Id1 regulates angiogenesis through transcriptional repression of thrombospondin-1

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Summary

Id proteins are helix-loop-helix transcription factors that regulate tumor angiogenesis. In order to identify downstream effectors of Id1 involved in the regulation of angiogenesis, we performed PCR-select subtractive hybridization on wild-type and *Id1* knockout mouse embryo fibroblasts (MEFs). Here we demonstrate that thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis, is a target of transcriptional repression by Id1. We also show that *Id1*-null MEFs secrete an inhibitor of endothelial cell migration, which is completely inactivated by depletion of TSP-1. Furthermore, in vivo studies revealed decreased neovascularization in matrigel assays in *Id1*-null mice compared to their wild-type littermates. This decrease was completely reversed by a TSP-1 neutralizing antibody. We conclude that TSP-1 is a major target for Id1 effects on angiogenesis.

Introduction

Basic helix-loop-helix (bHLH) DNA binding proteins regulate tissue-specific transcription within multiple cell lineages (Massari and Murre, 2000). The Id family of helix-loop-helix proteins does not possess a basic DNA binding domain and inhibits lineage commitment within multiple cell types through sequestration of bHLH transcription factors (reviewed in Norton, 2000). Over the past several years, Id proteins have been implicated in regulating a variety of cellular processes including cell growth, senescence, differentiation, and neoplastic transformation (Lasorella et al., 2001; Rivera and Murre, 2001; Zebedee and Hara, 2001). More recently, evidence has been mounting to suggest that Id1 and Id3 play a critical role in the regulation of angiogenesis during embryonic development and tumorigenesis (reviewed in Benezra et al., 2001). Lyden and colleagues have demonstrated that at least one copy of the *Id1* or *Id3* gene must be maintained in mice to prevent embryonic lethality and associated defects in neuronal differentiation and angiogenesis in the brain (Lyden et al., 1999). Moreover, loss of a single *Id1* or *Id3* allele has been shown to significantly impair the growth, progression, and/or metastasis of tumor xenografts in genetically altered mice (Lyden et al., 1999). Tumors that were able to grow or metastasize in *Id1*- or *Id3*-deficient mice showed defects in vascularization and extensive necrosis. This defective angiogenesis was attributed to impaired VEGF-dependent recruitment of precursor endothelial cells from the bone marrow to the newly developing tumor vasculature (Lyden et al., 2001), yet the precise mechanisms underlying this defective recruitment remain unclear. Several target genes for Id proteins have been identified largely based on the knowledge of promoters activated by bHLH proteins; however, to date no systematic search for Id-regulated genes has been performed. Given the variety of cellular processes affected by *Id* gene expression, we undertook an evaluation of Id target genes using a differential gene expression profiling system with embryo fibroblasts from wild-type and *Id1* knockout mice. Our studies have allowed us to identify several Id1 target genes involved in diverse biological functions such as matrix remodeling, intracellular signaling pathways, and angiogenesis. One of the angiogenesis-related targets is the endogenous inhibitor of neovascularization, thrombospondin-1 (TSP-1).

SIGNIFICANCE

Angiogenesis is critical for the development of all malignancies. Id transcription factors have been shown to regulate key steps in tumor growth and metastasis due to their effects on angiogenesis. Thus, identification of Id transcriptional targets will be useful for developing therapeutic interventions that disrupt Id-associated tumor growth and angiogenesis. Here we use a systematic genetic approach to identify Id1 target genes and have identified the angiogenesis inhibitor thrombospondin-1 as being a critical target of transcriptional repression by Id1. We show that mice null for *Id1* have defects in angiogenesis which are largely due to increased expression of thrombospondin-1, and therefore suggest that promotion of thrombospondin-1 anti-angiogenic activity in tumors with increased Id gene expression may be a useful therapeutic intervention in such malignancies.

TSP-1 is an extracellular matrix glycoprotein that inhibits tumor growth and metastases (Rodriguez-Manzaneque et al., 2001; Sheibani and Frazier, 1995; Streit et al., 1999; Volpert et al., 1998; Weinstat-Saslow et al., 1994), and was the first protein to be recognized as a naturally occurring inhibitor of angiogenesis (Good et al., 1990). TSP-1 has been shown to be a potent inhibitor of in vivo neovascularization and tumorigenesis. Targeted overexpression of TSP-1 in mice suppresses wound healing and tumorigenesis while the lack of functional TSP-1 results in increased vascularization of selected tissues (Rodriguez-Manzaneque et al., 2001; Stellmach et al., 1996; Streit et al., 1999, 2000). In addition, several oncogenes have been demonstrated to repress TSP-1 expression, including oncogenic *ras* (Rak et al., 2000), c-*myc* (Tikhonenko et al., 1996), v-*src* (Slack and Bornstein, 1994), and c-*jun* (Mettouchi et al., 1994), thus providing a potential mechanism for activation of the angiogenic switch in tumors. On the other hand, tumor suppressor proteins, such as p53 and PTEN, have been shown to maintain high, anti-angiogenic levels of TSP-1 (Dameron et al., 1994; Wen et al., 2001), and the loss of functional wild-type p53 was found to result in an angiogenic switch in vitro due to the transcriptional inactivation of TSP-1 expression. Here we demonstrate that Id1, a tumor-associated transcription factor, is a potent repressor of TSP-1 transcription. We also demonstrate that upregulated expression of TSP-1 leads to the suppression of angiogenesis in vivo in Id1 knockout mice and that this suppression is reversible upon antibody blockade of the functional TSP-1. We conclude that Id1 repression of TSP-1 is a major determinant of Id effects on tumor angiogenesis.

Results

TSP-1 is upregulated in *Id1*$^{-/-}$ mouse embryo fibroblasts

In order to identify downstream effectors of the helix-loop-helix protein Id1, we performed suppression subtractive hybridization on cDNAs from early-passage (P-3) *Id1* wild-type (+/+) and *Id1* knockout (−/−) mouse embryo fibroblasts (MEFs). Forward and reverse subtractions were repeated twice with two distinct pools of *Id1*$^{+/+}$ and *Id1*$^{-/-}$ MEF cDNAs. The initial screening of clones using forward subtracted and reverse subtracted library probes yielded over 125 cDNAs that were upregulated in *Id1*$^{-/-}$ MEFs (i.e., repressed by Id1). Sequence analysis identified several clones that were likely to affect endothelial cell migration and possibly angiogenesis (Table 1). One of the isolated clones encoded a 625 bp cDNA insert identical to a fragment of the murine TSP-1 gene with the exception of a 41 bp stretch within the matched sequences which was felt to represent differential splicing. Upregulation of the TSP-1 mRNA was confirmed by semi-quantitative RT-PCR and Northern analysis of *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mRNAs (Figure 1). Quantitative analysis of the Northern blots revealed a 3-fold increase in TSP-1 mRNA in *Id*$^{-/-}$ versus *Id1*$^{+/+}$ MEFs. This change in mRNA level was reflected in the level of secreted TSP-1 protein in conditioned media from *Id1*$^{-/-}$ MEFs, which was 3- to 5-fold greater than the level of TSP-1 in conditioned media from *Id1*$^{+/+}$ MEFs (Figure 4B).

Endothelial cell-associated expression of TSP-1 is elevated in *Id1*$^{-/-}$ mouse embryos

In order to determine whether in vivo expression of TSP-1 was altered in *Id1*$^{-/-}$ mice, day E13.5 embryos were evaluated for TSP-1 expression. We found that TSP-1 was expressed at higher levels in the vascular structures of *Id1*-null embryos as determined by colocalization of the immunostaining for TSP-1 and for the endothelial marker CD-31 (Figure 2). No obvious differences in the endothelial cell densities within individual vessels could be detected when *Id1*$^{-/-}$ versus *Id1*$^{+/+}$ embryonic tissues were compared.

Id-1 repression of TSP-1 promoter activity is independent of E boxes

In order to determine whether Id1 effects on TSP-1 expression occurred at the transcriptional level, we evaluated reporter activities of the full-length murine *TSP-1* promoter (−2800 bp to +48 bp) (Bornstein et al., 1990) and various promoter deletion mutants in the presence or absence of Id1 (Figure 3). Previous studies of the murine *TSP-1* promoter identified a serum-response element at −1210 bp that is critical for serum-regulated expression of TSP-1, as well as overlapping Egr-1 and Sp1 sites necessary for the constitutive TSP-1 expression at the −70 bp region (Shingu and Bornstein, 1994); however, no meaningful information has been available regarding the mouse *TSP-1* promoter region between positions −2800 and −480. Since Id proteins function primarily via repression of E box and Ets transcriptional activation sites (reviewed in Zebedee and Hara, 2001), we evaluated transcriptional activity of the *mTSP-1* promoter and deletion mutants of the same promoter which carried varying numbers of E boxes and Ets sites for repression by Id1. We observed a dose-dependent repression of the full-length *mTSP-1* promoter by Id1 (Figure 3A). In addition, we show continued *TSP-1* repression by Id1 of the mutants containing only the −160 bp to +48 bp fragment of the TSP-1 promoter (Figure 3B). Since the 160 bp mutant contains no E boxes or Ets sites and yet is repressible by Id1, we conclude that either Id1 is functioning via yet uncharacterized DNA consensus sites or is functioning through putative downstream effectors to inhibit *TSP-1* expression. Interestingly, we identified a strong region of transcriptional repression between −1120 bp and −1310 bp of the murine *TSP-1* promoter, which has not been previously described; however, deletion of this region did not affect repression by Id1.

TSP-1 is the key inhibitor of endothelial cell migration secreted by *Id1*-null MEFs

The in vitro endothelial cell migration assay provides a reproducible and quantitative measurement of the pro- or anti-angiogenic activity of compounds and consistently parallels angiogenic effects observed in vivo (Bouck et al., 1996; Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). In order to determine whether Id1 regulation of thrombospondin expression affected angiogenesis, we tested conditioned media (CM) from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ MEFs for their ability to induce migration of both bovine adrenal capillary endothelial cells (BACECs) and human dermal microvascular endothelial cells (HDMECs) (Figure 4A and data not shown). CM from *Id1*$^{+/+}$ MEFs were moderately angiogenic while CM from *Id1*$^{-/-}$ MEFs failed to induce endothelial cell migration over the background levels observed in the presence of BSA. Neutralizing TSP-1 antibody fully relieved the inhibition of endothelial cell chemotaxis by *Id1*$^{-/-}$ CM in both BACECs and HDMECs to the levels observed in *Id1*$^{+/+}$ CM while isotype control antibody had no effect, suggesting that impaired endothelial cell migration in the presence of *Id1*$^{-/-}$ CM

Table 1. Genes identified in PCR-Select screen for downstream targets of Id1

Clone	Genbank Accession Number	Function
Glutathione reductase	XM_132691	Reduces reactive oxygen species
Fibulin-2	X75285	ECM protein, endostatin binding protein
Ser-thr kinase receptor binding protein	AF096285	WD-domain protein involved in TGFβ signalling
Cathepsin B	S69034	Lysosomal enzyme
FISP-12	M70642	ECM protein, promotes endothelial cell adhesion and migration
Filamin A (actin-binding protein 280)	X53416	Couples integrins to cytoskeletal actin; assembles signalling complexes for cell motility/proliferation
Thrombospondin	XM_130459	ECM protein, potent inhibitor of angiogenesis
Lysyl oxidase	L04262	Extracellular Cu-aminooxidase; collagen/elastin X-linking, incr. in cell senescence; tumor suppressor fn.
Pro-alpha 2 (I) collagen	AF004877	ECM protein, upregulated during cellular senescence
Cytochrome C oxidase	L07096	Mitochondrial gene fn. in cellular oxidative phosphorylation; induced by p53
beta-5 integrin	AF022110	Forms heterodimers with alpha-v integrin, fn. in cell migration and growth factor-induced angiogenesis
Inhibin beta-A	M37482	Subunit of activin (TGFβ cytokine); involved in ALK receptor activation
Fibronectin	X93167	ECM protein, binds integrins

Genes listed are among those which were found to be upregulated in *Id1*$^{-/-}$ cells (i.e., repressed by Id1) using PCR-Select subtractive cloning. Listed genes have been identified as possible regulators of tumor angiogenesis.

was largely due to increased levels of TSP-1 secretion. Western blot analysis of CM from three independent experiments confirmed the elevated expression of TSP-1 in *Id1*$^{-/-}$ CM versus *Id1*$^{+/+}$ CM (Figure 4B). Since matrix metalloproteinases have been shown to influence endothelial cell migration and the angiogenic switch, we examined the function of metalloproteinases from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ CM. We demonstrate decreased activity of MMP-2 in CM from *Id1*$^{-/-}$ MEFs (Figure 4C) consistent with the loss of MMP-2 expression noted previously in *Id1*$^{-/-}$ mouse embryos (Lyden et al., 1999). Conversely, we note increased gelatinase activity of an unidentified 120 kDa protein product in CM from *Id1*$^{-/-}$ MEFs.

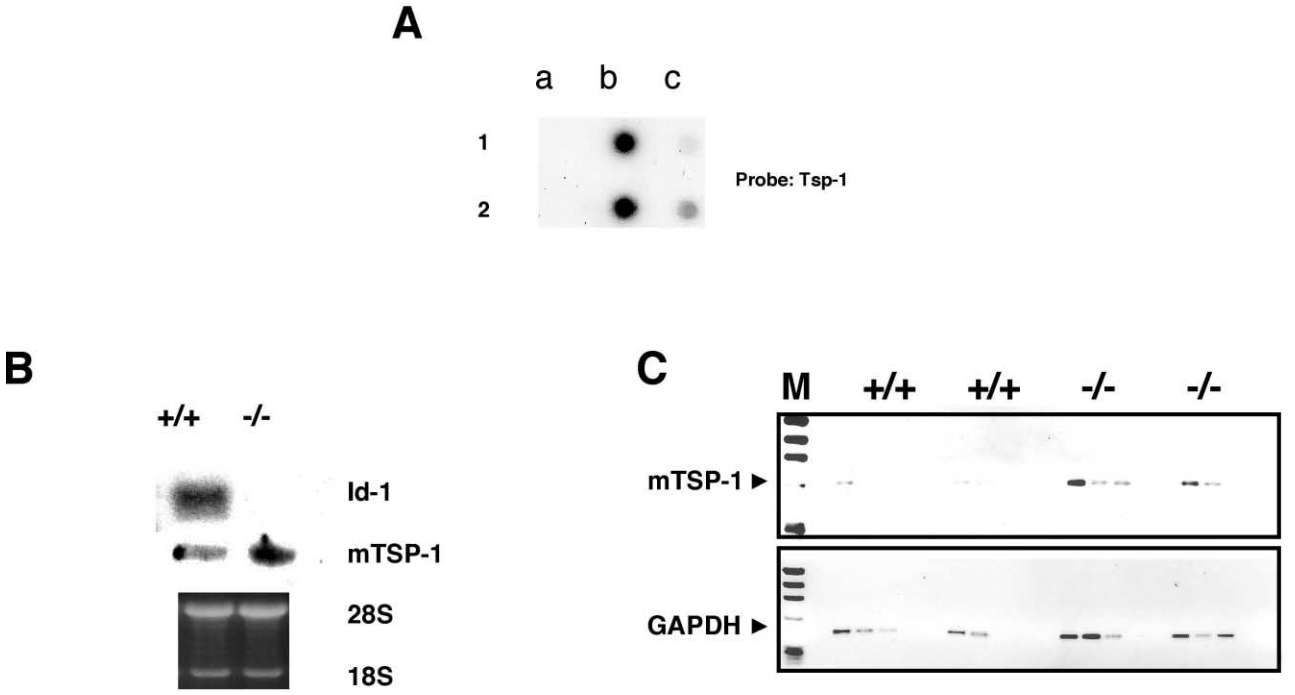


Figure 1. Thrombospondin-1 is upregulated in *Id1*$^{-/-}$ MEFs

A: Dot blot of subtraction libraries. A library of (*Id*$^{-/-}$ MEF cDNAs) − (*Id1*$^{+/+}$ MEF cDNAs) is spotted in 1b. A library of (*Id1*$^{+/+}$ MEF cDNAs) − (*Id1*$^{-/-}$ cDNAs) is spotted in 1c. *Id1*$^{-/-}$ MEF cDNA is spotted in 2b. *Id1*$^{+/+}$ MEF cDNA is spotted in 2c. Purified Id1 cDNAs are spotted in column a. Probe for dot blot is murine TSP-1.

B: Northern analysis of murine TSP-1 and Id1 in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ MEFs. Lower panel illustrates loading control on ethidium bromide-stained formaldehyde gel.

C: Semiquantitative RT-PCR of mTSP-1 and GAPDH from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ MEFs at P-3. M = ϕXHaeIII DNA size marker.

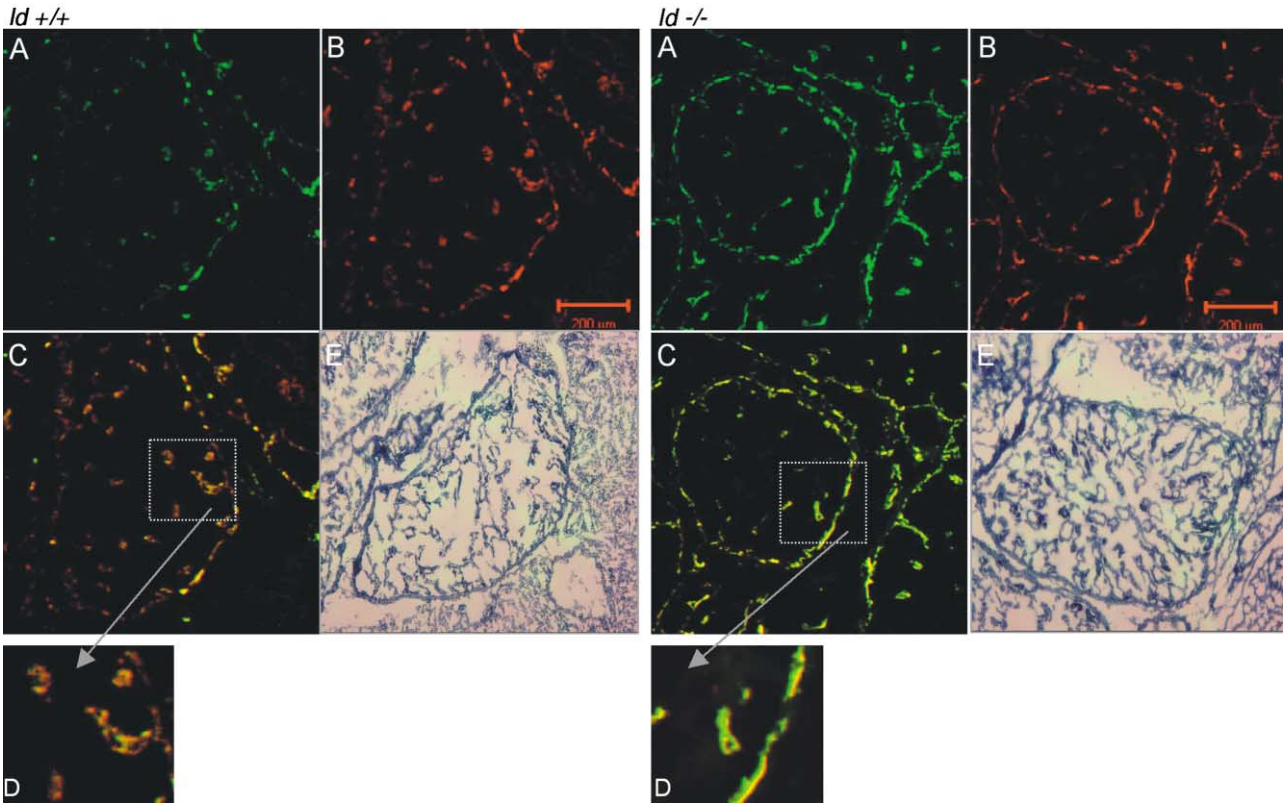


Figure 2. Endothelial cells of $Id^{-/-}$ embryos secrete higher TSP-1 levels in vivo

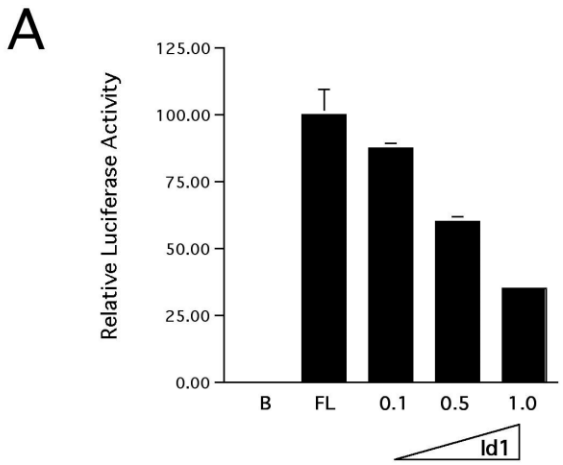
Immunostaining of $Id1^{+/+}$ and $Id1^{-/-}$ mouse embryos. Snap-frozen sections of $Id^{-/-}$ embryos and their wild-type littermates were stained for TSP-1 (**A**, green fluorescent label), and CD-31, to highlight endothelial structures (**B**, red fluorescent label) and analyzed by confocal microscopy. **C** shows a composite image of **A** and **B** where colocalized staining appears yellow. Note increased intensity of green fluorescence (TSP-1) in $Id^{-/-}$ embryo in **A** (TSP alone) and **C** (composite images) while red fluorescence intensity (capillary structures, panels **B**) remains similar. The change in green fluorescence intensity is even more obvious in the composite images at higher resolution (panels **D**). H&E sections are included for orientation (**E**).

Id1-null mice display defects in angiogenesis due to elevated levels of secreted TSP-1

Our in vitro assays suggested that TSP-1 is a likely target through which *Id1* exerts much of its effect on angiogenesis. To assess the relevance of *Id1*-mediated TSP-1 regulation in vivo, we performed matrigel assays in *Id1* wild-type and *Id1* knockout mice. The animals received subcutaneous matrigel plugs containing bFGF to trigger angiogenesis. When neovascularization was assessed in matrigel 10 days following injection, $Id1^{-/-}$ mice had significantly lower angiogenic activity compared to their wild-type littermates (Figure 5), consistent with earlier findings (Lyden et al., 1999, 2001). Seeking a link between impaired angiogenesis and higher TSP-1 levels in $Id1^{-/-}$ mice, the assays were repeated using matrigel plugs containing pro-angiogenic bFGF $\pm$ neutralizing TSP-1 antibody. Neutralization of TSP-1 activity resulted in a dramatic 1.9-fold increase in microvessel density in $Id1^{-/-}$ mice in our matrigel studies versus a modest 0.5-fold increase in $Id1^{+/+}$ mice, while isotype-matched control antibody had no effect (Figure 5). Similar experiments were performed using VEGF to stimulate angiogenesis in matrigel assays. These data confirmed the lower angiogenic activity in $Id1^{-/-}$ mice with increased angiogenic response noted in the presence of neutralizing anti-TSP-1 antibody in $Id1^{-/-}$ mice versus wild-type controls (data not shown). Microvascular density in tumor xenografts formed by Lewis lung carcinoma cells (Figure 6 and data not shown) was also lower in *Id1*-null mice versus wild-type controls ($\sim$1.3-fold, $p < 0.01$). Interestingly, when the vessels were stained for vascular smooth muscle cell (VSMC) actin, the number of CD31-positive vascular structures (mature or maturing vessels) was visibly higher in the tumors harbored by wild-type mice versus *Id1* knockout mice (Figure 6), suggesting that endothelial-associated TSP-1 may interfere with VSMC recruitment or, alternatively, block capillary formation before VSMC recruitment and vessel maturation are completed. Given the above data, we conclude that TSP-1 is a transcriptional target of Id1 in vivo and a major effector of its role in angiogenesis.

Discussion

Id genes have been implicated in the regulation of tumor angiogenesis: Id knockout mice are resistant to tumor growth and metastasis due to impaired angiogenesis although the mechanisms underlying the angiogenesis defects are only partially characterized (Lyden et al., 1999). Our data suggest that Id1 regulates tumor neovascularization via transcriptional repression of the angiogenesis inhibitor TSP-1. These results indicate that Id1 either acts indirectly on the mTSP-1 promoter or utilizes



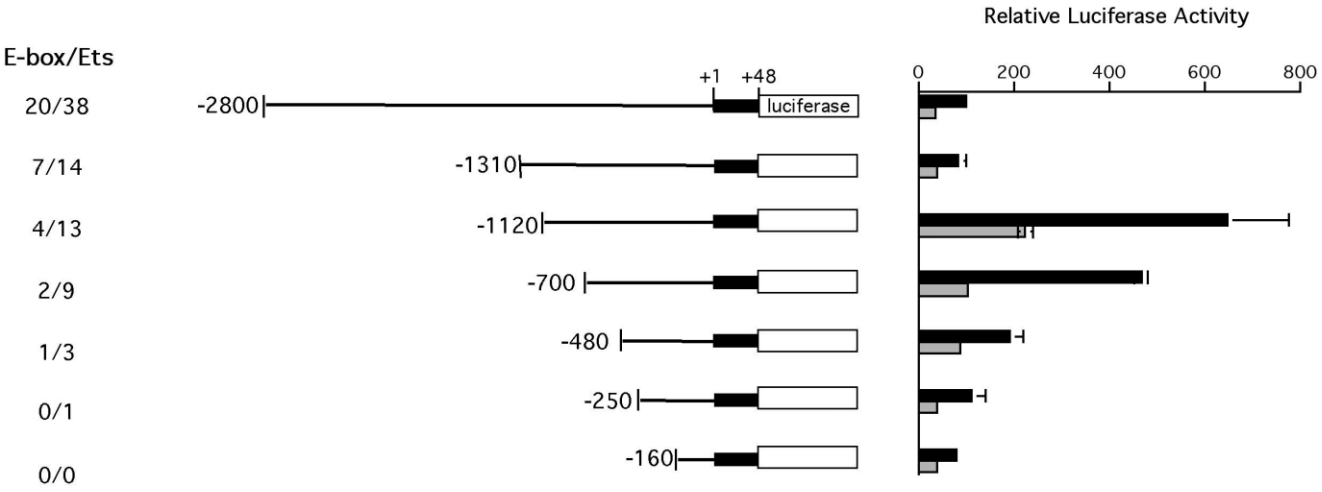


Figure 3. Id1 is a repressor of the mTSP-1 promoter

A: Dose-dependent inhibition of mTSP-1 promoter activity by Id1. B = basal luciferase activity of 1 μg of reporter construct. FL = promoter activity of 1 μg of full-length mTSP-1 promoter. Other data represent mTSP-1 promoter activity with addition of 0.1–1.0 μg of Id1.

B: Promoter activity of deletion mutants of FL mTSP1 promoter (−2800 bp–+48 bp). Number of E boxes/Ets sites is depicted on the left. Id1 inhibition of promoter activity of all mutants is depicted at the right where black bar is reporter activity alone and shaded bar is reporter activity +1 μg of Id1. Note continued Id1 repression with −160 bp–+48 bp mutant.

a novel regulatory element for this class of HLH proteins. Indeed, previous studies have identified the −239 to −135 bp region of the *mTSP-1* promoter as being critical for c-*jun*-mediated repression (Dejong et al., 1999). This repressive effect is indirect and requires activation of WT1 and enhanced WT1 binding to the *mTSP-1* promoter. We have been able to identify a region between −1310 and −1120 bp of the *mTSP-1* promoter that is strongly repressive in NIH3T3 cells under standard growth conditions; however, a similar constitutively repressible region of the human *TSP-1* promoter has not been identified to date. While the possibility to restore *TSP-1* promoter activity via such an element might be of therapeutic use to inhibit inappropriate angiogenesis, the small degree of homology between the human and murine TSP-1 promoters suggests that the transcriptional regulation of *TSP-1* may occur through differing mechanisms in these species.

Our data also show that TSP-1 is a target of Id1 repression in vivo and a potent mediator of Id1-associated effects on angiogenesis. We have shown that loss of Id1 expression in mice is associated with inhibition of bFGF- and VEGF-induced angiogenesis, which is reversed by inactivation of TSP-1 function. Thus, impaired tumor growth and metastases in Id-null mice are at least in part due to the increase in active thrombospondin-1 and subsequent inhibition of tumor angiogenesis. Much previous data supports the role of TSP-1 in inhibiting tumor angiogenesis. Overexpression of TSP-1 in tumor xenografts has been shown to inhibit tumor cell growth in vivo in a variety of tissue types (Bleuel et al., 1999; Sheibani and Frazier, 1995;

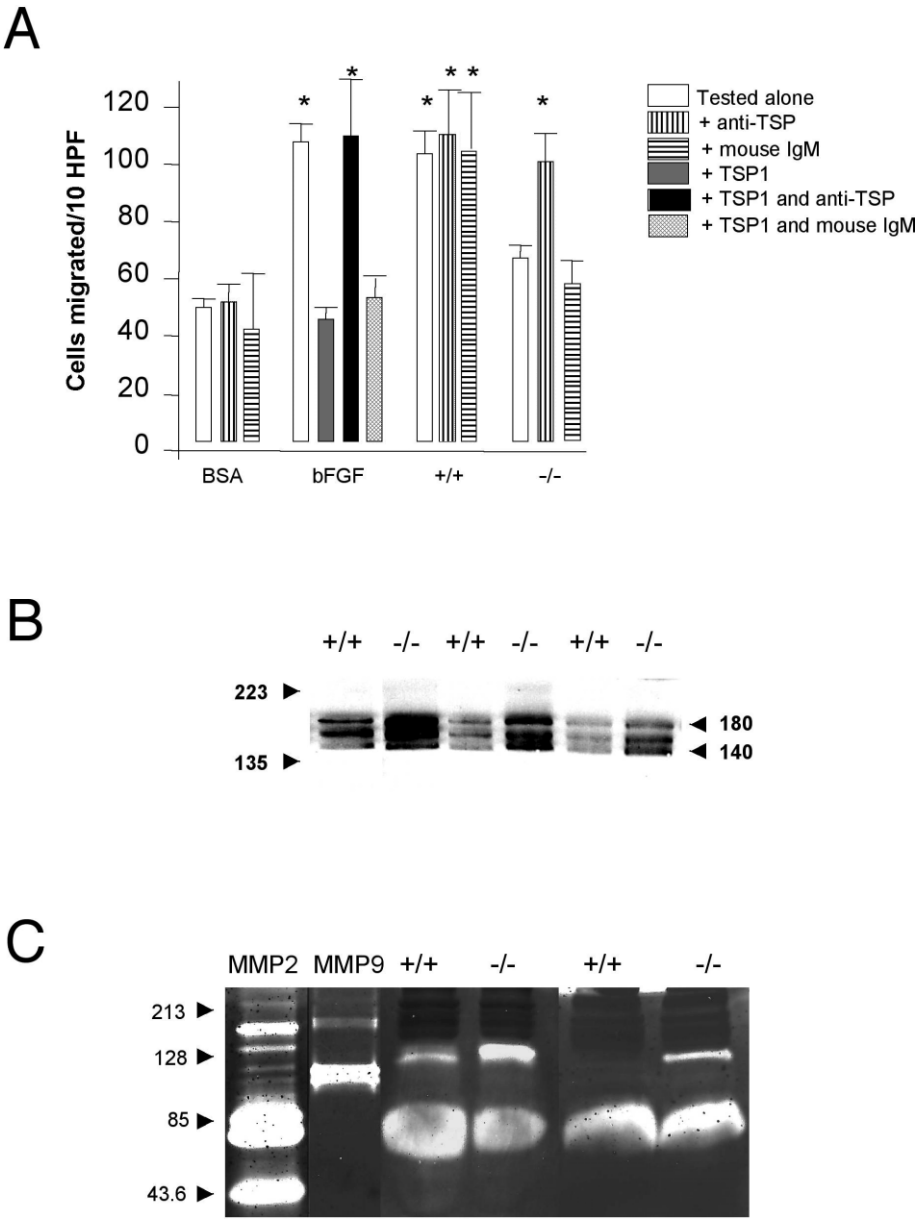


Figure 4. Inhibition of endothelial cell migration by *Id1*$^{-/-}$ MEFs is due to increased activity of thrombospondin-1

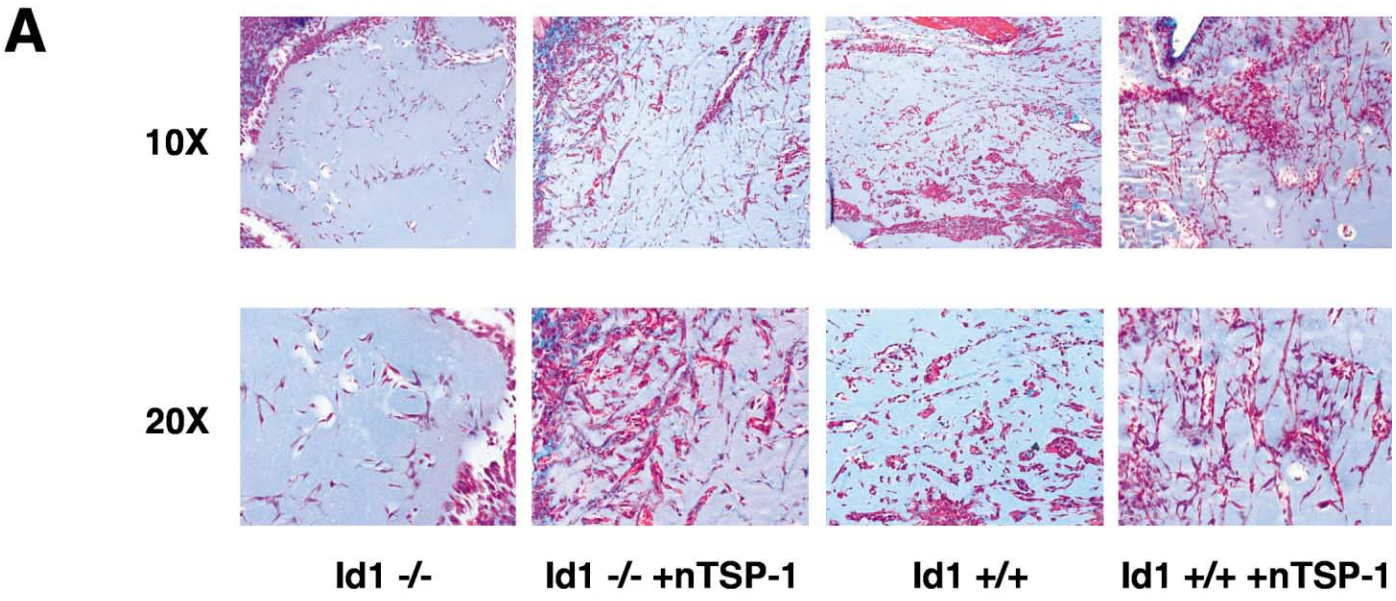
A: Endothelial cell migration assay. Human microvascular endothelial cells (HDMECs) were stimulated to migrate up a gradient of pro-angiogenic bFGF (10 ng/ml, clear bars) or media conditioned by wild-type MEFs (+/+), or MEF's null for *Id1* (−/−). Anti-TSP-1 neutralizing antibody (10 μg/ml) was added where indicated. Antibodies alone had no effect on the basal endothelial cell migration (BSA, hatched bars) and effectively blocked TSP-1 inhibitory effect on bFGF-induced chemotaxis (bFGF, black bars). Gray bars show inhibition of bFGF-induced migration by TSP-1. Note significantly decreased migration of endothelial cells in the presence of CM from *Id1*$^{-/-}$ MEFs versus *Id1*$^{+/+}$ MEFs. This difference is completely abrogated by neutralizing TSP-1 antbody but not affected by nonspecific control antibodies (mouse IgM). Bars marked with * are significantly different from basal migration with BSA ($p < 0.003$).

B: Western analysis of mTSP-1 expression in conditioned media from *Id1*$^{+/+}$ (+/+) and *Id1*$^{-/-}$ (−/−) MEFs.

C: Gelatinase activity of CM from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ MEFs. Controls for MMPs are included at left. Positive gelatinase activity is noted by clearing of coomassie-stained gelatin (white band). Size markers are depicted at far left.

Streit et al., 1999; Volpert et al., 1998), while expression of TSP-1 has been inversely correlated with malignant progression in breast and lung carcinomas and melanomas (Zabrenetzky et al., 1994). Moreover, mice with a genetic susceptibility to breast cancer showed decreased tumor formation in the presence of a breast-specific *TSP-1* transgene, while mice lacking TSP-1 showed increased tumor growth and progression (Rodriguez-Manzaneque et al., 2001). Thus, these data confirm the growth autoinhibitory properties of TSP-1 expressed in tumors; conversely, these data also demonstrate that loss of TSP-1 from tumors promotes malignant growth. In our system, however, we are specifically interested in the effect of matrix-associated TSP-1 expression since previous data suggest that it is the matrix, rather than the tumor itself, that precludes tumor growth in *Id1* and/or *Id3* knockout mice (Lyden et al., 1999). This is supported by experiments in which mice null for *TSP-1* and *p53* demonstrated an increased incidence of osteosarcomas versus mice lacking *p53* alone. In addition, tumor xenografts from B16F10 melanoma grew faster and exhibited higher vascular density in mice null for *TSP-1*, suggesting that TSP-1 can play the role of modifier or "landscaper" gene to suppress tumor growth (Lawler et al., 2001).

Recent studies showed that defective tumor angiogenesis in *Id1*$^{+/-}$, *Id3*$^{-/-}$ mice is due to altered host endothelial cell recruitment and migration (Lyden et al., 2001). In these studies, Id mutant mice were transplanted with wild-type bone marrow (BM), and tumor angiogenesis was restored to baseline through recruitment of exogenous BM-derived circulating endothelial cell precursors. The authors suggest that recruitment of BM-derived precursor cells is defective in *Id* mutant mice due to impaired induction of *Id* expression in response to VEGF; however, the identity of the downstream effectors related to VEGF



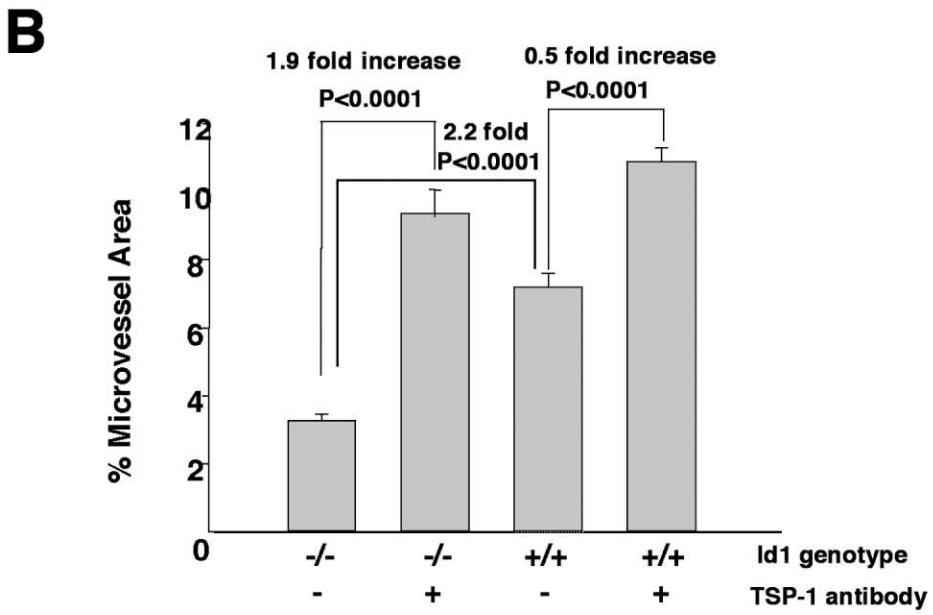


Figure 5. Inhibition of angiogenesis in *Id1*-null mice is due to increased activity of TSP-1

A: Matrigel assay for angiogenesis in $Id1^{-/-}$ mice versus $Id1^{+/+}$ mice with and without neutralizing TSP-1 antibody (nTSP-1). Note significant decrease in microvessel density in matrigel plugs from $Id1^{-/-}$ animals. This inhibition of angiogenesis is abolished in the presence of neutralizing TSP-1 antibody ($Id1^{-/-}$ + nTSP-1).

B: Calculated percent microvessel area of matrigel plugs in $Id1^{+/+}$ and $Id1^{-/-}$ animals with and without neutralizing TSP-1 antibody. Significant inhibition of angiogenesis is seen in $Id1^{-/-}$ mice, which is reverted to near wild-type control levels with neutralizing TSP-1 antibody. Low levels of endogenous TSP-1 are present in wild-type animals since neutralizing antibody increases microvessel area to some extent in these animals.

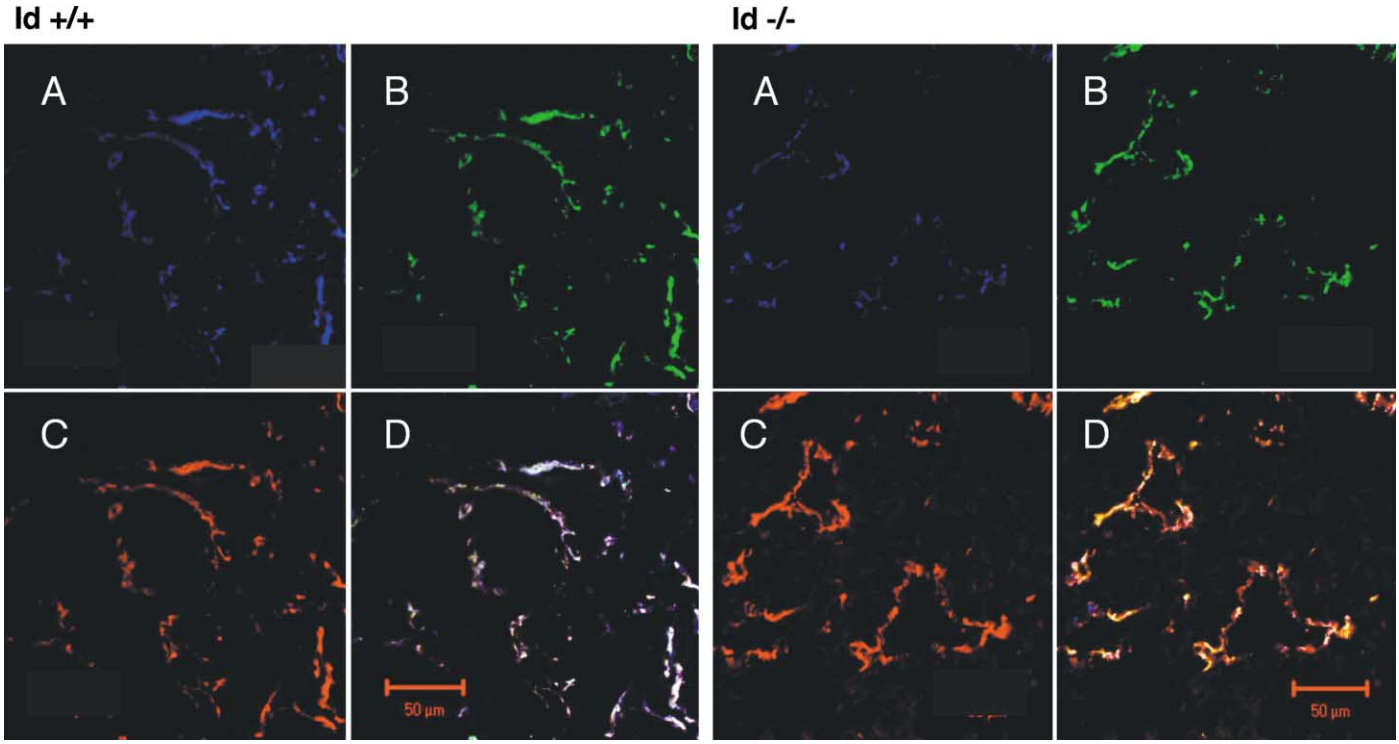


Figure 6. Altered angiogenesis in tumor xenografts in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice

Immunostaining of tumor xenografts in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice. Lewis lung carcinoma cells were implanted into *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice and evaluated for (**A**) vascular smooth muscle cell actin, (**B**) mTSP-1, and (**C**) CD31. **D** represents a merged image for all three panels where white is the composite overlap for all immunofluorescent stains used. Note decrease in vascular smooth muscle cell actin staining in tumors grown in *Id1*$^{-/-}$ mice.

induction of Id expression remained unclear. Here we also demonstrate defects in VEGF-induced angiogenesis in *Id1*$^{-/-}$ mice, which were reversed by inactivation of TSP-1. We therefore propose that recruitment of endothelial cells during tumor angiogenesis may be altered in *Id1*- and/or *Id3*-null mice due to increased TSP-1 expression in VEGF-responsive cells. This is likely because a higher proportion of endothelial precursor cells are positive for the TSP-1 anti-angiogenic receptor, CD36, compared to fully differentiated microcapillary endothelial cells (Kuzu et al., 1993; Regezi et al., 1993). Thus, increased levels of TSP-1 in *Id1*-null mice may influence the recruitment of CD36-rich endothelial precursors along with causing apoptosis of the CD36-positive mature microvascular cells of remodeling capillaries as previously noted (Jimenez et al., 2000). We also note the effect of loss of *Id1* on vessel maturation which may be related to TSP-1 expression. In our experiments, tumors grown in *Id1*$^{-/-}$ mice showed fewer endothelial structures that expressed vascular smooth muscle cell (VSMC) actin, a marker of vascular maturity and stability. Earlier studies showed that quiescent, mature vessels are less susceptible to apoptosis by anti-angiogenic factors than remodeling ones (Benjamin, 2000; Folkman, 1995; Hanahan and Folkman, 1996; Volpert et al., 2002). Thus, TSP-1 may perform an additional function in preventing VSMC recruitment during the remodeling of tumor vasculature, therefore rendering these vessels more susceptible to injury. Alternatively, reduced staining for VSMC may reflect dismantling of the capillaries in *Id1*-null mice due to TSP-induced endothelial cell apoptosis that precludes maturation (Guo et al., 1997; Jimenez et al., 2000).

We also identified several other genes of interest in our screen for Id1 effectors (Table 1) that may be of relevance to the defective tumor angiogenesis noted in *Id1*/*Id3* knockout mice. Among those was β5-integrin, whose repression by Id1 may also contribute to Id effects on tumor angiogenesis. Recent data demonstrate enhanced growth of tumor xenografts as well as enhanced pathological angiogenesis in mice lacking either β*3*- or β*5*-integrin (Reynolds et al., 2002). Since β5-integrin expression was upregulated in *Id1*$^{-/-}$ MEFs, it is possible that increased β5-integrin expression may be an additional factor contributing to decreased tumor angiogenesis in these mice. We await further information that may prove or disprove the role of this gene as an Id1 downstream effector and its function in tumor-associated angiogenesis.

Recent data have demonstrated a correlation between Id gene expression and tumor invasiveness in a variety of human malignancies (reviewed in Lasorella et al., 2001); however, the precise genetic targets for Id genes in these tumors remain unknown. Our data identify TSP-1, a potent inhibitor of angiogenesis, as an important downstream effector of Id1 in mice and a major regulator of Id1 effects on angiogenesis. Ongoing studies will identify other Id1 effectors, their role in angiogenesis and tumor growth, and their relationship to TSP-1 in these processes.

Experimental procedures

Generation and screening of subtracted cDNA library

Id1$^{-/-}$ mice were generated as previously described (Yan et al., 1997) and are of identical genetic background to *Id1*$^{+/+}$ *mice*. Mouse embryo fibroblasts (MEFs) at passage 3 (P-3) were prepared as previously described (Alani et al., 2001). Total cellular RNA was extracted using guanidinium isothiocyanate, and polyA RNA was prepared on an oligo dT column. Two micrograms of each RNA was reverse transcribed and used as both driver and tester in forward and reverse subtractions as outlined in the PCR-Select cDNA Subtraction Kit (Clontech). Each subtraction was repeated twice with two independent cell populations. Final pools of subtracted cDNAs were cloned into pBluescript SK+ (Stratagene). Differential screening of clones was performed according to manufacturers recommendations (PCR-Select, Clontech). Clones which were differentially expressed were then sequenced and further evaluated on Northern blots.

Northern analysis and semi-quantitative RT-PCR

Total cellular RNA was prepared from P-3 MEFs as above, separated on a 1.2% agarose gel, and transferred to nitrocellulose membranes. Blots were probed with ^{32}P-labeled probes encoding a full-length murine Id1 cDNA, murine Tsp-1 (66–681 bp), and murine GAPDH, and quantified using a phosphorimager (Molecular Dynamics). Semi-quantitative RT-PCR was performed as previously described (Jacobs et al., 1999) using the forward primer 5′ gggctagagaaaccccccac 3′ and reverse primer 5′ccaaagggagaaagtcc 3′ to amplify up 66-681bp of murine TSP-1.

Promoter analysis

Plasmids possessing −2800 to +48 bp of the murine thrombospondin promoter driving a luciferase reporter gene were kindly provided (Paul Bornstein, University of Washington, Seattle, Washington). Deletion mutants were constructed using PCR amplification of the regions of interest and KpnI/HindIII cloning sites for insertion into the reporter plasmid. All mutants constructed were sequenced in their entirety using standard techniques. pGL2-Basic (Promega) expression plasmids were used as negative controls. NIH 3T3 cells were cultured in DMEM 10% calf serum and plated into 35 mm dishes. Cells at 60% confluency were transfected with 1 ug of reporter construct and 0–1.0 μg of EMSV-*Id1* or EMSV plasmid using FuGENE 6 reagent (Roche) according to the manufacturer's protocol so that the total amount of DNA transfected was constant and totaled 2 μg. Transfections were normalized for efficiency using 0.01 ug of pRL (renilla luciferase plasmid). Cells were harvested 48 hr after transfection and reporter activity was measured using the Dual Luciferase (Promega) assay according to the manufacturer's recommendations.

Preparation of conditioned media

Conditioned media were prepared from P-3 MEFs in the folowing manner. Cells were grown to 90% confluence in complete media then washed twice with Ca^{2+}/Mg^{2+}-free PBS and placed in either serum-free DMEM or serum-free PC-1 (Clonetics) media for 48 hr (DMEM) or 24 hr (PC-1). The protease inhibitor AEBSF was added to a final concentration of 0.5 mM and media were concentrated in a Centricon plus-20 (Millipore) with a 10 kDa molecular weight cutoff for 2–3 hr at 4°C. Final protein concentrations of CM were brought to 5 mg/ml with basal media. To determine angioinhibitory activity, conditioned media were tested for their ability to block capillary endothelial cell migration induced by the known angiogenic factor basic fibroblast growth factor (bFGF).

Endothelial cell migration assays

Bovine adrenal capillary endothelial cells were a kind gift of Dr. Judah Folkman (Children's Hospital, Harvard Medical School) and grown in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum (Flow Laboratories, McLean, Virginia) with 1% endothelial cell mitogen (Biomedical Technologies, Inc., Stoughton, Massachusetts) and used between passages 14 and 15. Human dermal microvascular endothelial cells from Clonetics Cell Systems (San Diego, California) were grown in basal endothelial cell growth medium (MDCB131, GIBCO) with the supplement bullet kit (Clonetics) and 5% fetal bovine serum, and used at passage 9. Migrations were performed as described previously (Polverini et al., 1991). Endothelial cells were serum-starved overnight, harvested, resuspended in control media (basal media + 0.1% BSA), and plated at 3 × 10^4 cells/well on the bottom side of a gelatinized 5 μm (for bovine cells) or 8 μm (for human cells) porous membrane (Nucleopore Corp., Pleasanton, California) in an inverted, modified Boyden chamber (Neuroprobe Inc.) and incubated at 37°C for 2 hr to allow attachment. The chambers were then reinverted, 50 μl of test sample was placed in the top wells, and the cells were allowed to migrate toward the test samples for 4 hr at 37°C. TSP-1 was purified as previously described (Volpert et al., 1998) and used at a concentration of 5 nM. For assays with neutralizing antibody, the A4.1 anti-TSP-1 monoclonal antibody (NeoMarkers) was added at 10–20 μg/ml. Membranes were removed, fixed, and stained (DiffQuick, Fisher), mounted on glass slides, and the number of cells migrating to the top side of the membrane in ten high-powered fields were counted. Samples in each experiment were tested in quadruplicate and experiments were repeated at least three times. Statistical significance of the difference between sample means was determined using two-tailed Student's t test. Basal migration in the presence of 0.1% BSA was used as a negative control, migration toward a known angiogenic stimulus (bFGF, 10 ng/ml) as a positive control.

Matrigel angiogenesis assays

Mice were injected subcutaneously with 0.75 ml Matrigel supplemented with 150 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems) or VEGF 5 μg/ml as previously described (Passaniti et al., 1992). For assays with neutralizing antibody, the A4.1 anti-TSP-1 monoclonal antibody (NeoMarkers) was added to the matrigel at 20 μg/ml. Mice were sacrificed 10 days after the Matrigel injection. Gels were recovered by dissection, fixed in PBS-buffered 10% formalin containing 0.25% glutaraldeheyde, sectioned, and stained with Masson's Trichrome. The Image-Pro analysis system was used to quantify the area occupied by vessels in the histological sections. The mean vessel area/field from 10–20 fields/section/plug (X200 = 20× objective lens and 10× ocular lens: Zeiss Axioskop) was calculated and expressed as mean percentage area occupied by blood vessels ± SEM. The experiment was repeated twice. For fluorescent staining, Matrigel plugs were snap frozen in OCT compound, 5 μm thick cryosections obtained, and fixed in cold acetone, 1:1 acetone/chloroform, rinsed in PBS, and blocked in 5% donkey serum, 1% BSA. The slides were incubated overnight with rat anti-mouse CD31 antibody (PharMingen), washed, and re-blocked. PE-labeled anti-rat and FITC- or Cyan™- anti-hamster antibodies (Jackson Labs) were added in blocking solution for 1 hr (26°C), slides washed, and mounted in Mowiol (Fisher). Quantitative image analysis of digital images taken with Spot camera under inverted fluorescent microscope was performed using MetaView software. The number of CD31-positive structures was counted in a minimum of five high-powered (100×) fields. Statistical significance of the data was evaluated using two-tailed Student's t test.

Zymography

Samples were mixed 1:2 with sample buffer (62.5 mM Tris-CL, pH 6.8, 4% SDS, 25% glycerol, and 0.01% Bromophenol Blue), and run at 100 volts through a gel (10% polyacrylamide [wt/vol], 0.1% SDS, 0.1% gelatin). The gel was then placed in 2.5% Triton X-100 to allow for renaturation of the embedded proteins. Subsequently, the gel was placed in buffer (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 5mM $CaCl_2$, and 0.02% Brij-35) at 37°C for 16 hr to optimize metalloproteinase activity. The gel was stained for 1 hr in 40% methanol/10% acetic acid/0.5% (wt/vol) Coomassie brilliant blue G-250 and then destained in the same buffer without Coomassie brilliant blue. Proteinase activity was subsequently inferred by the presence of clear bands which appeared against a blue background. Molecular weights of the proteinases were determined by comparison to protein molecular weight standards (Bio-Rad) and metalloprotease controls (Chemicon International, Temecula, California). Control proteins were run at 5 ng/lane and were of human origin (MMP-2 and -9 were purified human proteins, and MMP-7 was human recombinant).

Western blotting

Conditioned media, collected as described, were loaded on 6% SDS-PAGE (7.5 ug total protein/lane) along with purified human TSP-1 (positive control) and transferred to PVDF membrane (Amersham). The blots were probed with anti-TSP monoclonal IgM (NeoMarkers), developed with HRP-conjugated secondary antibodies (RBI), and visualized by exposure with X-ray film.

Immunostaining of tissue specimens

Mouse embryos, tumor xenografts and Matrigel plugs were excized, snap-frozen in Tissue-Tek OCT (Sakura), and kept at −8°C. Five micrometer thick sections were prepared immediately before staining. The sections were fixed by consecutive treatments with ice-cold Acetone, Acetone/Chloroform mix (1:1), and Acetone. Endogenous biotin and avidin were blocked using the Blocking kit from Vector Labs, as recommended by manufacturer, followed by 1 hr incubation in MOM blocking solution (Ibid) containing Mouse Immunoglobulin Blocking Reagent. For colocalization of CD31 and TSP-1, the sections were then washed twice and incubated for 5 min in MOM diluent (protein block), and incubated 30 min at room temperature with primary antibodies in MOM at the following dilutions: anti-CD31 rat anti-mouse polyclonal (Santa Cruz) at 1:125 and anti-TSP-1 mouse monoclonal IgM (Neo-Markers) at 1:100. The sections were rinsed twice for 2 min in PBS and exposed for 15 min (room temperature) to the following dilutions secondary of the antibodies in MOM: donkey anti-rat RhodaminX conjugated (Jackson Labs) 1:200 and biotinylated anti-mouse AB (MOM kit, Vector Labs) 1:250. The samples were then washed in PBS and incubated for 5 min with Fluorescein Avidin D (Vector Labs) in PBS at 20 ug/ml. To visualize vascular smooth muscle, sections stained for CD31/TSP-1 were washed three times for 3 min each in PBS and endogenous biotin/avidin and mouse IgG re-blocked as above. Tissue sections were exposed for 30 min (room temperature) to mouse monoclonal IgG against vascular smooth muscle actin at 1:200 dilution in MOM, washed two times for two min each. Biotinylated anti-mouse antibodies (MOM kit) were applied as above, followed by 3 × 3 min washes in PBS and incubation with Streptavidin Cy5 (Jackson Labs, 1:1000) in PBS (15 min at room temperature). The slides were mounted and fluorescent images analyzed by confocal microscopy.

Tumor xenograft assay

2.0×10^6 Lewis lung carcinoma cells were injected subcutaneously into the flank of *Id1*$^{-/-}$ and wild-type control mice. Mice were observed on a daily basis and tumor measurements were taken 3×/week. Tumors were excised after 21 days and formalin fixed or snap frozen in Tissue-Tek OCT (Sakura) for histologic analysis.

Acknowledgments

We thank Bert Vogelstein, Stephen Baylin, Brad St. Croix, Phil Cole, and Joel Shaper for careful review of this manuscript and helpful discussions. We also thank Robert Benezra for providing Id1 mutant mice and Paul Bornstein for providing TSP-1 reporter constructs. R.M.A. is supported by NIH grant AR48246. O.V. is supported by American Cancer Society grant RSG-01-099-02-CSM, and NIH grant HL68003.

Received: July 9, 2002
Revised: November 8, 2002

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