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Wild-Type p53 and v-Src Exert Opposing Influences on Human Vascular Endothelial Growth Factor Gene Expression[1]

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ABSTRACT

Angiogenesis, the development of new capillaries, is tightly controlled by the balance of positive and negative regulatory pathways. A newly described angiogenic factor, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), binds exclusively to endothelial cells and promotes their proliferation. Here we have studied the role of p53, a tumor suppressor, and v-Src, an oncogene on VEGF regulation. Wild-type p53 down-regulated endogenous VEGF mRNA level, as well as VEGF promoter activity, in a dose-dependent manner, whereas mutant forms of p53 had no effect. Overexpression of v-Src, known to up-regulate VEGF expression, activated a VEGF promoter-luciferase construct in a dose-dependent manner. Moreover, v-Src, in the presence of wt-p53, was unable to activate transcription of the VEGF promoter. Collectively, these data suggest that wild-type p53 may play a role in suppressing angiogenesis.

INTRODUCTION

Angiogenesis is required for solid tumor growth as well as expansion of metastatic colonies (1–3). Growth factors with angiogenic activity include fibroblast growth factor, platelet derived growth factor B chain, and VEGF[3] (4–8). VEGF is a dimeric glycoprotein and has specific mitogenic activity for endothelial cells (6, 7). It is inducible by hypoxia and expressed in a subset of solid tumor cells distinguished by their immediate proximity to necrotic foci (6, 9). Recent studies have demonstrated that its cell surface receptor, Flk-1, is exclusively expressed in endothelial cells, and a retrovirus encoded a dominant negative mutant of Flk-1 prevents tumor growth *in vivo* (10). These observations suggest a critical role for VEGF in angiogenesis. Moreover, the progression from low-grade astrocytoma to the highly malignant glioblastoma involves the expansion and accumulation of genetic lesions in a clone of cells with *p53* (tumor suppressor gene) mutations (11). Since angiogenesis is an important factor in this conversion, we have explored a possible correlation between aberrant p53 expression and VEGF up-regulation.

In our recent studies, we showed that c-Src activation by hypoxia up-regulates VEGF expression, and constitutive v-Src increased VEGF mRNA (12). Here we describe a regulatory role for the tumor suppressor gene *p53* in angiogenesis by suppressing basal VEGF transcription. Using VEGF promoter constructs, we have extended our previous studies regarding up-regulation of VEGF gene expression by v-Src. Collectively, these data point to two regulatory VEGF pathways: an oncogenic one (v-Src) enhancing VEGF production, and a tumor suppressor (p53) signal suppressing VEGF.

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; wt, wild type; CMV, cytomegalovirus.

MATERIALS AND METHODS

Plasmids. The promoter region from a human VEGF genomic clone (−2362 to +296, relative to the transcription start site) was amplified by PCR from a plasmid DNA containing 3.4 kb genomic sequence upstream from the translation initiation site, using primers designed from the published human VEGF 5′-flanking region (this plasmid DNA and human VEGF cDNA were generous gifts from Judith Abraham, Scios Nova, Inc.; Ref. 13). The amplified products were subcloned into a promoterless vector (pGL2 Basic) containing the firefly luciferase gene as a reporter (Promega Corp.).

Human cytomegalovirus immediate-early promoter-driven wt and mutant p53 expression plasmids (generously provided by Arnold J. Levine, Princeton University, Princeton, NJ) were used in this study. wt-p53 contains wt-p53 cDNA, whereas p53-SCX3 is a single nucleotide mutation that results in a valine to alanine alteration at codon 143 in p53-wt (a p53 clone from a colon carcinoma xenograft), and p53–4.2N3 is a mutant human p53 cDNA derived from an epidermoid carcinoma cell line A431 (14, 15). This *p53* gene encodes an arginine to histidine change at codon 273. The Moloney murine leukemia virus long terminal repeat v-*Src* and c-*Src* expression vectors were described earlier (16).

Cell Culture and Transfection. Human glioblastoma-astrocytoma cells U-87 MG (U87; ATCC HTB-14), 293 cells (a well-characterized, adenovirus-transformed human fetal kidney cell line; ATCC CRL 1573), human fibrosarcoma cells (HT1080; ATCC CCL 121), and human osteosarcoma cells (U20S; a kind gift from Stephen Friend, Massachusetts General Hospital, Boston, MA) were maintained in DMEM with 10% fetal bovine serum (HyClone Laboratories) and 1 mM sodium pyruvate (only for U87 cells). Cells were transiently transfected at 50–70% confluence by the calcium phosphate method (17), with the indicated amounts of expression and reporter plasmids in 60-mm dishes. The transfection efficiency (as assessed by β-galactosidase histology) in our subclone of 293 cells was in the range of 50–70%. Transfection efficiency was normalized by β-galactosidase activity using a β-galactosidase gene under control of the CMV immediate-early promoter. For HT1080 cells, where β-galactosidase activity was very low, luciferase activity was normalized by total amount of cellular protein measured by the Bio-Rad protein assay kit. RNA was extracted from cells 36–40 h after transfection. For determination of luciferase activity, after removing medium, cells were washed twice with PBS buffer. Four hundred μl of reporter lysis buffer [25 mM Tris (pH 7.8), 4 mM EDTA, 2 mM DTT, 10% glycerol, and 1% Triton] were then added. After a 15-min room temperature incubation, the cell lysate was scraped and transferred to a microcentrifuge tube. The lysate was centrifuged for 15 s at room temperature. Twenty μl of room-temperature cell lysate were mixed with 100 μl of room-temperature luciferase assay reagent [20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2H_2O$), 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM CoA, 470 μM luciferin, and 530 μM ATP, final pH 7.8]; the reaction mixture was immediately placed in a luminometer (Turner TD-20e), and the light produced was measured for a period of 10 s.

Northern Blot Analysis. RNA, isolated by the single-step acid phenol extraction method (18), was separated on a formaldehyde-agarose gel, transferred to GeneScreen (DuPont) membrane using 10× SSC, and probed with random primer-labeled cDNAs in a solution containing 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA, and sonicated herring sperm DNA (50 μg/ml) at 68°C. Blots were washed three times with a solution containing 40 mM sodium phosphate (pH 7.2), 0.5% SDS, 0.5% BSA, and 1 mM EDTA at 68°C and then exposed to Kodak X-OMAT film. Quantitation of the Northern analysis was performed using a Hewlett Packard scanner (Scan Jet IIc).

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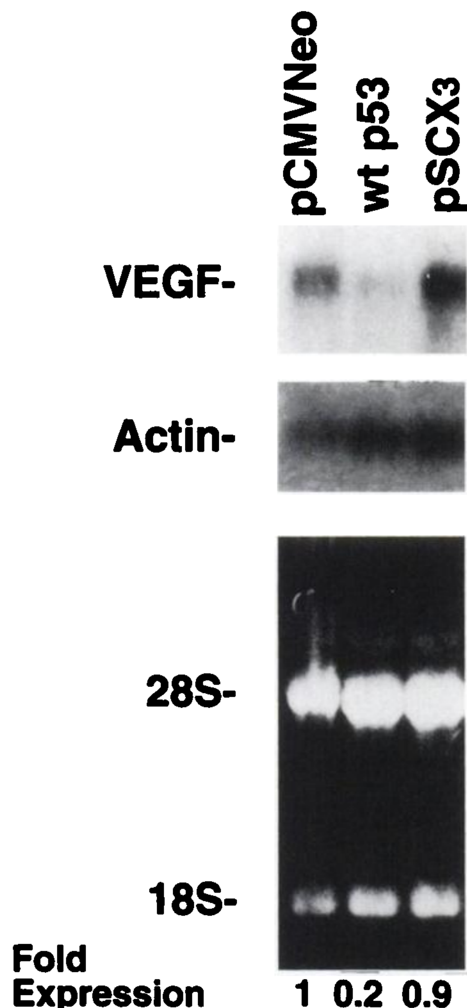

Fig. 1. Inhibition of VEGF gene expression by wt-p53. Five μg of total RNA were extracted from 293 cells transiently transfected with 1.0 μg of empty expression vector (*Lane 1*), wt-p53 (*Lane 2*), and mutant p53 (*Lane 3*) expression vectors. The Northern blot was hybridized to a human VEGF cDNA probe. This probe recognizes a VEGF transcript of approximately 4.0 kb. After probing for VEGF mRNA, the blot was stripped and hybridized with a β-actin probe (for normalization). *Lower panel*, ethidium staining of the RNA samples prior to transfer. Similar results were obtained in two independent experiments.

RESULTS

Effects of wt and Mutant p53 on VEGF Expression. VEGF gene expression is up-regulated in many human solid tumors compared to normal cells from which the tumor originates (19).[4] Hypoxia is clearly one key inducer of VEGF mRNA (9). However, little is known about other regulators of basal VEGF levels. To investigate whether p53 exerts an effect on VEGF expression, we initially used wt-p53 or mutant p53 expression vectors transfected into 293 cells. Fig. 1 shows that wt-p53, but not mutant p53, has a pronounced suppressive effect on VEGF gene expression (80% inhibition). No effect was seen on β-actin mRNA levels. It is known that wt-p53 expressed at very high levels can repress transcription of promoters containing a TATA box but lack a p53 binding site (20). The β-actin promoter does not have a p53 binding site but contains a TATA box. Since the amount of wt-p53 DNA used in our experiments could not suppress β-actin gene expression, we suggest that the effect of wt-p53 on VEGF gene expression is a specific effect.

[4] D. Mukhopadhyay *et al.*, unpublished observations.

Effect of wt and Mutants of p53 on VEGF Promoter Activity. The human VEGF promoter has been identified and characterized (13). The promoter contains no TATA or CCAAT boxes or putative p53 binding sites but has five Sp1-binding sites. We made a VEGF promoter-luciferase construct as described in "Materials and Methods." We used the 2.6-kb promoter-luciferase construct to analyze the effect of wt or mutant p53 on this promoter by cotransfection assays. Fig. 2*A* shows that wt-p53 inhibited VEGF promoter activity in a dose-dependent manner in both U87 and 293 cells. In the presence of 0.5 μg of wt-p53 plasmid DNA, the promoter activity in 293 cells was reduced by 70% compared to an empty expression vector control. For U87 cells, similar data was obtained (80% inhibition). The pCMV β-gal activity was unaffected at this concentration of expression vector. Moreover, wt-p53 had no effect on pGL2 Basic, the backbone luciferase plasmid into which the VEGF promoter fragments were cloned (data not shown). We tested VEGF promoter activity in other cell types in the presence of 0.5 μg of wt-p53 plasmid DNA. Similar effects (Fig. 2*B*) were noted. Since these studies were in cell lines that contain different endogenous p53 levels and in different functional states (*e.g.*, 293 cells express E1A that binds p53), the effect of overexpression of wt-p53 on the VEGF promoter is generalizable. Furthermore, we studied the effect of overexpression of p53 mutants (known to convert p53 from a tumor suppressor gene to an oncogene) on the VEGF promoter. Fig. 3 shows that in both cell types (U87 and 293 cells), the mutants of p53 did not affect promoter activity. These results suggest that wt-p53 has a specific effect on the VEGF promoter by suppressing its transcription.

Effect of v-Src on VEGF Promoter. Recently, we have shown that c-Src is activated by hypoxia and up-regulates VEGF expression under hypoxic conditions, whereas v-Src can up-regulate VEGF mRNA levels in the absence of hypoxia (12). Here we investigated the role of v-Src on the VEGF promoter. Fig. 4*A* shows that v-Src up-regulates VEGF promoter activity in a dose-dependent manner in U87 and 293 cells. In the presence of 1.5 μg of v-*Src* expression vector, the VEGF promoter activity increases up to 4- to 5-fold. This observation corroborates our previous *in vivo* experiments in which endogenous VEGF message was increased by v-Src overexpression and suggests that transcriptional activation by v-Src plays a role in increased VEGF gene expression. Note that cotransfection with c-*Src* expression vector did not show any significant effect on this promoter activity (Fig. 4*B*), also correlating with data on VEGF mRNA.

Effect of p53 on v-Src-mediated VEGF Induction. Next, we addressed the question of whether p53 overexpression can abrogate the effect of v-Src on VEGF promoter activity. We cotransfected overexpressing vectors of v-*Src* and wt-p53 (with empty vectors as appropriate controls) and the VEGF promoter construct. In the presence of wt-p53, the VEGF induction by v-Src is completely repressed (Fig. 5). Significantly, the two previously used oncogenic mutants of p53 do not inhibit v-Src-mediated VEGF promoter activity. This result suggests that in VEGF regulation, wt-p53 has a role "downstream" of v-Src.

DISCUSSION

Angiogenesis is a central mechanism for tumor cell growth and metastasis (1, 2). VEGF is the most important directly acting angiogenic protein (6, 7, 21, 22). Although VEGF is produced by numerous cell types, its receptor is exclusively localized to endothelial cells (23). Considerable attention has focused in the field of angiogenesis on post-receptor events mediating the VEGF action. Little is known about the regulation of VEGF itself, although this topic is clearly of considerable importance. One factor regulating the expression of VEGF is hypoxia, a signal that plays a critical role in tumors as well

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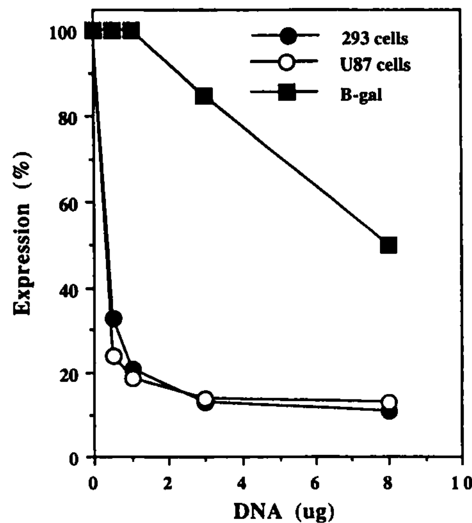

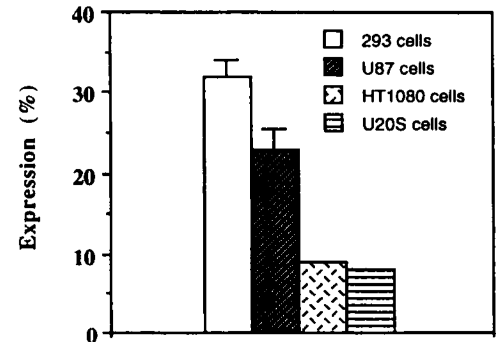

Fig. 2. Effect of wt-p53 protein on transcription from the VEGF promoter. *A*, 293 cells and U87 cells were cotransfected with 1.0 μg of a 2.6-kb VEGF promoter construct or 1.0 μg of a CMV promoter-driven, β-galactosidase gene plasmid (in 293 cells) and different amounts of p53 expression vectors. The ordinate is relative luciferase activity expressed as a percentage of activity measured after cotransfection of the VEGF promoter construct with the empty expression vector, pCMVneo. *B*, 1.0 μg of 2.6-kb VEGF promoter construct and 0.5 μg of wt-p53 expression vector DNA were cotransfected in different cell lines. Data with error bars (SD) were derived from at least three experiments; the remaining data were the average of two independent experiments.

as in diabetic retinopathy (6, 7, 9, 24, 25). It is likely that other mechanisms of regulation exist for this important angiogenic protein. In particular, since the angiogenic phenotype is one that probably results from the accumulation of sequential mutations in multiple genes, it seemed reasonable to ask whether the activation of oncogenes or the ablation of a tumor suppressor gene can affect VEGF expression. The tumor suppressor wt-p53 inhibits proliferation of normal as well as transformed cells by interacting with viral and cellular oncoproteins (26, 27). Overexpression of wt-p53 can inhibit transcription of many cellular and viral promoters (28–32). One of the critical events in the conversion of an astrocytoma to a glioblastoma is the inactivation of p53 (11). Importantly, glioblastomas tend to be well vascularized, and VEGF expression levels in glioblastoma are considerably higher than those in astrocytomas (6). Therefore, we hypothesized that p53 might regulate VEGF expression.

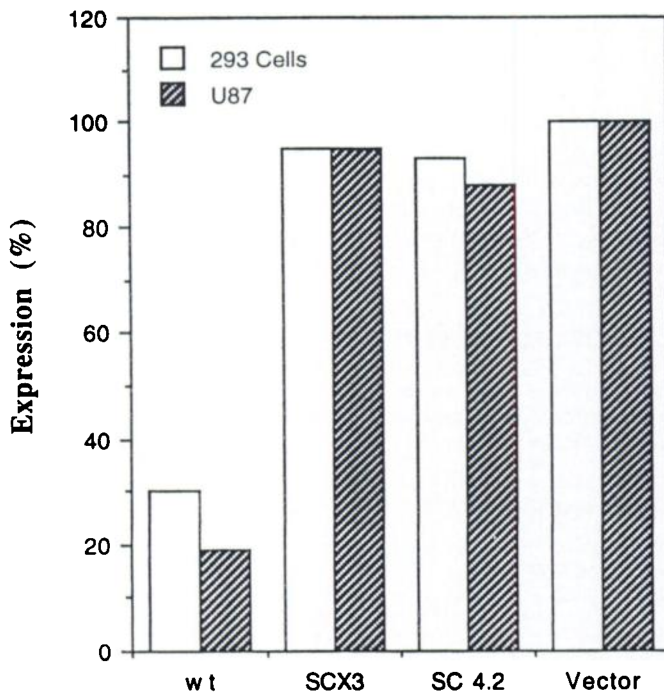

Fig. 3. Effect of mutant p53 on transcription from the VEGF promoter. U87 cells and 293 cells were cotransfected with the 1.0 μg of 2.6-kb VEGF promoter construct and 0.5 μg of wt-p53 expression vector DNA. Luciferase activity was measured as described earlier. The results are the average of two independent experiments.

In Fig. 1, we demonstrated that wt-p53 down-regulated endogenous VEGF gene expression, but a mutant of p53 had no effect. Moreover, this effect was specific for VEGF gene expression, since other genes, such as β-actin and transforming growth factor β1 (data not shown), were not affected by overexpression of wt-p53. Recently, Keiser *et al.* (33) have reported that a mutated form of the murine p53 (ala 135 to val), but not wt-p53, induced expression of VEGF mRNA and potentiated 12-*O*-tetradecanoylphorbol-13-acetate-mediated VEGF mRNA expression. In our data, using pSCX3 plasmid (ala 143 to val), a mutated form of the human p53, we did not find any up-regulation of endogenous VEGF levels, whereas wt-p53 down-regulated VEGF expression. Moreover, wt-p53 down-regulates the transcription of VEGF promoter activity in a dose-dependent manner in both U87 and 293 cells (Fig. 2*A*). We examined results in different cell types, irrespective of the endogenous status of p53 (Fig. 2*B*). These data suggested that wt-p53 acts as repressor of VEGF transcription but that mutants of p53 do not affect promoter activity (Fig. 3). This observation correlates well with the effects of wt-p53 and mutant p53 on VEGF mRNA levels, suggesting that transcriptional repression by the VEGF gene may account largely for the down-regulation of the endogenous message.

Mack *et al.* (20) observed that both *in vivo* and *in vitro* expression of wt-p53 specifically repressed the activity of TATA promoters that do not contain a wt-p53 DNA-binding sequence. They also showed that promoters whose transcription is directed by a pyrimidine-rich initiator element are unaffected by wt-p53. The VEGF promoter does not possess a TATA box or a wt-p53 DNA-binding sequence. Thus, the repression we see might be mediated by indirect means, *e.g.*, other factor(s) which are activated by wt-p53. Of importance, Dameron *et al.* (34) showed that in fibroblasts, wt-p53 inhibits angiogenesis through up-regulation of thrombospondin-1, a potent inhibitor of angiogenesis. It is tempting to speculate that thrombospondin-1 somehow down-regulates VEGF expression. On the other hand, the possibility of a new p53 binding site in the VEGF promoter cannot be ignored. Further deletion analysis of the VEGF promoter is in progress to localize *cis*-acting elements responsible for this repression. Collectively, these data suggest a role for wt-p53 on VEGF regulation, either directly interacting with the VEGF promoter or by an indirect pathway.

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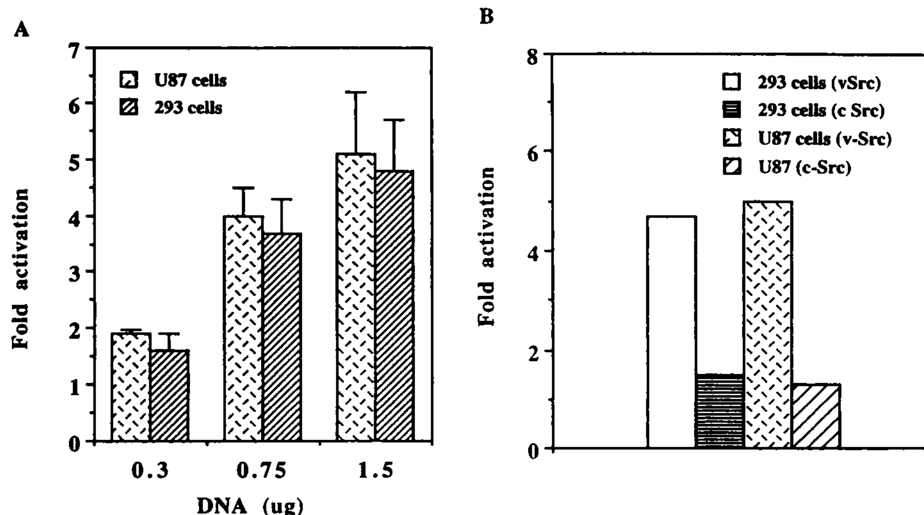

Fig. 4. Effect of v-Src on transcription from the VEGF promoter. U87 cells and 293 cells were cotransfected with 1.0 μg of the 2.6-kb VEGF promoter construct and: *A*, a different amount of v-*Src* expression vector DNA; and *B*, 1.5 μg of v-*Src* or 1.5 μg of c-*Src* expression vector DNA. Relative transcription was calculated using the empty expression vector (pEVX) DNA as a control. Data with error bars (SD) were derived from at least three experiments; the remaining data were the average of two independent experiments.

Previous findings on Src as a critical intermediary in the hypoxia-mediated VEGF response also prompted us to ask whether there is a role for the activated version of Src, namely v-Src, in regulating VEGF. In particular, increased vascularity has been noted in polyomavirus middle-T antigen transformed tumors (hemangiomas; Refs. 35–37). Several lines of evidence indicate that activated Src plays an important role in middle T-induced protein phosphorylation and cell transformation (38–42). It was, therefore, reasonable to ask whether v-Src might be regulating VEGF in the absence of hypoxia.

Our previous studies showed that v-Src induces endogenous VEGF expression. In this report, we have demonstrated that v-Src transactivates the VEGF promoter in a dose-dependent manner in both U87 and 293 cells (Fig. 4*A*). However, when we cotransfected v-*Src* and p53 plasmid DNA, the VEGF promoter activity diminished dramatically, whereas mutants of p53 did not reduce the v-Src-mediated activation. We also tested the effect of c-Src on the VEGF promoter (Fig. 4*B*). c-Src had no significant effect on VEGF promoter activity, corroborating our previous results that c-Src overexpression has no effect on endogenous VEGF mRNA level. We have also addressed the question of whether hypoxia can up-regulate VEGF promoter activity. Interestingly, there was no significant up-regulation of VEGF promoter activity, and recently we have shown that VEGF mRNA induction by hypoxia is not mediated solely by transcriptional activation, based on nuclear run-on experiments (data not shown; Ref. 43). Therefore, v-Src-mediated induction and hypoxia-mediated up-regulation of VEGF mRNA seem to occur through different pathways; v-Src-mediated activation is a transcriptional event, whereas activation through hypoxia may be mostly a posttranscriptional event.

Our studies on p53 and v-Src suggest that there is a fine interplay between a tumor suppressor gene and an oncogene with regard to VEGF regulation. It is likely that other tumor suppressors and tumor generators may have similar effects. It is also interesting to note that wt-p53 can, in fact, override the effects of v-Src when introduced in the appropriate amounts, suggesting that the protective effect of p53 lies downstream of the deleterious effects of v-Src. Significantly, mutants of p53 cannot block the ability of v-Src to induce VEGF. We have as yet to explore the role of hypoxia and how it synergizes with the effects of p53 and v-Src.

DNA (μg)					
pEVX	-	-	-	-	1
pEVXvSrc	1	1	1	1	-
pCMVneo	0.5	-	-	-	-
pCMVp53	-	0.5	-	-	0.5
pCMVSCX3	-	-	0.5	-	-
pCMVSC4.2	-	-	-	0.5	-

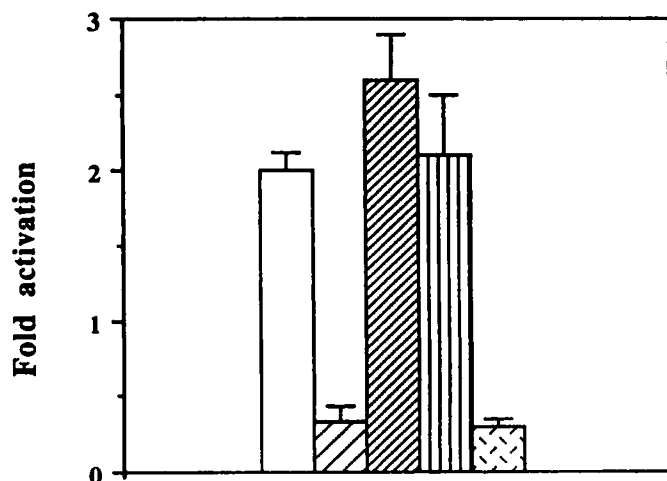

Fig. 5. Effect of wt-p53 on v-Src-mediated transcription from the VEGF promoter. 293 cells were cotransfected with the indicated amount of plasmid DNAs, and fold transcriptional activation was assessed by densitometric scanning of the resulting Northern blot.

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