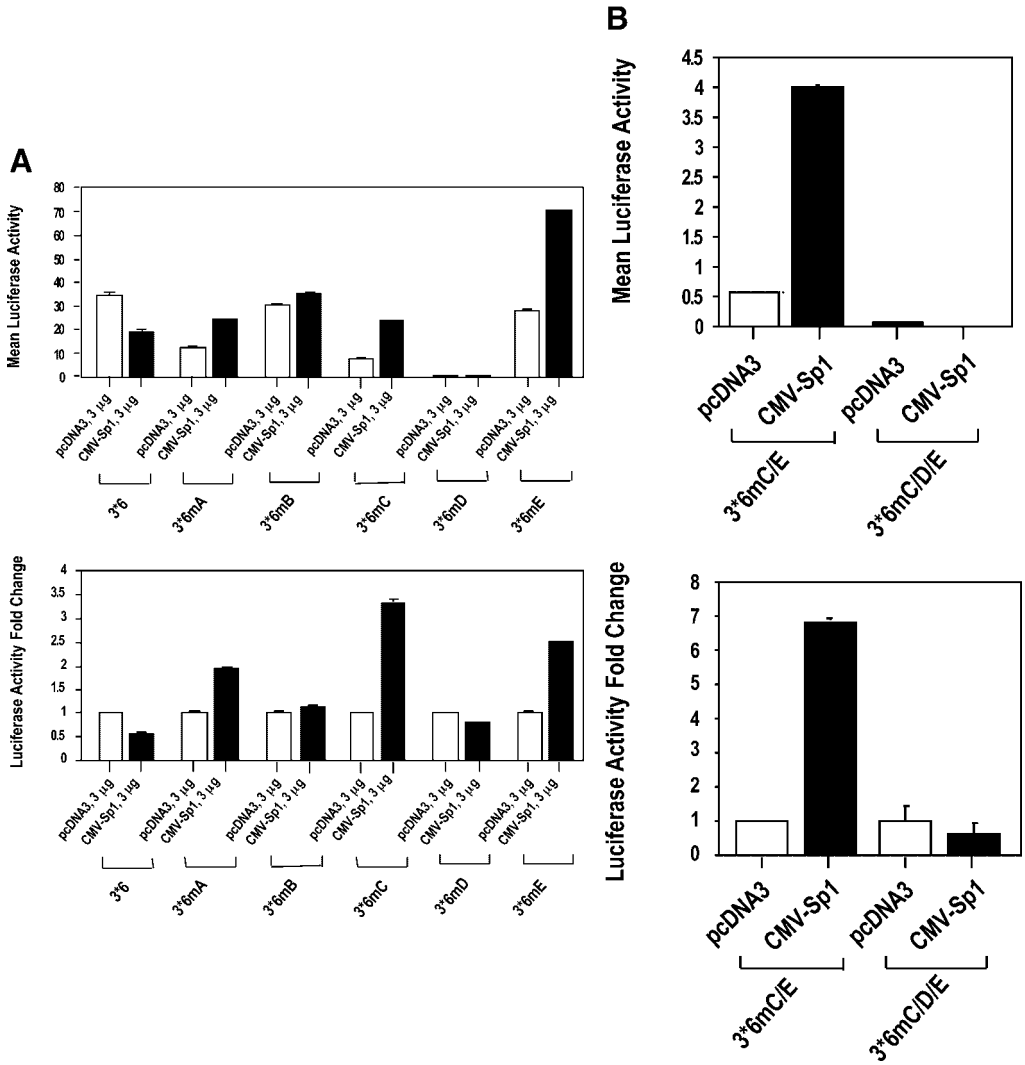

Fig. 4. Mutation of Sp1-binding sites in 3*6 reverts $p21^{WAF1/Cip1}$ promoter repression to activation following Sp1 overexpression in WKY12-22 SMCs. **A**: Co-transfection of Sp1 with 3*6 containing mutations in Sp1-binding elements. **B**: Double mutation (elements C and E; 3*6mC/E) leads to superactivation of the $p21^{WAF1/Cip1}$ promoter. Triple mutation (elements C, D, and E; 3*6mC/D/E) results in loss of promoter activity. **Upper panel** demonstrates mean luciferase activity. **Lower panel** demonstrates fold change in luciferase activity. Transient transfection analysis in WKY12-22 cells expressing Sp1 together with mutant promoter constructs. The empty expression vector (pcDNA3) has no affect. Firefly luciferase activity was normalized to Renilla activity. Error bars represent standard errors of the mean. The data is representative of two independent determinations.

To account for changes in basal expression of mutant constructs, data from Figure 4 was plotted in two ways: (i) mean luciferase activity (upper panel) and (ii) fold change in luciferase activity (lower panel). Figure 4A clearly demonstrates loss of basal $p21^{WAF1/Cip1}$ promoter activity (upper panel, open bars) when sites A, C, or D were mutated. No change in activity is observed when sites B or E were mutated (upper panel, open bars). Sp1 could no longer repress when the integrity of even one of these elements was disrupted (Fig. 4A). Mutations in some sites (elements A, C, and E) following Sp1 overexpression, even resulted in activation of

Fig. 3. (*Overleaf*) Endogenous SMC Sp1 interacts with all Sp1 elements in the $p21^{WAF1/Cip1}$ promoter. **A**: Sp1 binds elements A and B. EMSA using ^{32}P-$p21^{WAF1/Cip1}$ [−83/−48] and WKY12-22 SMC NE. Mutation in elements A and B abrogate Sp1 binding. **B**: Endogenous Sp1 binds elements C and D. EMSA using ^{32}P-$p21^{WAF1/Cip1}$ [−44/−10] and WKY12-22 SMC NE. Mutations in elements C and D impedes binding of Sp1. **C**: Sp1 binds element E. EMSA using ^{32}P-$p21^{WAF1/Cip1}$ [−29/+9] and WKY12-22 SMC NE. Mutation of element E abrogates Sp1 binding. Arrow denotes Sp1 supershift. Sp1 antibody is denoted by Sp1α.

the promoter (Fig. 4A, lower panel). When a double mutation (of elements C and E) was introduced into construct 3*6 (to produce construct 3*6mC/E; Fig. 1A), Sp1 activated the promoter almost 7-fold (Fig. 4B, lower panel). A triple mutation (of elements C, D, and E; Fig. 1B) in construct 3*6mC/D/E completely inactivated the promoter (Fig. 4B, upper panel). The presence of all proximal Sp1 elements is therefore critical for Sp1 repression of the p21$^{WAF1/Cip1}$ promoter in WKY12-22 SMCs. We have accordingly termed these Sp1 binding sites negative Sp1-response elements (NSREs).

Endogenous Endothelial Sp1 Binds to and Activates the p21$^{WAF1/Cip1}$ Promoter via Sites C, D, and E

The preceding binding and functional studies in WKY12-22 SMCs revealed that Sp1 interacts with all five NSREs in the p21$^{WAF1/Cip1}$ promoter and that disruption in any of these elements results in the loss of Sp1 repression. Because Sp1 activates the p21$^{WAF1/Cip1}$ promoter (Fig. 2B) in endothelial cells, we next investigated the role of these Sp1 binding elements in this cell type. EMSA revealed that endothelial nuclear Sp1 interacts with elements C and D (Fig. 5B) as demonstrated by a subtle, but reproducible Sp1 supershift and the loss or significant decrease in complex formation upon mutation of either site. The supershift may be due to the occupancy of other proteins in the complex which partially mask the antibody's epitope in Sp1. Moreover, while a classic "supershift" (i.e., slower electrophoretic mobility) is not observed for element E (Fig. 5C), the complex is totally eliminated by the Sp1 antibody, indicating therefore, the presence of functional Sp1 in the complex. In contrast, we found that elements A and B were not bound by Sp1 in endothelial cell extracts (Fig. 5A), despite equivalent levels of DNA-binding activity by the zinc finger transcription factor YY1 in WKY12-22 and endothelial cells (data not shown). This was demonstrated by the lack of a supershiftable complex in the presence of a rabbit-Sp1 antibody (Fig. 5A). This lack of a supershiftable complex was not due to: (i) an antibody of inferior quality, since the same antibody was used in all our EMSA studies, (ii) poor quality of extract, since Sp1 was supershifted in Figure 5B and C, nor (iii) the probe, since p21$^{WAF1/Cip1}$ [−44/−10] produced an Sp1 supershift in Figure 3B using SMC extracts. Therefore we conclude that in marked contrast to our findings in WKY12-22 SMCs, endogenous endothelial Sp1 only binds elements C, D, and E.

Co-transfection analysis in this cell type using the mutant constructs revealed loss of p21$^{WAF1/Cip1}$ promoter activity with elements C, D, and E, consistent with the observed Sp1 binding to these sites (Fig. 6A, upper panel). Mutation of any of these elements (A–E) was insufficient to ablate Sp1 activation of the p21$^{WAF1/Cip1}$ promoter (Fig. 6A). Interestingly, the p21$^{WAF1/Cip1}$ promoter construct bearing a double mutation in sites C and E (3*6mC/E; Fig. 1A) was activated 26-fold by Sp1 (Fig. 6B, lower panel). These findings indicate that p21$^{WAF1/Cip1}$ promoter elements C and E in endothelial cells, as in WKY12-22 SMCs, serve as NSREs, since loss of the capacity of Sp1 to bind to these elements results in superactivation of the p21$^{WAF1/Cip1}$ promoter (Figs. 4A and 6A). In endothelial cells, element D is the only site in 3*6mC/E able to interact with Sp1. This suggests that element D mediates Sp1 activation when elements C and E are inactivated. Therefore, element D serves as a positive Sp1 response element (PSRE) in endothelial cells since basal expression of the p21$^{WAF1/Cip1}$ promoter is significantly reduced, and Sp1 activation is abolished in the triple mutant (3*6mC/D/E; Fig. 1B) (Fig. 6B, upper panel).

We recently demonstrated that Sp1 suppression of p21$^{WAF1/Cip1}$ blocked the formation of p21$^{WAF1/Cip1}$–cyclin D1–Cdk4 complex assembly, preventing phosphorylation of the checkpoint tumor suppressor Rb, and subsequently inhibiting proliferation of vascular SMCs [Kavurma and Khachigian, 2003]. The present study, using WKY12-22 SMCs as a model, provides the first evidence that endogenous Sp1 interacts directly with distinct *cis*-acting elements in the p21$^{WAF1/Cip1}$ promoter resulting in repression of p21$^{WAF1/Cip1}$ gene expression. The integrity of all elements is critical, since not only are there relatively large changes in basal expression upon mutation of the Sp1 binding motifs, but mutation of a single Sp1 response element was able to revert promoter repression in this cell type to activation. Altered basal p21$^{WAF1/Cip1}$ promoter expression upon mutation of the Sp1 binding sites suggests that Sp1 plays a direct regulatory role in the promoter and may play an indirect role through co-operative interactions with other factors. In marked contrast in endothelial cells, Sp1

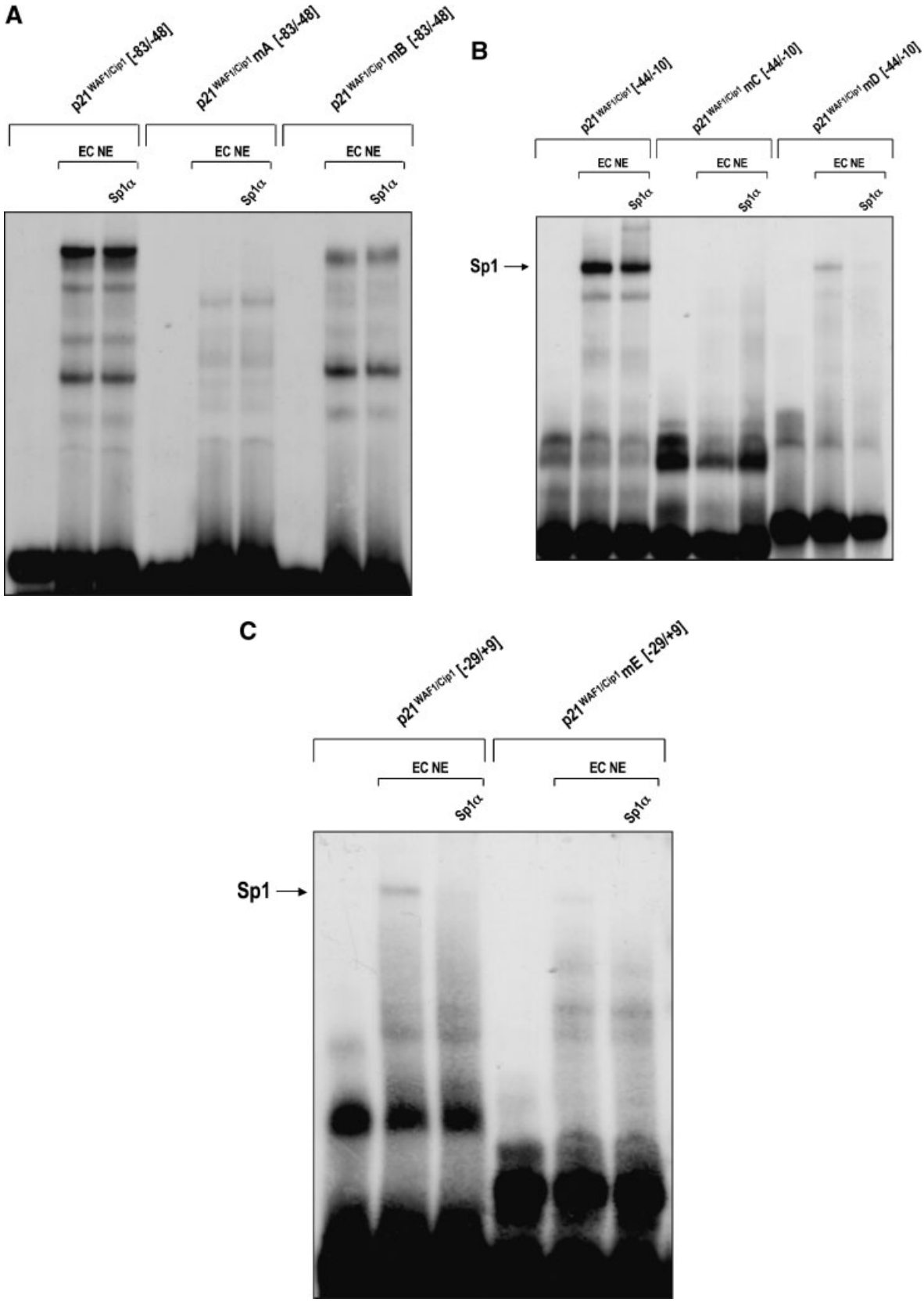
A
p21WAF1/Cip1 [-83/-48]
p21WAF1/Cip1 mA [-83/-48]
p21WAF1/Cip1 mB [-83/-48]
EC NE
Sp1α
B
p21WAF1/Cip1 [-44/-10]
p21WAF1/Cip1 mC [-44/-10]
p21WAF1/Cip1 mD [-44/-10]
Sp1
C
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Fig. 5.

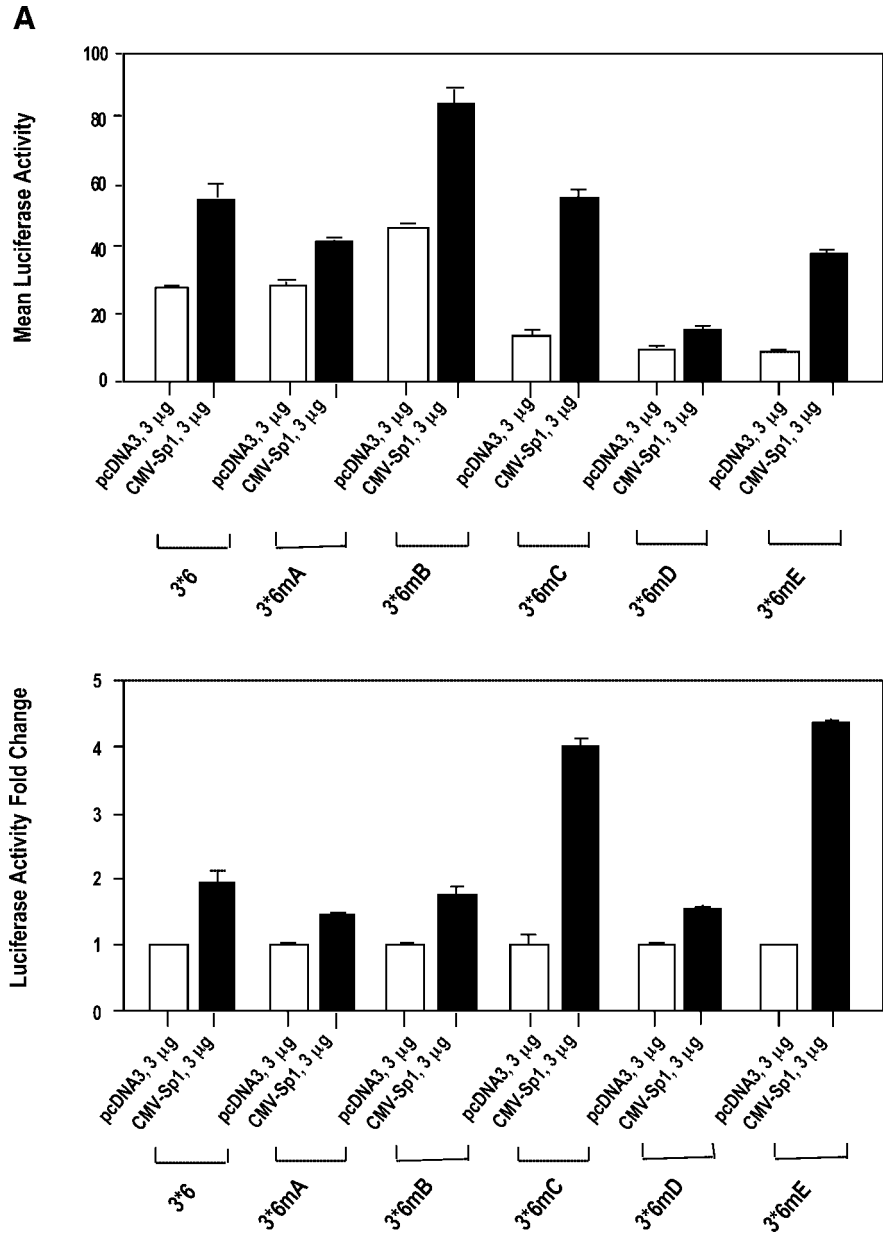

Fig. 6. Sp1 activation of the p21$^{WAF1/Cip1}$ promoter in endothelial cells involves elements C, D, and E. **A**: serial mutation of Sp1-elements in 3*6 do not lead to loss of p21 promoter activity following Sp1 overexpression. **B**: Double mutant (elements C and E; 3*6mC/E) leads to a 26-fold increase in p21$^{WAF1/Cip1}$ activation following Sp1 overexpression (**left panel**). Triple mutant (elements C, D, and E; 3*6mC/D/E) inhibits p21$^{WAF1/Cip1}$ promoter activity following Sp1 overexpression (**right panel**). **Upper panel** demonstrates mean luciferase activity. **Lower panel** demonstrates fold change in luciferase activity. Transient transfection analysis in endothelial cells overexpressing CMV-Sp1 together with mutant constructs. pcDNA3 has no effect. Firefly luciferase activity was normalized to Renilla activity. Error bars represent standard errors of the mean. The data is representative of two independent determinations.

Fig. 5. Endogenous endothelial nuclear Sp1 binds elements C, D, and E, but not A or B. **A**: Endogenous Sp1 does not bind elements A and B. EMSA using ^{32}P-p21$^{WAF1/Cip1}$ [−83/−49] and endothelial cell (EC) NE. **B**: Endogenous Sp1 binds elements C and D. EMSA using ^{32}P-p21$^{WAF1/Cip1}$ [−44/−10] and endothelial cell NE. Mutation of elements C and D impede Sp1 binding. **C**: Sp1 binds element E. EMSA using ^{32}P-p21$^{WAF1/Cip1}$ [−29/+9] and endothelial cell NE. Mutation of element E abrogates binding of Sp1. Arrow, denotes Sp1 supershift. Sp1 antibody incubation with endothelial cell NE is denoted by Sp1α.

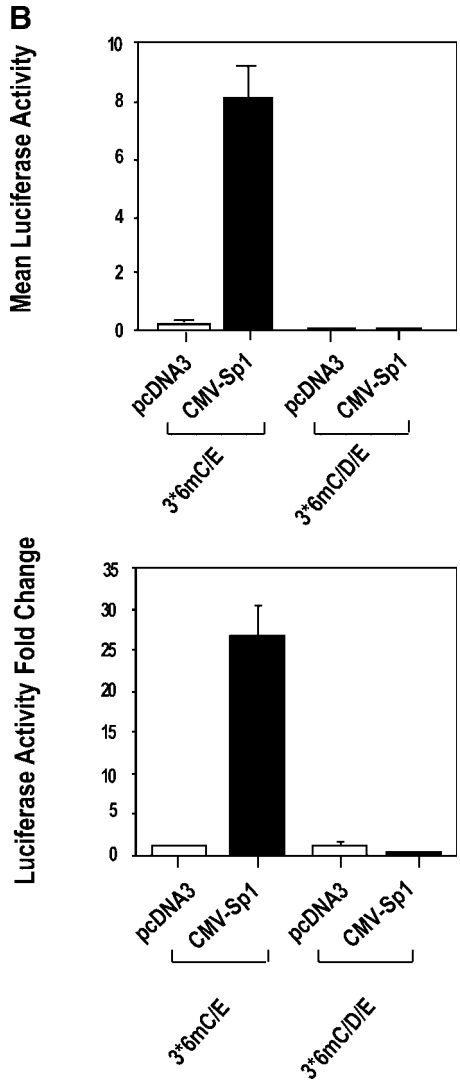

Fig. 6. (*Continued*)

interacts only with elements C, D, and E and mediates increased $p21^{WAF1/Cip1}$ promoter activity. Mutation in a single Sp1 site is not sufficient to ablate this effect. The single PSRE (element D) in the wild type $p21^{WAF1/Cip1}$ promoter in endothelial cells would appear to be overwhelmed by the net negative effect of neighbouring NSREs, C and E. In contrast, element D serves as a NSRE in WKY12-22 SMCs when all other sites are occupied, but switches to a PSRE when its flanking NSREs are inactive. These findings thus demonstrate first, that elements C, D, and E are essential for Sp1 activation of $p21^{WAF1/Cip1}$ transcription in endothelial cells, and elements A and B are not bound by Sp1 in this cell type. Second, elements C and E serve as NSREs in endothelial cells and third, that all five elements are bound by Sp1 in WKY12-22 SMCs and that each of these sites are critical for Sp1 repression of the $p21^{WAF1/Cip1}$ promoter. These findings also demonstrate cooperative interactions between Sp1 and different recognition elements within the $p21^{WAF1/Cip1}$ promoter.

Negative regulation of promoter activity by Sp1 has been the subject of previous investigations. For example, Sp1 through GC elements represses the protamine promoter [Jankowski and Dixon, 1987], the megakaryocyte-specific α_{IIb} promoter [Shou et al., 1998], transobalamin promoter [Li et al., 1998], and the ANT2 promoter [Zaid et al., 2001]. In fact, positive and negative effects of Sp1 on the regulation of the *ANT2* gene has been shown to involve separate GC elements in the proximal promoter [Li et al., 1996]. The present study, however, is the first to demonstrate the capacity of cell type-specific utilization of Sp1 binding sites to influence promoter activity.

Atherosclerosis is a complex arterial disease that initially involves endothelial cell activation, SMC migration and proliferation, and inflammation. Activated endothelial cells secrete growth factors which act in a paracrine manner on underlying SMCs and stimulate cell movement, growth, and the eventual formation of a neointima. The pattern of gene expression between these cell types is different. In the case of SMCs, this pattern will even be heterogenous in the one cell type. Transcription factors interacting with key promoter elements mediate spatial and temporal changes in gene expression. These interactions, in turn, regulate cell growth. This study demonstrates that $p21^{WAF1/Cip1}$ transcription, which selectively regulates endothelial and SMC phenotype [Kavurma and Khachigian, 2003], is exquisitely dependent upon the integrity of specific positive and negative Sp1 regulatory elements in the $p21^{WAF1/Cip1}$ promoter.

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