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Control of Angiogenesis in Fibroblasts by p53 Regulation of Thrombospondin-1

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As normal cells progress toward malignancy, they must switch to an angiogenic phenotype to attract the nourishing vasculature that they depend on for their growth. In cultured fibroblasts from Li-Fraumeni patients, this switch was found to coincide with loss of the wild-type allele of the *p53* tumor suppressor gene and to be the result of reduced expression of thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis. Transfection assays revealed that p53 can stimulate the endogenous TSP-1 gene and positively regulate TSP-1 promoter sequences. These data indicate that, in fibroblasts, wild-type p53 inhibits angiogenesis through regulation of TSP-1 synthesis.

Angiogenesis is controlled by the local balance between factors that stimulate new vessel growth and factors that inhibit it. In most normal tissues, inhibitory influences predominate, and cells derived from these tissues usually do not stimulate angiogenesis. In contrast, tumor cells, which must attract new vessels in order to grow and metastasize efficiently (1), are potently angiogenic as a result of decreased production of inhibitors and increased secretion of inducers (2). As normal cells undergo genetic changes that lead to malignancy they must switch from an inhibitory to an angiogenic phenotype. In this report we examine the role that the loss of the tumor suppressor gene *p53* plays in enabling human fibro-

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blasts to become angiogenic.

The p53 protein is mutated in human tumors more frequently than any other known oncogene or suppressor gene [(3) and references therein]. In normal cells it is hypothesized to act as a transcriptional regulator (4), enhancing the expression of genes that contain specific p53-binding sites and interacting with a variety of transcription factors to inhibit the expression of other genes (4, 5). Wild-type p53 protein can mediate a number of cellular activities, including apoptosis, the maintenance of genetic stability, G_1 growth arrest, cell differentiation, and the suppression of tumorigenicity (3, 5). The up-regulation by p53 of the gene encoding p21, an inhibitor of G_1 cyclin-dependent kinases, appears to be responsible for p53-mediated growth arrest (6). The p53-regulated effector molecules responsible for the other cellular phenotypes dependent on wild-type p53 remain to be identified.

To examine the effect of p53 on angiogenesis, we used fibroblasts cultured from Li-Fraumeni patients (7). These individuals have inherited one wild-type (wt) and one mutant allele of the *p53* gene and as a result have an elevated risk of developing sarcomas and other tumors in which the remaining wt allele is inactivated (8). At early passage, the Li-Fraumeni fibroblasts are diploid and have one mutant and one wt *p53* allele. On continued passage in culture, however, they spontaneously lose their wt allele, become aneuploid and immortal (although not tumorigenic), and retain only the mutant allele of *p53* (7).

We previously showed (9) that hamster fibroblasts could switch to an angiogenic phenotype upon loss of an unidentified tumor suppressor gene by decreasing their se-

Table 1. Effect of Li-Fraumeni fibroblast conditioned media on corneal neovascularization. Serum-free conditioned media described in Fig. 1 were tested for the ability to induce neovascularization when incorporated into a noninflammatory pellet and implanted into the normally avascular rat cornea (17). Simultaneous controls showed that antibodies to TSP-1 were not angiogenic when tested alone (0/3 corneas positive) and not inhibitory when tested with bFGF (3/3 corneas positive).

Media source	Anti–TSP-1	Positive corneas/number implanted for patient		
		041	172	087
Early-passage cells	−	0/8	0/3	0/9
	+	3/3	3/3	5/7
Late-passage cells	−	4/4	3/3	11/11
Mix of early- and	−	0/7	0/3	0/13
late-passage cells	+	3/4	3/3	4/4
Early-passage cells	−	0/3	0/3	0/4
(+ bFGF)	+	3/3	3/3	4/4

cretion of thrombospondin-1 (TSP-1), a large, multifunctional matrix glycoprotein (10) that is a potent inhibitor of neovascularization (9, 11). Fibroblasts cultured from Li-Fraumeni patients underwent a similar angiogenic switch when they lost their wt p53 allele and became immortal (Fig. 1). Cells from three patients, each harboring a different p53 mutation, were found to secrete high amounts of TSP-1 into the media at early passage when they still retained a wt p53 allele; in contrast, at late passage when the wt p53 allele had been lost, the cells secreted reduced amounts of TSP-1 (Fig. 1A). The mRNA levels [measured with a probe made from TSP-1 complementary DNA (cDNA) that may cross-react with TSP-2] also declined 14- to 17-fold after loss of wt p53 and remained low when cells progressed to tumorigenicity (12, 13). Of the five different thrombospondin-type proteins identified to date (10), only TSP-1 and TSP-2 contain domains likely to influence angiogenesis (11), and of these two, only TSP-1 is regulated by p53. Northern (RNA) blot analysis with probes specific for the 3′ untranslated region of the mRNAs of TSP-1 and TSP-2 showed that TSP-1 was expressed in Li-Fraumeni fibroblasts at a 7- to 20-fold excess over TSP-2 and that only TSP-1 decreased when the cells lost wt p53 (13).

Coincident with the loss of wt p53 and the decline in TSP-1 expression, media from the Li-Fraumeni fibroblasts lost the ability to inhibit angiogenesis in vitro (Fig.

1B) and in vivo (Table 1). Inhibitory activity was relieved when neutralizing antibodies to TSP-1 [see (18)] were added to

the assays, suggesting that secreted TSP-1 was responsible for the inhibitory activity of early-passage cells and that, in the absence
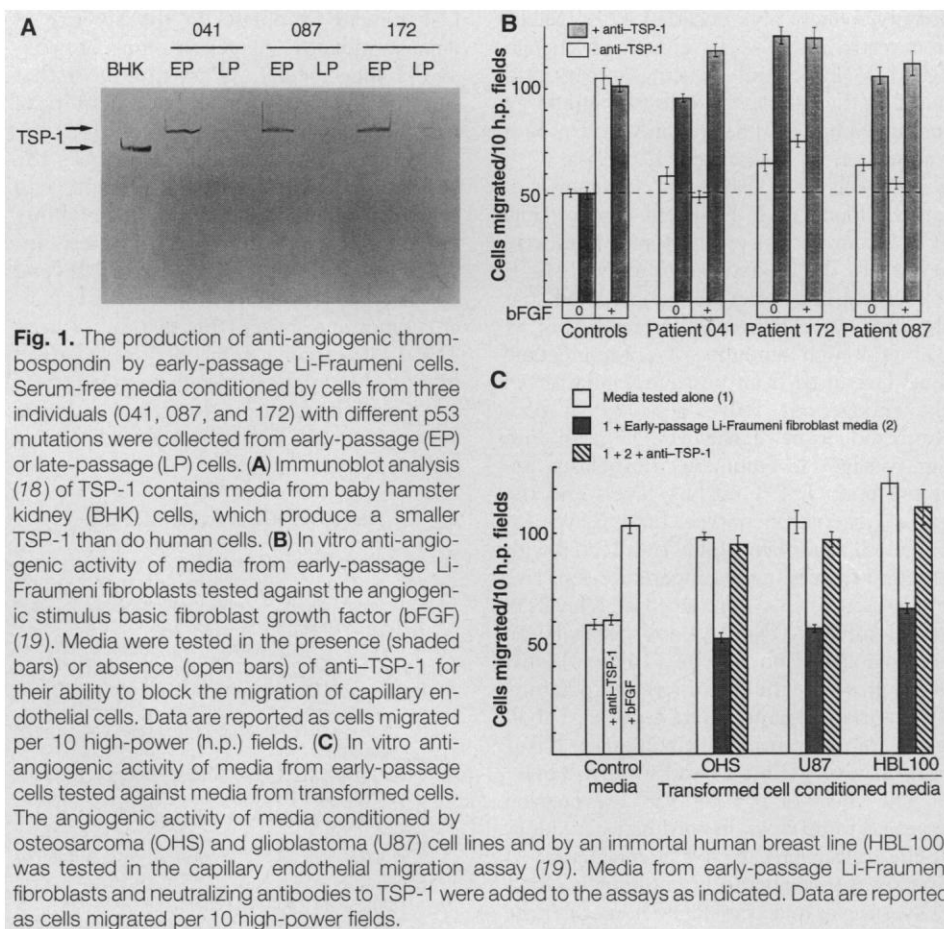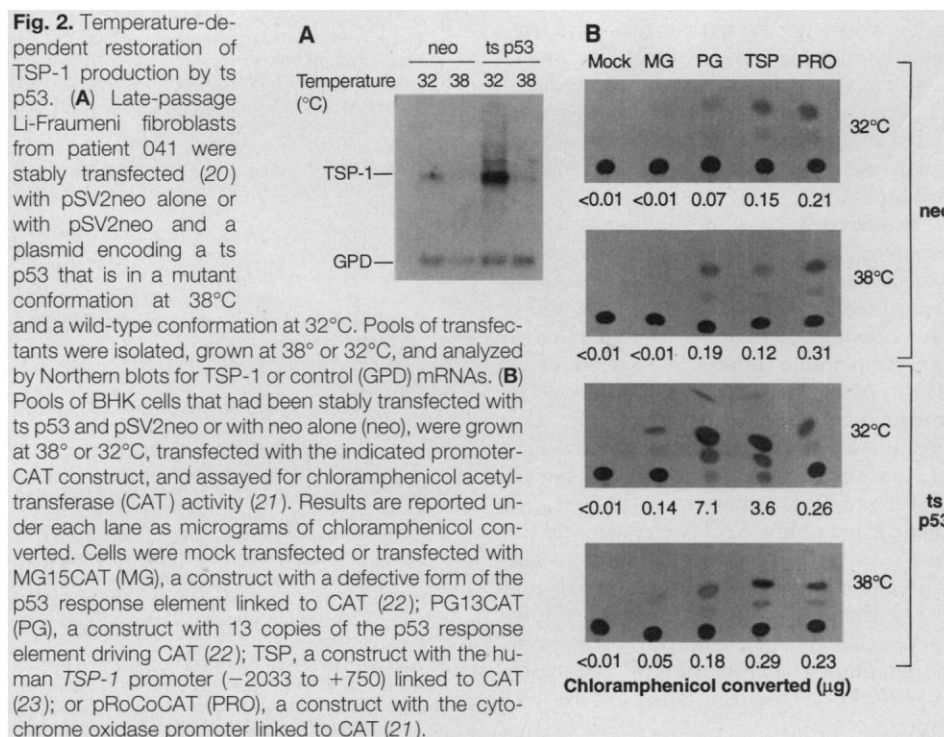

Fig. 1. The production of anti-angiogenic thrombospondin by early-passage Li-Fraumeni cells. Serum-free media conditioned by cells from three individuals (041, 087, and 172) with different p53 mutations were collected from early-passage (EP) or late-passage (LP) cells. (**A**) Immunoblot analysis (18) of TSP-1 contains media from baby hamster kidney (BHK) cells, which produce a smaller TSP-1 than do human cells. (**B**) In vitro anti-angiogenic activity of media from early-passage Li-Fraumeni fibroblasts tested against the angiogenic stimulus basic fibroblast growth factor (bFGF) (19). Media were tested in the presence (shaded bars) or absence (open bars) of anti–TSP-1 for their ability to block the migration of capillary endothelial cells. Data are reported as cells migrated per 10 high-power (h.p.) fields. (**C**) In vitro anti-angiogenic activity of media from early-passage cells tested against media from transformed cells. The angiogenic activity of media conditioned by osteosarcoma (OHS) and glioblastoma (U87) cell lines and by an immortal human breast line (HBL100) was tested in the capillary endothelial migration assay (19). Media from early-passage Li-Fraumeni fibroblasts and neutralizing antibodies to TSP-1 were added to the assays as indicated. Data are reported as cells migrated per 10 high-power fields.

Table 2. Temperature-dependent restoration of anti-angiogenic activity by ts p53 in late-passage Li-Fraumeni fibroblasts. Serum-free conditioned media were collected from late-passage cells growing at the indicated temperature that had been stably transfected with neo or neo and ts p53. Media were tested with the additions indicated for the ability to induce neovascularization in the rat cornea (17). Dashes indicate that no other reagents were added.

Trans-fected gene	Temp. (°C)	Addition	Positive corneas/number implanted
Media from transfected 041 cells			
ts p53*	32	—	0/3
ts p53*	32	Anti–TSP-1	3/3
ts p53*	32	bFGF	0/3
ts p53*	38	—	3/3
neo*	32	—	3/3
neo*	38	—	3/3
Media from transfected 087 cells			
ts p53†	32	—	0/4
ts p53†	32	bFGF	0/2
ts p53†	32	bFGF + anti–TSP-1	2/2
ts p53†	32	Media from 087 cells expressing ts p53 grown at 38°C	0/3
ts p53†	38	—	6/6

*Pools of >30 transfected clones.   †Single transfected clone.

Fig. 2. Temperature-dependent restoration of TSP-1 production by ts p53. (**A**) Late-passage Li-Fraumeni fibroblasts from patient 041 were stably transfected (20) with pSV2neo alone or with pSV2neo and a plasmid encoding a ts p53 that is in a mutant conformation at 38°C and a wild-type conformation at 32°C. Pools of transfectants were isolated, grown at 38° or 32°C, and analyzed by Northern blots for TSP-1 or control (GPD) mRNAs. (**B**) Pools of BHK cells that had been stably transfected with ts p53 and pSV2neo or with neo alone (neo), were grown at 38° or 32°C, transfected with the indicated promoter-CAT construct, and assayed for chloramphenicol acetyltransferase (CAT) activity (21). Results are reported under each lane as micrograms of chloramphenicol converted. Cells were mock transfected or transfected with MG15CAT (MG), a construct with a defective form of the p53 response element linked to CAT (22); PG13CAT (PG), a construct with 13 copies of the p53 response element driving CAT (22); TSP, a construct with the human TSP-1 promoter (−2033 to +750) linked to CAT (23); or pRoCoCAT (PRO), a construct with the cytochrome oxidase promoter linked to CAT (21).

of TSP-1, these cells display an angiogenic phenotype (Fig. 1B).

The switch to an angiogenic phenotype depended on a reduction in TSP-1 rather than on an increase in angiogenesis-inducing activity, which also occurred (14). Media from early-passage cells contained enough TSP-1 to inhibit all inducing activity produced by the late-passage cells (Table 1), by a tumor cell line (12) derived in vitro by H-ras transfection of Li-Fraumeni fibroblasts (13), and by cell lines derived from tumors frequently found in Li-Fraumeni families (Fig. 1C). In vitro assays on the tumor lines were verified by the in vivo cornea assay (14).

Several lines of evidence indicated that the correlation between the presence of wt p53 and high amounts of antiangiogenic TSP-1 resulted from positive regulation of the endogenous TSP-1 gene by wt p53. Re-introduction of the wt p53 gene into late-passage Li-Fraumeni fibroblasts restored both TSP-1 mRNA levels and the antiangiogenic phenotype. Late passage Li-Fraumeni fibroblasts stably transfected with a plasmid encoding a temperature-sensitive p53 protein (ts p53) produced high levels of TSP-1 mRNA (Fig. 2A) and displayed an antiangiogenic phenotype (Table 2) only when grown at the permissive temperature. The antiangiogenic phenotype was abolished by neutralizing antibodies to TSP-1 both in vivo (Table 2) and in vitro (14).

The effect of p53 on TSP-1 expression appeared to be rapid. In baby hamster kidney (BHK) cells expressing ts p53, mRNA recognized by a probe derived from human TSP-1 cDNA began to accumulate 6 hours after the shift to a permissive temperature; this delay is consistent with the time required for wt p53 to translocate to the nucleus in similar temperature-shift experiments (15). The induction was not a consequence of p53-induced G_1 arrest, as normal fibroblasts that were arrested at confluence or by aphidicolin showed a decrease rather than an increase in mRNA levels (13).

Positive regulation of the human TSP-1 promoter by wt p53 was demonstrated in transient transfection assays. Expression of a reporter gene driven by the human TSP-1 promoter (-2033 to $+750$) responded to the temperature-induced activation of wt p53 in parallel to a reporter gene driven by known p53 response elements (Fig. 2B). Positive regulation by wt p53 was also seen (13) with constructs containing human TSP-1 promoter sequences (-38 to $+750$) that carried only a TATA element and the first intron. This intron (16) contains two adjacent sequence elements at $+516$ and $+538$ that are similar to known p53 response elements (4); however, it remains to be determined whether the TSP-1 promoter is a direct or indirect target of the p53 protein.

Our results show that p53 inhibits the angiogenic phenotype in fibroblasts by stimulating their production of TSP-1. As effective antiangiogenic amounts of TSP-1 are secreted by normal and early-passage Li-Fraumeni fibroblasts in the absence of damage-induced overexpression of p53, TSP-1 may mediate a constitutive rather than an induced antineoplastic activity of p53.

It is not yet clear if TSP-1 blocks the development of an angiogenic phenotype in cells other than fibroblasts. But the ability of this protein to inhibit angiogenesis induced by cell lines derived from several tumor types characteristic of the Li-Fraumeni syndrome raises the possibility that TSP-1 or antiangiogenic peptides derived from it (11) might be developed into effective prophylactic agents for delaying or preventing tumors in individuals who carry only one wt p53 allele.

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17. For the cornea assays [P. J. Polverini, N. P. Bouck, F. Rastinejad, *Methods Enzymol.* **198**, 440 (1991)] we incorporated conditioned media (1 to 3 µg) and, where indicated, basic fibroblast growth factor (bFGF; 0.15 µM) and anti-TSP-1 (40 µg/ml) into a pellet of ~5 µl and implanted it into the cornea of an anesthetized rat. Vigorous growth of vessels from the limbus by 7 days was scored as a positive response. Care of rats was in accordance with institutional guidelines.
18. We collected serum-free media conditioned by skin fibroblasts from Li-Fraumeni patients. "Early-passage" cells were used after 10 to 21 passages; "late-passage" cells were used after more than 207 passages [for MDAH041 cells, which carry a frameshift mutation at codon 184 resulting in a stop codon at position 246 (7)], after more than 62 passages [for MDAH087 cells, which carry an Arg to Trp mutation at codon 248 (7)], or after more than 106 passages [for MDAH172 cells, which carry an Arg to His mutation at codon 175 (M. A. Tainsky, unpublished data)]. The media were concentrated with Amicon membranes with a 10-kD cutoff, and samples containing 6 µg of protein were subjected to electrophoresis on polyacrylamide gels. The separated proteins were transferred to Hybond-N membrane and probed with monoclonal antibody A4.1 (anti-TSP-1) (9), which recognizes an undefined epitope on the stalk region of human TSP-1. It neutralizes the antiangiogenic activity of TSP-1 both in vitro and in vivo (9, 11) and was used throughout these experiments. Antibody A4.1 recognizes human TSP-2 poorly, if at all, on protein immunoblots (13), but the degree to which it may cross-react with the native TSP-2 protein is unknown. Nor is it known whether the TSP-2 protein is actually capable of inhibiting angiogenesis. Therefore, in assays where A4.1 is used to neutralize angiogenic activity, there is a formal possibility that, in addition to neutralizing the activity of TSP-1, A4.1 will also neutralize the activity (should it exist) of the small amount of TSP-2 that is present.
19. Capillary endothelial cell migration assays (9) were performed in a modified Boyden chamber with bovine-adrenal capillary endothelial cells. To each 50-µl well of Dulbecco's modified Eagle's medium (DMEM) containing 0.1% bovine serum albumin (BSA), we added 0.5 to 1.0 µg of conditioned media and, where indicated, anti-TSP-1 (2 µg). DMEM with 0.1% BSA served as a negative control and bFGF (0.5 ng) as a positive control. Endothelial cells treated in parallel showed no toxicity as monitored by trypan blue exclusion. U87 and HBL100 cells were obtained from the American Type Culture Collection.
20. Cells were transfected with pLSVp53cGVal135 [D. Michalovitz, O. Halevy, M. Oren, *Cell* **62**, 671 (1990)] (ts p53) and pSV2neo at a 5:1 molar ratio by the lipofectin (Gibco-BRL) or polybrene (12) methods. G418-resistant clones expressing the murine ts p53 were identified by immunoblotting or by Northern blot analysis. Incubators at the nonpermissive temperature for all clones expressing ts p53 were kept between 38° and 38.5°C. These cells and control cells transfected with pSV2neo alone were grown for 5 to 7 days at 38° or 32°C. Total RNA was then analyzed on Northern blots with probes specific for the 3' untranslated region of human TSP-1 [T. L. LaBell, D. J. McGookey Milewicz, C. M. Disteche, P. H. Byers, *Genomics* **12**, 421 (1992)] and for the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene [J. Y. Tso, X.-H. Sun, T.-H. Kao, K. S. Reese, R. Wu, *Nucleic Acids Res.* **13**, 2485 (1985)], which is not regulated by p53 [M. Harvey *et al.*, *Oncogene* **8**, 2457 (1993)].
21. BHK cells (9) were transfected with pLSVp53cGVal135, and pools of stable transfectants containing more than 100 clones were grown overnight at 38° or 32°C. These cells were then transfected with reporter constructs, and extracts were assayed for CAT activity 56 hours later. To control for temperature-dependent variation in the rate of protein synthesis, we performed parallel transfections with constructs containing the cytochrome oxidase promoter driving the CAT gene [J. V. Virbasius and R. C. Scarpulla, *Mol. Cell. Biol.* **11**, 5631 (1991)]. Enzyme activity was calculated from reactions adjusted to give <30% total conversion.
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