Advances in Brief

Recombinant Adenovirus Expressing Wild-Type *p53* Is Antiangiogenic: A Proposed Mechanism for Bystander Effect[1]

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Abstract

Angiogenesis is required for the growth and progression of malignancies. Recent studies have demonstrated that genetic alterations may accompany acquisition of the angiogenic phenotype. The tumor suppressor gene *p53* is most frequently mutated in human cancers and is also known to be a transcriptional regulator of a variety of genes. Here, we investigated the antiangiogenic effect of the wild-type *p53* (wt-*p53*) gene transfer on a human non-small cell lung cancer cell line. Mutant *p53*-expressing H226Br non-small cell lung cancer cells were transduced with the wt-*p53* gene using a recombinant adenoviral vector (Ad5CMVp53) and applied to semiquantitative reverse transcription-PCRs for the detection of altered mRNA expression of angiogenic and/or antiangiogenic factors. *In vivo* neovascularization assay of Ad5CMVp53-infected cells was then performed using a membrane-diffusion chamber system s.c. transplanted in *nu/nu* mice. We also evaluated the effect of Ad5CMVp53-infected H226Br cells on nontransduced tumor cells *in vivo* by s.c. inoculating mixture of cells into *nu/nu* mice. Ad5CMVp53 infection markedly inhibited the expression of an angiogenic factor, vascular endothelial growth factor, and increased the expression of a novel antiangiogenic factor, brain-specific angiogenesis inhibitor 1, resulting in reduced neovascularization *in vivo*. Mixing experiments showed that tumor cells transduced with the wt-*p53* gene inhibited the *in vivo* tumor growth of adjacent nontransduced cells. Our data suggest that a recombinant adenovirus expressing the wt-*p53* gene is antiangiogenic, which may explain, in part, the mechanism of the bystander effect induced by the wt-*p53* gene transfer on adjacent tumor cells.

Introduction

Angiogenesis is the development of new capillaries from preexisting capillary blood vessels, and it is necessary for the growth of solid tumors beyond 1–2 mm in diameter (1). The occurrence of the angiogenic phenotype in tumor cells causes rapid expansion of primary tumors and increases the risk of distant metastasis; the mechanism of the angiogenic switch, however, remains unknown. Angiogenesis consists of dissolution of the basement membrane, migration, and proliferation of endothelial cells, canalization, branching and formation of vascular loops, and formation of a basement membrane (2). These steps might be regulated by the local balance between the amount of angiogenic stimulators and inhibitors (3–5). In normal tissue, endogenous angiogenic inhibitors such as platelet factor 4, TSP1,[3] angiostatin, and endostatin constitutively suppress angiogenesis, while, as cells undergo malignant transformation, angiogenic mitogens, which include bFGF, VEGF, and platelet-derived epithelial cell growth factor, become dominant, which causes the aberrant angiogenesis. These results suggest that tumorigenesis is associated with angiogenesis and that tumor growth may be alleviated by inhibiting angiogenesis.

The mutation of the tumor suppressor gene *p53* occurs in many inherited and sporadic forms of human malignancy (6). We previously reported that restoration of wt-p53 function caused a significant antitumor effect in human NSCLC cells and in human colorectal cancer cells that expressed the homozygously mutated or deleted *p53* gene (7–10). The p53 protein mediates various normal cellular activities as a transcriptional factor. VEGF, also known as vascular permeability factor, is a

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[3] The abbreviations used are: TSP, thrombospondin; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; wt-*p53*, wild-type *p53*; NSCLC, non-small cell lung cancer; BAI1, brain-specific angiogenesis inhibitor 1; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection.

M_r 34,000–50,000 dimeric glycoprotein and an angiogenic stimulator of endothelial cells (11). Recent studies have demonstrated that the expression of VEGF is positively regulated by oncogenic transformation with activated forms of c-*Src*, v-*Src*, and H-*ras* (12); physiological stress of hypoxia (13); increased cell density (14); and inactivation of the *p53* gene (15). A novel antiangiogenic factor, BAI1, is a p53 target molecule (16). The p53-induced *BAI1* gene product contained five TSP type I repeats and inhibited *in vivo* neovascularization induced by bFGF in the rat cornea. These findings lead to the hypothesis that a mechanism of the *in vivo* antitumor effect of the wt-*p53* gene transfer is through its potent antiangiogenic function.

Here, we demonstrated that wt-*p53* gene transfer downregulated the increased amount of VEGF expression and upregulated the BAI1 expression in mutant *p53*-expressing human NSCLC cells. We also provided the evidence that changed VEGF and BAI1 expression after introduction of the wt-*p53* gene caused the inhibition of *in vivo* tumor angiogenesis using a diffusion chamber system, which consisted of cellulose ester membrane filters. Finally, the ability of tumor cells, which were transduced by the wt-*p53* gene, to suppress the tumor formation of mixed nontransduced cells was determined to characterize the bystander effect of the wt-*p53* gene transfer on adjacent cells.

Materials and Methods

Cells and Culture Conditions. A human NSCLC cell line H226Br, which has a homozygously mutated *p53* gene at codon 254, was routinely propagated in monolayer culture in RPMI 1640 supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO). The H226Br cell line is a variant of the NCI-H226 cell line derived from a brain metastasis in a *nu/nu* mouse (gift of I. J. Fidler, The University of Texas M. D. Anderson Cancer Center, Houston, TX). The transformed embryonic kidney cell line 293 was grown in DMEM (Life Technologies, Inc., Grand Island, NY) with high glucose (4.5 g/liter), supplemented with 10% FCS.

Recombinant Adenoviruses. The production and *in vitro* characterization of a recombinant adenoviral vector, which contained the human wt-*p53* cDNA, were reported previously (17). Briefly, the plasmid containing the CMV promoter and the wt-*p53* cDNA was cotransfected with pJM17 into 293 cells by liposome/DNA coprecipitation to produce an adenoviral wt-*p53* expression vector. The resultant virus was named Ad5CMVp53. The adenoviral vector containing luciferase cDNA (Ad5RSVLuc) was used as a control vector. These viruses were produced by lysing infected 293 cells. Titers of the viral stock were determined using a plaque-forming assay with 293 cells. The viruses were aliquoted and stored at −80°C.

Reverse Transcription-PCR Analysis. Total RNA was isolated from H226Br cells infected with Ad5RSVLuc or Ad5CMVp53 using RNAzol (Cinna/Biotecx, Friendswood, TX) in a one-step phenol extraction method and used as the PCR templates. Reverse transcription was performed using 0.5 μg of total/cytoplasmic RNA per reaction to ensure that the amount of amplified DNA was proportional to that of the specific mRNA in the original sample. PCR with specific primers was performed for 25–35 cycles in a 50-μl volume according to the manufacturer's protocol (PCR kit; Perkin-Elmer/Cetus, Norwalk, CT) using a Perkin-Elmer (Foster City, CA) thermal cycler. Specific primers were used for the promoter/p53 region of Ad5CMVp53 (sense, 5′-GGT GCA TTG GAA CGC GGA TT-3′; antisense, 5′-CAA ATC ATC CAT TGC TTG GGA-3′), VEGF (sense, 5′-CAC ATA GGA GAG ATG AGC TTC-3′; antisense, 5′-CCG CCT CGG CTT GTC ACA T-3′; Ref. 18), BAI1 (sense, 5′-ACT CAT CCT GCG ACG GTG TG-3′; antisense, 5′-TCC CTC AGG TCC TTC ATG CG-3′; Ref. 16), bFGF (sense, 5′-GCC TTC CCG CCC GGC CAC TTC AAG G-3′; antisense, 5′-GCA CAC ACT CCT TTG ATA GAC ACA A-3′; Ref. 19), TSP1 (sense, 5′-CGT CCT GTT CCT GAT GCA TG-3′; antisense, 5′-GGC AGG ACA CCT TTT TGC AGA-3′; Ref. 20), and GAPDH (sense, 5′-CAG CCG AGC CAC ATC-3′; antisense, 5′-TGA GGC TGT TGT CAT ACT TCT-3′), which served as a control. The amplification reactions involved denaturation at 93°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min (VEGF); denaturation at 96°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min (BAI1); denaturation at 97°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 3 min (bFGF); or denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min (TSP1). The PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. The density of products were quantified using an image analyzer with GAPDH as the internal control.

Immunohistochemistry. Immunohistochemical staining of mock- or Ad5CMVp53-infected monolayers of H226Br cells was performed using a Histofine kit (Nichirei, Tokyo, Japan). The slides were blocked with 1% BSA for 30 min and incubated with the primary antibody overnight at 4°C in a humidified chamber. After rinsing, the slides were incubated with biotinylated rabbit antimouse IgG for 10 min and then with avidin-biotin-peroxidase complex for 10 min. Peroxidase activity was determined using 3,3′-diamino-benzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide. The slides were counterstained with methyl green. The primary antibody used was anti-p53 antibody (Oncogene Science, Manhasset, NY).

Western Blot Analysis. Parental or viral vector-infected H226Br cells were washed twice in cold phosphate saline buffer and lysed in SDS solubilization buffer [62.5 mM Tris-HCl (pH 6.5) containing 10% glycerol, 5% β-mercaptoethanol, and 2% SDS; Sigma]. Equal amounts of proteins were boiled for 5 min and electrophoresed under reducing conditions on 12.5% (w/v) polyacrylamide gel. Proteins were electrophoretically transferred to Immobilon-P polyvinylidene difluoride transfer membrane (Millipore, Bedford, MA) and incubated with the primary antibody against VEGF (Santa Cruz Biochemicals, Santa Cruz, CA) or p53 (Oncogene Science, Manhasset, NY), followed by peroxidase-linked secondary antibody. The Amersham ECL Chemiluminescent Western System (Amersham, Arlington Heights, IL) was used to detect secondary probes.

***In Vivo* Assay for Tumor Angiogenesis.** *In vivo* angiogenesis was determined using the dorsal air-sac method (21). Briefly, 1×10^7 parental or viral vector-infected H226Br cells were suspended in PBS and put into round-shaped chambers, which consisted of a ring covered with cellulose ester filters (pore size, 0.45 μm; Millipore) on both sides. These chambers were implanted into a dorsal air sac produced in 4–5-week-old female BALB/c *nu/nu* mice by the injection of 10 ml of air. Five

mice in each group were killed on day 5, and the formation of a dense capillary network in s.c. regions was examined under a dissecting microscope. All experiments involving mice were performed under protocols and guidelines approved by the Okayama University Animal Care and Use Committee.

***In Vivo* Tumor Growth and Determination of Microvessels.** Female BALB/c *nu/nu* mice were s.c. implanted with parental H226Br cells mixed with Ad5CMVp53-infected H226Br cells at the indicated ratio. The s.c. H226Br tumors were measured with calipers to determine the perpendicular diameter and tumor volume was calculated as $(\text{width}^2) \times (\text{length})/2$. Development of the tumors was monitored over 21 days. Tumor tissue was frozen in O.C.T. compound and then cut at 5-μm thickness. Immunohistochemical staining using a rat antimouse monoclonal antibody against CD31 (PharMingen, San Diego, CA) was performed. The number of vessels in each tumor was determined by counting the positive microvessels in a ×200 field.

Statistical Analysis. Differences in the tumor volumes for the treatment groups were compared using the Student's *t* test. Statistical significance was defined as $P < 0.05$.

Results

Recombinant Adenovirus-mediated wt-*p53* Gene Transfer. A recombinant adenovirus with human wt-*p53* cDNA controlled by the CMV immediate early gene promoter (Ad5CMVp53) was used. A human NSCLC cell line H226Br was transduced *in vitro* with the human wt-*p53* gene by the exposure to Ad5CMVp53 at a MOI of 30 for 36 h. Reverse transcription-PCR analysis using sense primer for the CMV promoter and antisense primer located within *p53* exon 3 was performed to determine whether the transduced *p53* gene was expressed in H226Br cells. The CMV/p53 segment of anticipated size was detected in cells transduced with wt-*p53* but not in cells incubated with the viral vehicle or control Ad5RSVLuc vector (Fig. 1*A*).

Immunohistochemistry demonstrated the wt-p53 protein, which was identified by intensive staining, compared to H226Br cells, which expressed the mutant p53 protein in most infected cells (Fig. 1*B*). A previous study demonstrated that the transduced wt-p53 protein was produced a day after infection, peaked after 3 days, and was maintained for up to 7 days (17). These results suggest that the adenovirus-mediated gene transfer and expression were markedly effective on human NSCLC cells. There was no apparent toxicity produced by this adenovirus concentration.

We previously reported that wt-*p53* gene transfer can induce apoptotic cell death in H1299 NSCLC cells (9); H226Br cells, however, were not sensitive to *p53*-mediated apoptosis. As shown in Fig. 1*C*, the growth of H226Br cells that were infected with 30 MOI of Ad5CMVp53 was significantly inhibited, compared to mock-infected and control Ad5RSVLuc-infected cells. Cell cycle profiles demonstrated a relatively high proportion of G_1 cells but not sub-G_1 cells, which indicates apoptosis (data not shown), suggesting that wt-*p53* gene transfer could have a temporary cytostatic effect instead of inducing cytotoxicity in H226Br NSCLC cells.

Regulation of Angiogenesis-related Factor Expression by wt-*p53* Gene Transfer. To investigate how the wt-*p53* gene transfer into H226Br cells changes their angiogenic function, we studied the mRNA expression of angiogenic stimulators, including VEGF and bFGF, and angiogenic inhibitors, including TSP1 and BAI1, after Ad5CMVp53 transduction using reverse transcription-PCR with specific primers for each factor (Fig. 2, *A* and *B*). Ad5CMVp53 infection resulted in a 77, 69, and 75% decreases in the 121, 165, and 189 VEGF transcripts, respectively, within 36 h after infection, compared to the mock-infected H226Br cells. bFGF mRNA expression was not significantly affected by Ad5CMVp53 infection, although bFGF transcription was expressed in parental H226Br cells. Parental H226Br cells expressed a low amount of TSP1 mRNA and down-regulation by Ad5CMVp53 infection occurred. Ad5CMVp53-infected H226Br cells expressed a significant amount of amplification product for BAI1 mRNA compared to mock-infected cells (2.9-fold increase).

To determine whether reduced VEGF mRNA expression caused by wt-*p53* gene transfer was consistent with VEGF protein change, VEGF protein was quantified by Western blot analysis. H226Br cells transduced by the wt-*p53* gene decreased 65 and 77% in VEGF protein expression 24 and 48 h after Ad5CMVp53 infection, respectively, compared to mock-infected control cells. This down-regulation of VEGF protein expression was inversely correlated with the overexpression of exogenously transduced wt-p53 protein (Fig. 2*C*). The kinetic study demonstrated that decreased VEGF protein expression returned to the level of the nontransduced H226Br cells after 7 days (data not shown) because the adenovirus-mediated exogenous gene expression was transient.

Inhibition of Tumor Cell-induced Angiogenesis by wt-*p53* Gene Transfer. We next assessed whether transduction of the wt-*p53* gene inhibited *in vivo* angiogenesis induced by H226Br NSCLC cells. To address this issue, mock-infected H226Br cells or H226Br cells infected with Ad5RSVLuc (30 MOI) or Ad5CMVp53 (30 MOI), which were packed into membrane chambers, were implanted into a dorsal air sac that was produced in *nu/nu* mice. The chambers consisted of membranes that allowed the passage of macromolecules such as VEGF and BAI1 but not cells. Five days after implantation, neovascularization, as demonstrated by the development of capillary networks and curled microvessels in addition to the preexisting vessels, occurred in the dorsal subcutis touched by the chamber, which contained mock-infected or Ad5RSVLuc-infected H226Br cells; infection with Ad5CMVp53, however, caused the preexisting vessels to become smaller as well as less tortuous and reduced the development of curled microvessels, which were characteristic for tumor neovasculature. Examples of the different grades of angiogenesis induced individually with the various treatments are shown in Fig. 3.

Inhibition of Nontransduced Tumor Cell Growth by Mixed wt-*p53* Gene-transduced Cells. The finding that restoration of wt-p53 function suppressed the neovascularization *in vivo* prompted us to study whether antiangiogenesis, which was caused by wt-*p53*-transduced H226Br cells, affected tumor growth and tumor angiogenesis of nontransduced H226Br cells

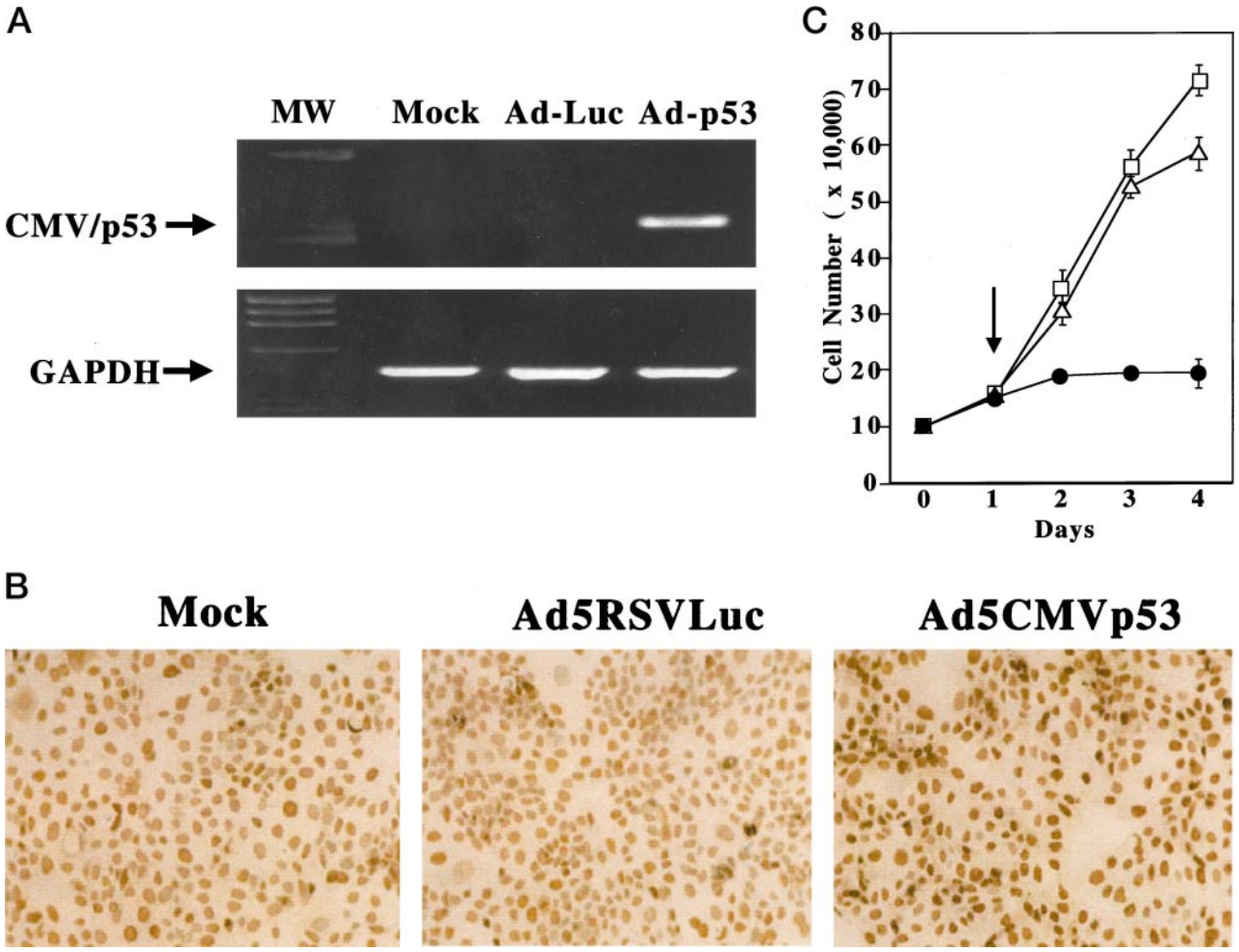

Fig. 1 *A*, detection of transduced *p53* gene expression in H226Br cells. Noninfected H226Br cells (2×10^6) and cells infected with Ad5RSVLuc or Ad5CMVp53 at a MOI of 30 were collected 36 h after infection and subjected to reverse transcription-PCR analysis. First-strand DNA generated from RNA was amplified using CMV promoter/p53 exon 3 primers or GAPDH primers. The PCR products were visualized on an ethidium bromide gel. *B*, efficient adenovirus-mediated wt-*p53* gene transfer in H226Br lung cancer cells. H226Br cells were infected with Ad5RSVLuc or Ad5CMVp53 at 30 MOI and then stained with anti-p53 antibody 36 h after infection. *Left*, mock-infected H226Br cells; *middle*, Ad5RSVLuc-infected H226Br cells; *right*, Ad5CMVp53-infected H226Br cells. *C*, effect of Ad5CMVp53 on the growth of H226Br human lung cancer cells. H226Br cells (10^4 cells) were plated in each well of triplicate 24-well plates and infected with Ad5RSVLuc or Ad5CMVp53 at a MOI of 30 for 36 h. Cell viability was determined by trypan blue staining on daily. Mock-infected cell growth was used as a control. *Data points*, means; *bars*, SD. *Arrow*, time of infection. □, mock; △, Ad5RSVLuc; ●, Ad5CMVp53.

in vivo. When 10^6 H226Br cells were injected s.c. into *nu/nu* mice, palpable tumors appeared in almost 100% of the mice 2 weeks after tumor injection; injection of H226Br cells infected with Ad5CMVp53 *in vitro*, however, caused the development of few relatively small tumors, which suggested that Ad5CMVp53-infected NSCLC cells caused less tumorigenicity, as reported previously (17).

We next performed mixed experiments using parental nontransduced H226Br cells and Ad5CMVp53-transduced H226Br cells. Nontransduced H226Br cells (1×10^6 cells/mouse) were mixed with 2×10^5, 5×10^5, or 1×10^6 H226Br cells that were previously exposed to Ad5CMVp53 at a MOI of 30 for 36 h *in vitro* and injected s.c. into *nu/nu* mice. Tumor formation was determined on days 14, 21, 28, and 35 by measuring the perpendicular diameter. As shown in Table 1, when compared with tumors composed of nontransduced H226Br cells alone, the tumor growth was significantly inhibited by the mixture of Ad5CMVp53-infected H226Br cells. Preliminary experiments demonstrated that the *in vivo* growth of control AdRSVLuc-infected H226Br tumors was similar to that of mock-infected H226Br tumors (data not shown). These results suggest that tumor cells transduced with the wt-*p53* gene might affect the growth of neighboring nontransduced tumor cells, presumably by inhibiting angiogenesis, thereby leading to tumor growth inhibition.

Finally, to further confirm this paradigm, tumor sections were immunohistochemically stained with the antibody for CD31 antigen as an endothelial cell marker. The staining revealed that H226Br tumors mixed with Ad5CMVp53-transduced cells displayed very few and extremely small blood

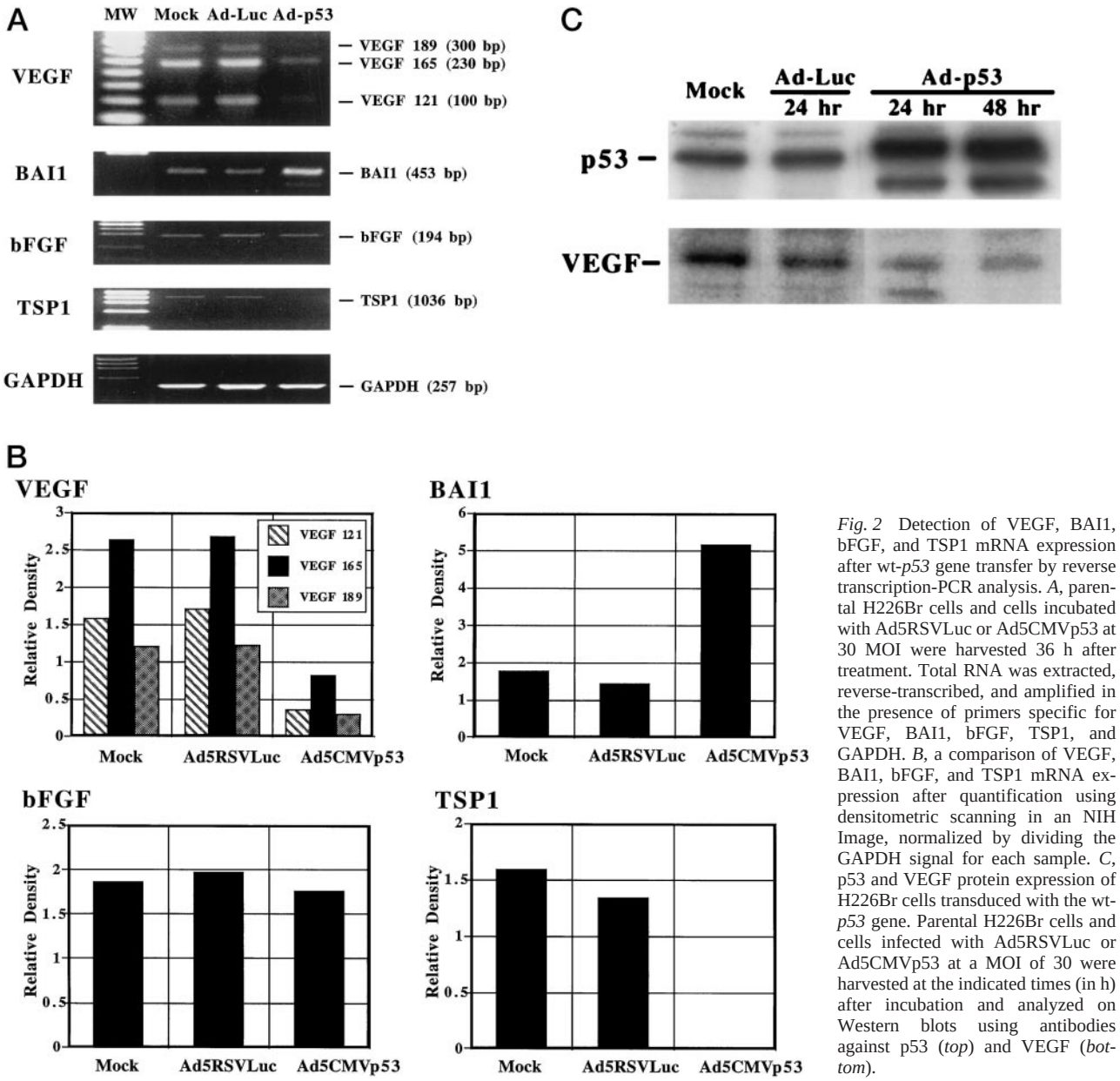

Fig. 2 Detection of VEGF, BAI1, bFGF, and TSP1 mRNA expression after wt-*p53* gene transfer by reverse transcription-PCR analysis. *A*, parental H226Br cells and cells incubated with Ad5RSVLuc or Ad5CMVp53 at 30 MOI were harvested 36 h after treatment. Total RNA was extracted, reverse-transcribed, and amplified in the presence of primers specific for VEGF, BAI1, bFGF, TSP1, and GAPDH. *B*, a comparison of VEGF, BAI1, bFGF, and TSP1 mRNA expression after quantification using densitometric scanning in an NIH Image, normalized by dividing the GAPDH signal for each sample. *C*, p53 and VEGF protein expression of H226Br cells transduced with the wt-*p53* gene. Parental H226Br cells and cells infected with Ad5RSVLuc or Ad5CMVp53 at a MOI of 30 were harvested at the indicated times (in h) after incubation and analyzed on Western blots using antibodies against p53 (*top*) and VEGF (*bottom*).

vessels (Fig. 4). The difference in vascularity was also determined by vessel counting using light microscopy. These *in vivo* studies demonstrated that inhibition of angiogenesis might be an important mechanism of the bystander effect that was caused by wt-*p53*-expressing tumor cells.

Discussion

p53 gene therapy, a novel form of cancer therapy based on the genetic abnormalities in tumor cells, is currently being studied in clinical trials (22). Reconstitution of wt-*p53* expression can suppress the growth of different tumor cells in culture by inducing cell cycle arrest and/or apoptosis (7–10). Recombinant adenovirus was used as the vector for the delivery and expression of the wt-*p53* gene because of its high-transduction efficiency compared to other viral and nonviral vectors (23); the adenovirus-mediated strategy, however, is not efficient enough to accomplish complete transduction of all tumor cells *in vivo*. The incomplete transduction efficiency may be a significant limitation of the currently available delivery system. To overcome this problem, we focused on the p53-regulated autocrine/paracrine factors responsible for the other cellular activities as well as tumor microenvironments. We have hypothesized that *p53* gene therapy might not need to completely transduce the tumor cells, if the wt-*p53* gene transfer not only induces the direct effect in the individual transduced cell but also causes

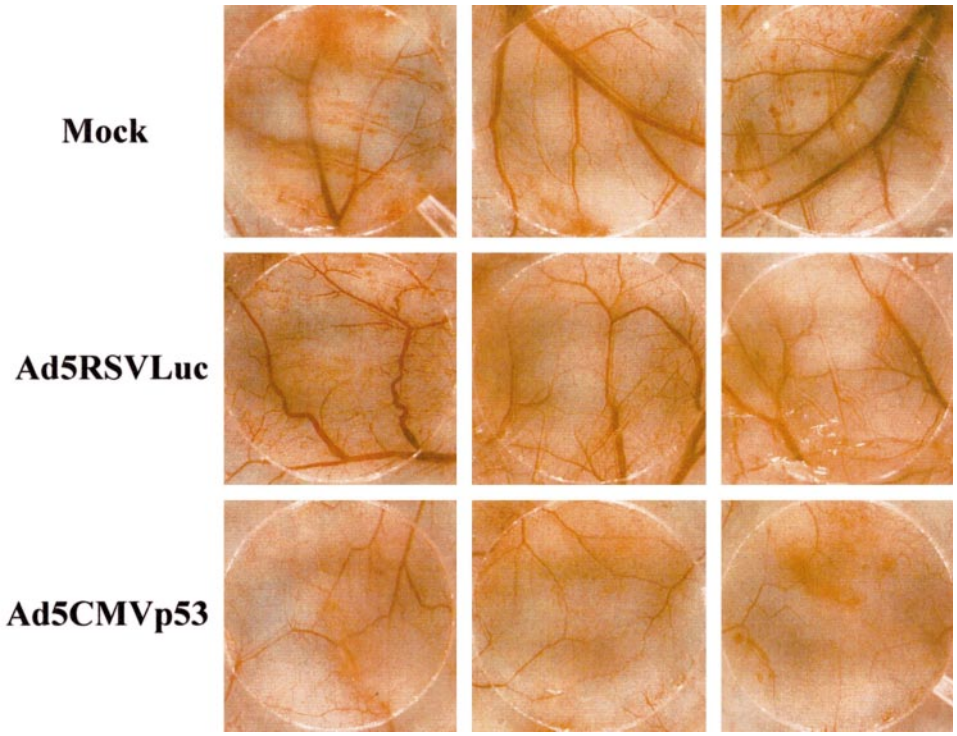

Fig. 3 Inhibition of H226Br tumor cell-induced angiogenesis by Ad5CMVp53 infection. Mock-infected (*Mock*; *top*), Ad5RSVLuc-infected (*Ad5RSVLuc*; *middle*), or Ad5CMVp53-infected (*Ad5CMVp53*; *bottom*) H226Br lung cancer cells prepared at a density of 1×10^7 were placed in a diffusion chamber, and the chamber was implanted into a dorsal air space produced in BALB/c *nu/nu* mice on day 0. Mice were killed on day 5, and the chamber was removed from the s.c. tissue. A new ring without filters was placed on the same site to mark the position of the chamber. The capillary networks developed inside the rings were photographed to determine the effect of Ad5CMVp53 transduction on angiogenesis.

Table 1 Inhibition of nontransduced H226Br tumor cell growth by mixed wt-*p53* gene-transduced cells

No. of mice with tumor/ total no. of mice	Cell no.[a]		Mean volume ± SD (mm^3)[b]			
			Days after tumor inoculation			
	H226Br	H226Br + Ad-p53	14	21	28	35
6/6	10^6		100.1 ± 53.8	266.2 ± 140.0	561.5 ± 339.7	1111.0 ± 635.1
5/5	10^6	2×10^5	53.8 ± 26.1	118.6 ± 52.6	282.2 ± 98.8	551.5 ± 227.0
5/5	10^6	5×10^5	40.2 ± 17.1	74.3 ± 31.2	167.0 ± 74.0	331.7 ± 150.5[c]
6/6	10^6	10^6	24.1 ± 18.9	37.6 ± 30.7	115.8 ± 56.9	269.5 ± 125.6[c]
2/6		10^6	0	0	33.9 ± 13.7	71.9 ± 26.5[d]

[a] Nontransduced H226Br cells were mixed with H226Br cells infected with Ad5CMVp53 (30 MOI).
[b] Mixed H226Br cells were s.c. injected into *nu/nu* mice. Perpendicular diameters were measured, and tumor volume was calculated at the indicated time.
[c] $P < 0.05$ by Student's *t* test when compared to the control group.
[d] $P < 0.01$ by Student's *t* test when compared to the control group.

the growth suppression of bystander, nontransduced cells via other mechanisms, such as angiogenesis inhibition. A recent study demonstrating that liposome-mediated wt-*p53* gene transfer into human breast cancer cells reduced the number of blood vessels supports our hypothesis (24).

Clinical studies support an association between the ability of tumor cells to induce blood vessel formation and their ability to grow as a solid tumor (1). During tumor development, transformed cells not only accumulate genetic alterations in proto-oncogenes and/or tumor suppressor genes, but they also need to produce angiogenesis. Recent studies have indicated that the angiogenic switch is caused by a change in the balance of angiogenic inducers and angiogenic inhibitors (3–5). Over the last decade, a number of distinct proteins have been characterized that can drive physiological and pathological angiogenesis. One of the most promising ways to therapeutic intervention of human cancer is to identify a molecule that can regulate the expression of factors related to angiogenesis and manipulate its expression, leading, in turn, to inhibition of angiogenesis. This study may address this issue.

Our results demonstrated that overexpression of the wt-*p53* gene could attenuate VEGF expression and activate BAI1 expression in H226Br NSCLC cells. VEGF is a selective mitogen for endothelial cells, and it directly stimulates the growth of blood vessels via its high affinity tyrosine kinase receptor, Flk-1/KDR, present exclusively on endothelial cells (11), whereas BAI1 is a specific inhibitor of endothelial cell migration and contains several discrete functional domains that can inhibit experimental angiogenesis (16). Transcriptional repression of VEGF after wt-*p53* introduction

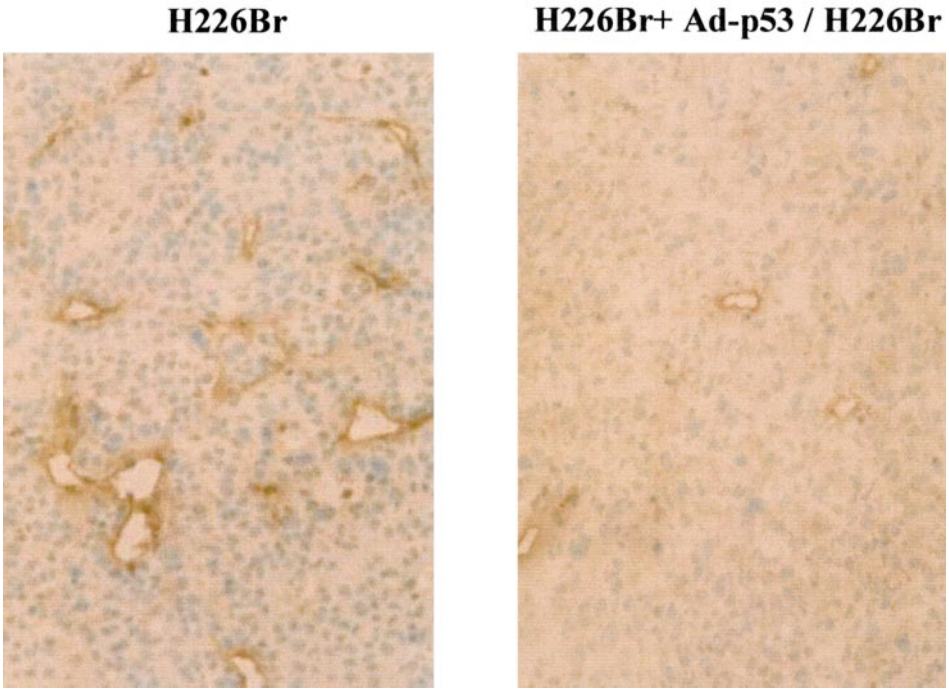

Fig. 4 Blood vessel formation in H226Br tumors mixed with Ad5CMVp53-infected H226Br cells. BALB/c *nu/nu* mice were s.c. injected with 1×10^6 parental H226Br cells mixed with Ad5CMVp53-transduced cells at the ratio of 1:1. As a control, 1×10^6 H226Br cells alone were inoculated into mice. Mice with tumors were killed on day 28, and frozen tissue sections of the tumors were probed with an antibody against CD31. Magnification, ×200.

is likely to be mediated by indirect means because the VEGF promoter has no specific p53-binding sequences. Recent studies have reported that a mutant form of the murine p53 protein (Ala-135 to Val) induced VEGF mRNA expression (25). Moreover, our observation that wt-p53 down-regulated endogenous VEGF expression only in mutant *p53*-expressing H226Br cells but not in *p53*-deleted H358 cells (data not shown) suggests that the interaction between the mutant and wt-p53 protein might regulate the VEGF promoter activity. In contrast, wt-p53 seems to act as a direct activator of BAI1 transcription, because the p53-binding site has been identified in the intron 9 of the *BAI1* genomic structure (16). Indeed, overexpression of the wt-*p53* gene resulted in transcriptional activation of BAI1 in tumor cell lines differing in their *p53* status, although the amount of activation varied among the cell lines (data not shown).

These molecular manipulations, secondary to wt-*p53* gene transfer, inhibited tumor neovascularization, as determined by the dorsal air sac method in *nu/nu* mice. Although various methods have been developed to study *in vivo* tumor angiogenesis, the effect of factors secreted into the microenvironment can be determined using this diffusion chamber system without cell-to-cell or cell-to-matrix binding (21). The pathology of the tissue of human cancer subjects demonstrated that VEGF expression is correlated with microvessel density (26). Consistent with this finding, a decreased amount of VEGF inhibited the development of capillary networks and curled microvessels. However, it is interesting to note that mRNA expression of bFGF, which is another important inducer of angiogenesis, was not affected by wt-*p53* gene transfer. These results suggest that major angiogenic factors may vary among cell types. For example, many lung cancer cell lines express detectable levels of VEGF mRNA (27), whereas VEGF is barely detectable in malignant melanomas (28); VEGF may predominantly contribute to the angiogenesis of NSCLC cells. The loss of both alleles of *p53* gene caused a reduction of secreted levels of TSP1, which is an angiogenic inhibitor, in fibroblasts cultured from Li-Fraumeni patients (29) and introduction of the wt-*p53* gene into *p53*-deficient fibroblasts restored the expression of TSP1 (30); our results, however, showed that transduced wt-*p53* gene caused decreased TSP1 mRNA expression in H226Br tumor cells. This unexpected effect might have occurred because of the differential regulation of the TSP1 transcription between normal and neoplastic cells. In addition, the observation that the BAI1 protein contains five TSP-type I repeats but TSP1 contains three suggests that BAI1 may be a dominant inhibitor of angiogenesis instead of TSP1 in H226Br cells.

A Phase I trial of the replication-defective retrovirus-mediated *p53* gene therapy of NSCLC has been reported (22). Local tumor regression was observed in three of nine lung cancer patients who had not responded to conventional therapy. The magnitude of the therapeutic response exceeded the effect expected from the transduction efficiency of retrovirus vector, which suggests that the bystander effect contributed to tumor progression. To evaluate the efficacy of the bystander effect, a more clinically relevant model was used: wt-*p53*-transduced tumor cells mixed with nontransduced cells were injected into *nu/nu* mice. The initial tumor growth was inhibited by mixed wt-*p53*-expressing tumor cells; *in vitro* mixing experiments, however, demonstrated an insignificant bystander effect (data not shown), indicating that the interaction between the tumor cells and surrounding tissue

components such as vascular beds may be involved in this phenomenon. In concordance with this finding, tumors that contained wt-*p53*-transduced cells formed less blood vessels than nontransduced tumors, suggesting that inhibiting angiogenesis by the wt-*p53* gene transfer elicits the bystander effects.

It remains to be studied whether tumor cells transduced with the wt-*p53* gene are capable of inhibiting the growth of distant tumors. Circulating angiogenesis inhibitors such as angiostatin and endostatin can suppress the growth of remote metastases (31, 32). Because VEGF is not present in the circulation, it is difficult to expect that decreased VEGF secretion may interfere with the distant tumor growth. However, the observation that GST-BAI1 fusion protein, which contains the middle three TSP type I repeats of BAI1, was antiangiogenic suggests that BAI1 can be enzymatically cleaved and secreted into the circulation as a soluble form, similar to other angiogenesis inhibitors (18). Increased BAI1 expression, therefore, may inhibit the growth of remote metastases. This question is being currently investigated in our laboratory.

In conclusion, we report a novel antiangiogenic property of *p53* gene therapy mediated by the altered expression of the angiogenic inducer VEGF and the angiogenic inhibitor BAI1. Therapeutic efforts aimed at targeting the upstream regulator of factors necessary for angiogenesis, instead of targeting any one specific factor, may be most successful at inhibiting angiogenesis and thereby inducing tumor regression. Our results suggest that wt-*p53* gene transfer can attenuate tumor cell-induced neovascularization *in vivo* and suppress the growth of neighboring tumor cells.

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References

1. Folkman, J. Tumor angiogenesis. Adv. Cancer Res., *43:* 175–203, 1985.

2. Ausprunk, D. H., and Folkman, J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc. Res., *14:* 53–65, 1977.

3. Fidler, I. J., and Ellis, L. M. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell, *79:* 85–88, 1994.

4. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., *1:* 27–31, 1995.

5. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenesis switch during tumorigenesis. Cell, *86:* 353–364, 1996.

6. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.

7. Fujiwara, T., Cai, D. W., Georges, R. N., Mukhopadhyay, T., Grimm, E. A., and Roth, J. A. Therapeutic effect of a retroviral wild-type p53 expression vector in an orthotopic lung cancer model. J. Natl. Cancer Inst. (Bethesda), *86:* 1458–1462, 1994.

8. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Zhang, W-W., Owen-Schaub, L. B., and Roth, J. A. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type *p53* gene. Cancer Res., *54:* 2287–2291, 1994.

9. Kagawa, S., Fujiwara, T., Hizuta, A., Yasuda, T., Zhang, W-W., Roth, J. A., and Tanaka, N. p53 expression overcomes $p21^{WAF1/CIP1}$-mediated G_1 arrest and induces apoptosis in human cancer cells. Oncogene, *15:* 1903–1910, 1997.

10. Ogawa, N., Fujiwara, T., Kagawa, S., Nishizaki, M., Morimoto, Y., Tanida, T., Hizuta, A., Yasuda, T., Roth, J. A., and Tanaka, N. Novel combination therapy for human colon cancer with adenovirus-mediated wild-type p53 gene transfer and DNA-damaging chemotherapeutic agent. Int. J. Cancer, *73:* 367–370, 1997.

11. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science (Washington DC), *246:* 1306–1309, 1989.

12. Mukhopadhyay, D., Tsiokas, L., Zhou, X., Foster, D., Brugge, J. S., and Sukhatme, V. P. Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature (Lond.), *375:* 577–581, 1995.

13. Shweki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (Lond.), *359:* 843–845, 1992.

14. Koura, A. N., Liu, W., Kitadai, Y., Singh, R. K., Radinsky, R., and Ellis, L. M. Regulation of vascular endothelial growth factor expression in human colon carcinoma cells by cell density. Cancer Res., *56:* 3891–3894, 1996.

15. Mukhopadhyay, D., Tsiokas, L., and Sukhatme, V. P. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Res., *55:* 6161–6165, 1995.

16. Nishimori, H., Shiratsuchi, T., Urano, T., Kimura, Y., Kiyono, K., Tatsumi, K., Yoshida, S., Ono, M., Kuwano, M., Nakamura, Y., and Tokino, T. A novel brain-specific p53-target gene, *BAI1*, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. Oncogene, *15:* 2145–2150, 1997.

17. Zhang, W-W., Fang, X., Mazur, W., French, B. A., Georges, R. N., and Roth, J. A. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. Cancer Gene Ther., *1:* 1–10, 1994.

18. Anthony, F. W., Wheeler, T., Elcock, C. L., Pickett, M., and Thomas, E. J. Identification of a specific pattern of vascular endothelial growth factor mRNA expression in human placenta and cultured placental fibroblasts. Placenta, *15:* 557–561, 1994.

19. Morrison, R. S. Suppression of basic fibroblast growth factor expression by antisense oligonucleotides inhibits the growth of transformed human astrocytes. J. Biol. Chem., *266:* 728–734, 1991.

20. Bertin, N., Clezardin, P., Kubiak, R., and Frappart, L. Thrombospondin-1 and -2 messenger RNA expression in normal, benign, and neoplastic human breast tissues: correlation with prognostic factors, tumor angiogenesis, and fibroblastic desmoplasia. Cancer Res., *57:* 396–399, 1997.

21. Tanaka, N., Sakamoto, N., Inoue, K., Korenaga, H., Kadoya, S., Ogawa, H., and Osada, Y. Antitumor effect of an antiangiogenic polysaccharide from an *Arthrobacter* species with or without steroid. Cancer Res., *49:* 6727–6730, 1989.

22. Roth, J. A., Nguyen, D., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Ferson, D. Z., Hong, W. K., Komaki, R., Lee, J. J., Nesbitt, J. C., Pisters, K. M. W., Putnum, J. B., Schea, R., Shin, D. M., Walsh, G. L., Dolormente, M. M., Han, C-I., Martin, F. D., Yen, N., Xu, K., Stephens, L. C., McDonnell, T. J., Mukhopadhyay, T., and Cai, D. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. Nat. Med., *2:* 985–991, 1996.

23. Kozaesky, K. F., and Wilson, J. M. Gene therapy: adenovirus vectors. Curr. Opin. Genet. Dev., *3:* 499–503, 1993.

24. Xu, M., Kumar, D., Srinvas, S., Detolla, L. J., Yu, S. F., Stass, S. A., and Mixson, A. J. Parenteral gene therapy with p53 inhibits human breast tumors *in vivo* through a bystander mechanism without evidence of toxicity. Hum. Gene Ther., *8:* 177–185, 1997.

25. Keiser, A., Weich, H. A., Brandner, G., Marme, D., and Kolch, W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene, *9:* 963–969, 1994.

26. Maeda, K., Chung, Y-S., Ogawa, Y., Takatsuka, S., Kang, S-M., Ogawa, M., Sawada, T., and Sowa, M. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. Cancer (Phila.), *77:* 858–863, 1996.

27. Ohta, Y., Endo, Y., Tanaka, M., Shimizu, J., Oda, M., Hayashi, Y., Watanabe, Y., and Sasaki, T. Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. Clin. Cancer Res., *2:* 1411–1416, 1996.

28. Wang, Y., and Becker, D. Antisense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth. Nat. Med., *3:* 887–893, 1997.

29. Volpert, O. V., Dameron, K. M., and Bouck, N. Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. Oncogene, *14:* 1495–1502, 1997.

30. Dameron, K. M., Volpert, O. V., Tainsky, M. A., and Bouck, N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science (Washington DC), *265:* 1582–1584, 1994.

31. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell, *79:* 315–328, 1994.

32. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277–285, 1997.

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