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Thrombospondin-2 plays a protective role in multistep carcinogenesis: a novel host anti-tumor defense mechanism

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The angiogenic switch during tumorigenesis is thought to be induced by a change in the balance of pro-angiogenic and anti-angiogenic factors. To elucidate the biological role of the endogenous angiogenesis inhibitor thrombospondin-2 (TSP-2) during multistep carcinogenesis, we subjected TSP-2-deficient and wild-type mice to a chemical skin carcinogenesis regimen. Surprisingly, TSP-2 expression was strongly upregulated in the mesenchymal stroma of wild-type mice throughout the consecutive stages of tumorigenesis whereas the angiogenesis factor, vascular endothelial growth factor, was induced predominantly in tumor cells. TSP-2 deficiency dramatically enhanced susceptibility to skin carcinogenesis and resulted in accelerated and increased tumor formation. The angiogenic switch occurred in early stages of pre-malignant tumor formation, and tumor angiogenesis was significantly enhanced in TSP-2-deficient mice. While TSP-2 deficiency did not affect tumor differentiation or proliferation, tumor cell apoptosis was significantly reduced. These results reveal upregulation of an endogenous angiogenesis inhibitor during multistep tumorigenesis and identify enhanced stromal TSP-2 expression as a novel host anti-tumor defense mechanism.

Keywords: angiogenesis/cancer/skin/thrombospondin-2

Introduction

Increasing evidence indicates a pivotal role for epigenetic, cell–cell and extracellular influences in tumorigenesis (Hanahan and Weinberg, 2000). Induction of angiogenesis, as an epigenetic event, is generally considered essential for tumor growth (Folkman, 1992). Acquisition of an angiogenic phenotype ('angiogenic switch') (Hanahan and Folkman, 1996) may even precede the development of other traits that contribute to the malignant phenotype, and it has been demonstrated that inhibition of angiogenesis impairs *de novo* tumor growth (Parangi *et al*., 1996; Bergers *et al*., 1999). The switch to the angiogenic phenotype during tumorigenesis is thought to be induced by a change in the balance of positive and negative regulators of endothelial cell growth (Hanahan and Folkman, 1996). The controlling mechanisms, however, remain incompletely understood. Vascular endothelial growth factor (VEGF) is thought to be the major angiogenesis factor in malignant tumor growth (Kim *et al*., 1993; Ferrara, 1995; Brown *et al*., 1997), and transgenic mice overexpressing VEGF in skin were characterized by accelerated papilloma formation in chemically induced skin carcinogenesis (Larcher *et al*., 1998). While these findings suggest that increased activity of pro-angiogenic factors may lead to enhanced susceptibility to carcinogenesis, the biological role of endogenous inhibitors of angiogenesis in multistage carcinogenesis has remained unknown.

Several naturally occurring angiogenesis inhibitors have been identified, including thrombospondin-1 (TSP-1) (Iruela-Arispe *et al*., 1991), TSP-2 (Volpert *et al*., 1995; Bornstein *et al*., 2000), angiostatin (O'Reilly *et al*., 1994), endostatin (O'Reilly *et al*., 1997) and vasostatin (Pike *et al*., 1998). TSP-2 is a 420 kDa homotrimeric glycoprotein that plays an important role in a variety of biological processes, including cell–cell and cell–matrix interactions (Bornstein *et al*., 2000; Lawler, 2000). In adult mouse skin, low level TSP-2 expression is associated predominantly with dermal fibroblasts (Kyriakides *et al*., 1998b). TSP-2 inhibits the angiogenic activity of basic fibroblast growth factor (Volpert *et al*., 1995) and the formation of focal adhesions in bovine endothelial cells *in vitro* (Murphy-Ullrich *et al*., 1993). Moreover, we recently found that transfected TSP-2 inhibited tumor growth and angiogenesis of human squamous cell carcinomas transplanted onto nude mice, with a greater potency than the related molecule TSP-1 (Streit *et al*., 1999a). To characterize the biological role of endogenous TSP-2 in multistage skin carcinogenesis, we subjected TSP-2-deficient mice (Kyriakides *et al*., 1998a) and age-matched wild-type mice to a standard two-step chemical skin carcinogenesis protocol, using 7,12-dimethylbenz-[α]anthracene (DMBA) for tumor initiation and phorbol 12-myristate 13-acetate (PMA) to promote tumor growth (DiGiovanni, 1992).

Here we report that stromal TSP-2 expression was highly upregulated in the mesenchymal tumor stroma of wild-type mice throughout the consecutive stages of skin tumorigenesis. TSP-2 deficiency dramatically enhanced the susceptibility to experimental skin carcinogenesis and was associated with increased tumor angiogenesis and decreased tumor cell apoptosis. Our findings suggest that stromal upregulation of the endogenous angiogenesis inhibitor TSP-2 plays a protective role in controlling *de novo* tumor growth as part of host anti-tumor defense mechanisms.

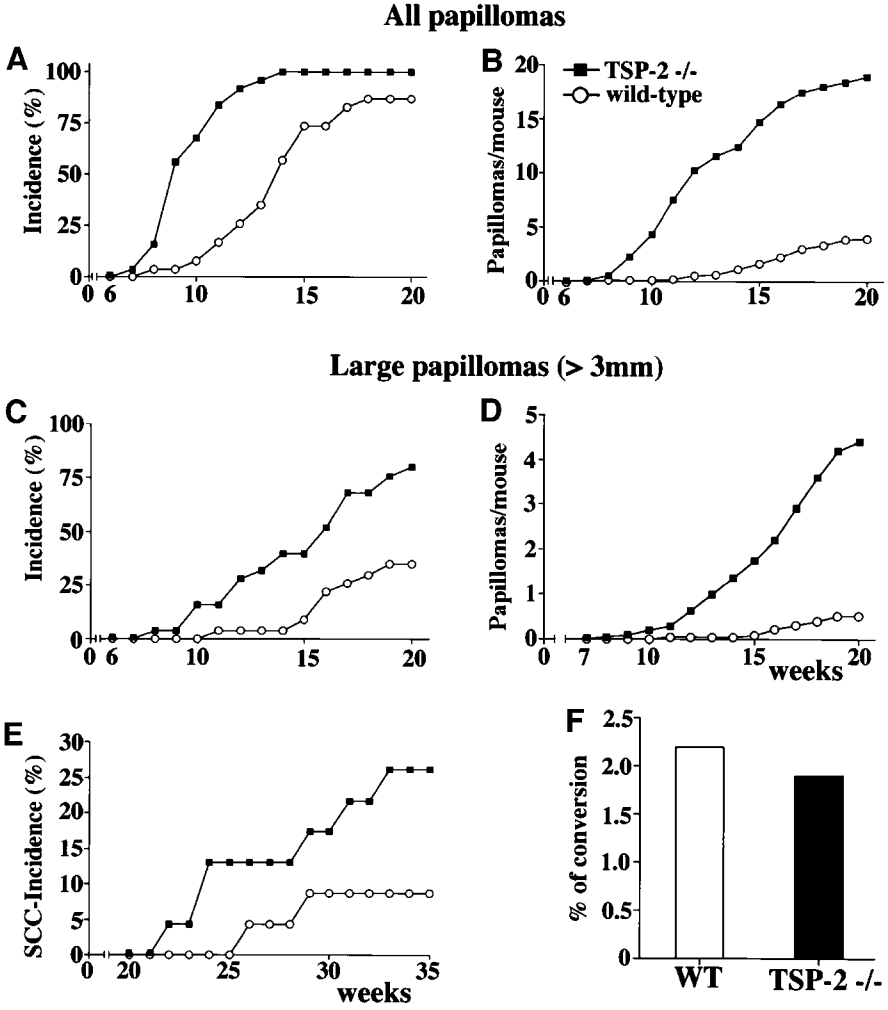

Fig. 1. Accelerated and increased skin carcinogenesis in TSP-2-deficient mice. (**A**) Earlier and increased incidence of papillomas (>1 mm) in TSP-2-deficient mice (n = 24; filled squares), as compared with wild-type mice (n = 25; open circles). Incidence is expressed as the percentage of mice with detectable tumor formation during the 20 weeks of topical PMA application. (**B**) Significantly increased frequency of papilloma (>1 mm) formation, expressed as the average number of papillomas per mouse, in TSP-2-deficient mice (P <0.001 after 10 weeks of PMA treatment). (**C**) Earlier and increased incidence of large papillomas (>3 mm) in TSP-2-deficient mice. (**D**) Increased average number of large papillomas (> 3 mm) per mouse in TSP-2-deficient mice; P <0.01 after 13 weeks; P <0.001 after 17 weeks. (**E**) Increased incidence of squamous cell carcinomas (SCC) in TSP-2-deficient mice. (**F**) Comparable rate of malignant conversion of papillomas to SCC in TSP-2-deficient and wild-type (WT) mice.

Results

Accelerated and increased skin carcinogenesis in TSP-2-deficient mice

We subjected wild-type and TSP-2-deficient mice to a standard two-step skin carcinogenesis protocol by using topical application of a single subcarcinogenic dose of DMBA for initiation, followed by 20 weekly topical applications of PMA for tumor promotion. TSP-2-deficient mice showed accelerated formation of skin papillomas, with an average latency period of 9 weeks after the first PMA application, as compared with 14 weeks in wild-type mice (Figure 1A). At 14 weeks, 100% of TSP-2-deficient mice showed visible tumor formation, as compared with only 57% of wild-type mice (Figure 1A). Moreover, the number of epithelial tumors was significantly increased in TSP-2-deficient mice, as compared with wild-type mice (P <0.001). After 20 weeks of PMA promotion, TSP-2-deficient mice had developed >19 papillomas per mouse, in comparison with the development of <5 papillomas per wild-type mouse (Figure 1B). These effects were even more pronounced when only larger papillomas (>3 mm) were compared. In TSP-2-deficient mice, the first large papilloma developed after 8 weeks of PMA promotion, 3 weeks earlier than in wild-type mice (Figure 1C). Following 20 weeks of PMA promotion, the incidence of large papillomas was 80% in TSP-2-deficient mice, as compared with only 35% in wild-type mice (Figure 1C). The average number of large papillomas was increased 7.5-fold in TSP-2-deficient mice (Figure 1D; P <0.001), indicating that papillomas grew significantly faster in the absence of TSP-2. No papillomas or carcinomas were observed in wild-type or TSP-2-deficient mice treated with DMBA or with PMA alone (data not shown). Papillomas were prone to regression in both genotypes after termination of the PMA treatment at 20 weeks. At week 24, the average number of papillomas per wild-type mouse was 3.2, as compared with 4.4 at week 20. In TSP-2-deficient mice, the average number of papillomas per mouse was 15.3 at week 24, as compared

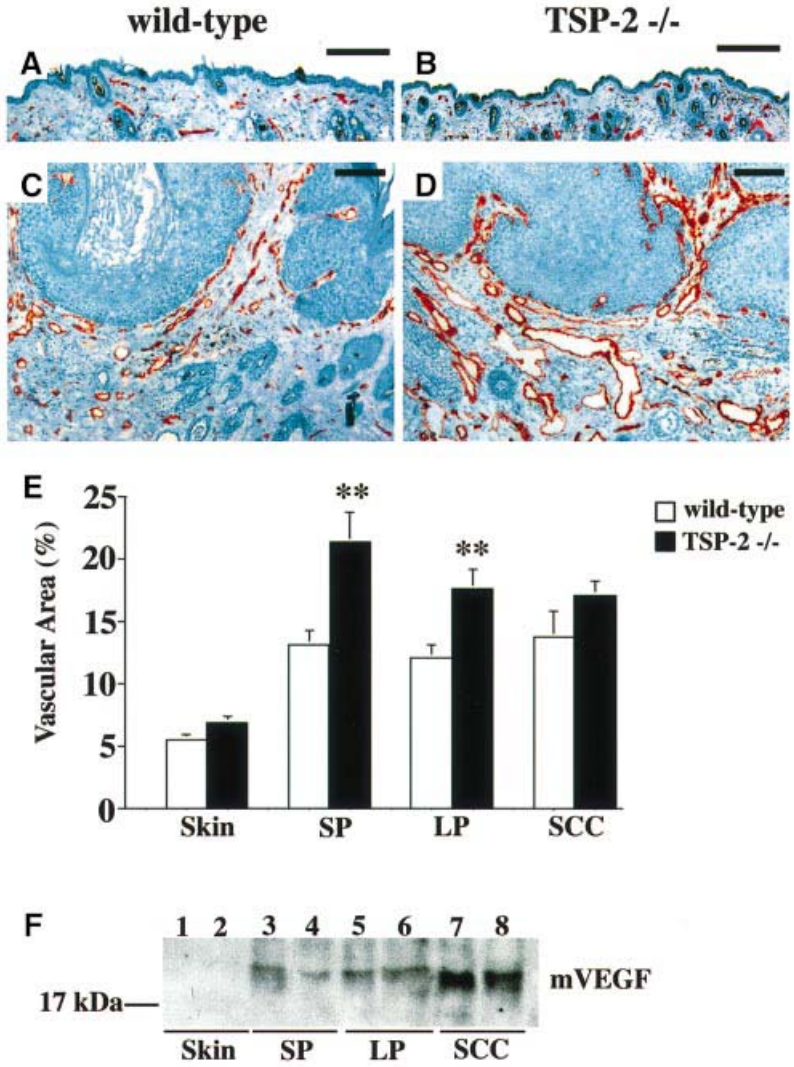

Fig. 2. Enhanced angiogenesis at early stages of skin carcinogenesis in TSP-2-deficient mice. CD31 immunostains of blood vessels in untreated skin (**A** and **B**) and in early papillomas (**C** and **D**) of wild-type (A and C) and TSP-2-deficient (B and D) mice demonstrate strongly increased vascularization of papillomas, as compared with untreated skin, in both genotypes. Vascularization was much more pronounced in TSP-2-deficient papillomas (D) than in wild-type papillomas (C), with an increased number of enlarged blood vessels. Bar = 100 μm. (**E**) Computer-assisted quantitative image analysis of CD31-stained sections demonstrated that the average vascular area was significantly increased in TSP-2-deficient mice (filled bars) already at the stage of early papilloma formation and remained higher than in wild-type mice (open bars) throughout the progressive stages of skin carcinogenesis. Skin, untreated skin (n = 3); SP, small papillomas (⩽3 mm; n = 7); LP, large papillomas (>5 mm; n = 6); SCC, squamous cell carcinomas (n = 2). Data are expressed as mean values ± SEM. $^{**}P$ <0.01. (**F**) Western blot analysis revealed comparable upregulation of VEGF expression in small (SP) and large (LP) papillomas and in SCC of both wild-type (lanes 1, 3, 5 and 7) and TSP-2-deficient (lanes 2, 4, 6 and 8) mice, as compared with untreated skin.

with 19.0 at week 20. Malignant conversion of papillomas to squamous cell carcinomas (SCC) was first seen at 22 weeks after PMA promotion in TSP-2-deficient mice and after 26 weeks in wild-type mice (Figure 1E). Twenty-six percent of TSP-2-deficient mice but only 9% of wild-type mice had developed SCC at 35 weeks (Figure 1E). However, no differences in the percentage of malignant conversion of papillomas to SCC were found between wild-type and TSP-2-deficient mice (Figure 1F). In TSP-2-deficient mice, regional lymph node metastases were first detected at 30 weeks after initiation of PMA promotion, and 33.3% of SCC-bearing mice developed metastases by week 35. No metastases were found in wild-type mice after 35 weeks.

Increased tumor angiogenesis in TSP-2-deficient mice

To analyze tumor-associated vascularization, frozen sections of untreated skin, papillomas of varying sizes and SCC were stained for the endothelial junction molecule CD31 (Dejana *et al*., 1995). The 'angiogenic switch' from vascular quiescence to upregulation of angiogenesis was observed in the early stages of skin carcinogenesis in wild-type mice, as shown by immunohistochemical analysis of CD31-stained blood vessels in untreated skin (Figure 2A) and in small papillomas (Figure 2C). This effect was more striking in TSP-2-deficient mice, which showed an increased number of enlarged blood vessels (Figure 2B and D). Computer-assisted morphometric image analysis

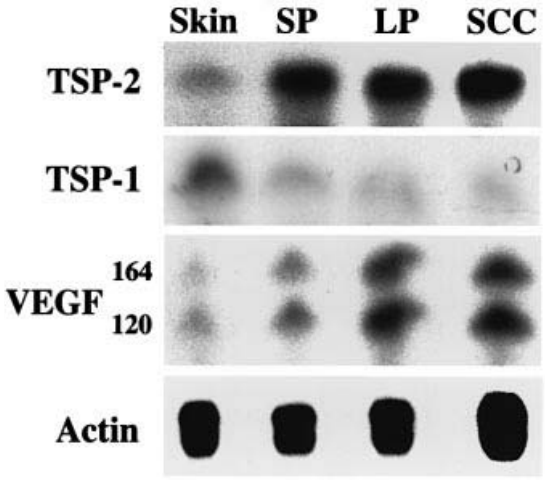

Fig. 3. Upregulation of TSP-2 expression during skin carcinogenesis. RNase protection assay of RNA obtained from untreated skin, small papillomas (SP; ⩽3 mm), large papillomas (LP; >3 mm) and squamous cell carcinomas (SCC) of wild-type mice revealed significant upregulation of TSP-2 mRNA expression during early tumor formation in chemically induced skin carcinogenesis, whereas TSP-1 mRNA expression was downregulated. Expression of both VEGF120 and VEGF164 mRNAs was upregulated most prominently in large papillomas and in SCC. A β-actin riboprobe served as loading control.

revealed that both the vessel density and the average vessel size were increased in early and late papillomas in TSP-2-deficient mice, as compared with wild-type mice, resulting in a significant increase in the relative area occupied by tumor blood vessels (Figure 2E). These differences were most pronounced in early-stage small TSP-2-deficient papillomas that showed a >3-fold increase in vascularity, as compared with untreated skin. Although the vascularity of TSP-2-deficient SCC was also increased over wild-type levels, no significance levels could be calculated due to the small number of SCC in wild-type mice. We found only minor heterogeneity of vascularization between different samples within each genotype. Importantly, the differences in vascularization were not due to effects of TSP-2 deficiency on VEGF expression, since western blot analyses of lysates obtained from untreated skin and from different stages of epithelial carcinogenesis did not reveal any major difference in VEGF protein levels between wild-type and TSP-2-deficient mice (Figure 2F).

Enhanced stromal TSP-2 expression during skin carcinogenesis

RNase protection assays of total RNA extracted from skin and tumors of wild-type mice revealed that TSP-2 mRNA expression was upregulated substantially in early-stage papillomas, as compared with low expression levels in untreated skin (3.9-fold induction; Figure 3). No further major increase was observed during tumor progression to large papillomas (4.5-fold induction) and SCC (4.8-fold induction). In contrast, the expression of TSP-1 mRNA was downregulated during epithelial carcinogenesis (Figure 3). We also found upregulation of VEGF mRNA expression in early papillomas, with a further induction of expression in late-stage papillomas and in SCC, but little VEGF mRNA was expressed in untreated skin (Figure 3). Both VEGF164 and VEGF120 were upregulated, whereas we were unable to detect other VEGF isoforms. To investigate whether the enhanced expression of TSP-2 mRNA in skin tumors was derived from epithelial tumor cells themselves or from the mesenchymal tumor stroma, we performed *in situ* hybridization in tissue samples obtained from wild-type mice. When compared with untreated skin (Figure 4A and B), TSP-2 mRNA expression was strongly induced in mesenchymal stroma cells of papillomas (Figure 4C and D) and of SCC (Figure 4E and F), whereas no TSP-2 expression was detected in tumor cells. No major differences in the amount of TSP-2 mRNA expression were found between papillomas and SCC. Conversely, VEGF mRNA was expressed strongly in tumor cells, with little or no expression in the tumor stroma (Figure 4G and H). Control hybridizations with a sense riboprobe revealed very low background signal (Figure 4I and J). These results indicate an important role for stroma-derived TSP-2 in the control of skin tumorigenesis, possibly counteracting the angiogenic effects of tumor-derived VEGF.

TSP-2 deficiency does not affect epidermal differentiation or proliferation

Skin carcinogenesis is associated with major alterations in epidermal differentiation. To investigate whether TSP-2 deficiency, in addition to its prominent effects on angiogenesis, might also directly affect epidermal or tumor differentiation, we compared the expression of markers for early and late epidermal differentiation during skin carcinogenesis in wild-type and TSP-2-deficient mice. Comparable expression of keratin 10 (K10), an early marker for terminal keratinocyte differentiation (Roop *et al.*, 1988), was found in the suprabasal layers of untreated epidermis (Figure 5A and B) of both wild-type and TSP-2-deficient mice. Consistent with previous findings (Huitfeldt *et al.*, 1991), K10 staining was generally weaker in papillomas than in normal epidermis, and the onset of K10 expression was delayed to a similar extent in both genotypes (Figure 5C and D). Loricrin, a marker for late terminal keratinocyte differentiation (Mehrel *et al.*, 1990), was expressed in the granular layer of TSP-2-deficient untreated skin (Figure 5F) and of epidermal tumors (Figure 5H) in a pattern indistinguishable from that of the wild-type mice (Figure 5E and G).

The hyperproliferation-associated keratin K6 (Weiss *et al.*, 1984) was induced comparably in skin tumors of both wild-type and TSP-2-deficient mice (Figure 5K and L), whereas normal skin, with the exception of hair follicles, did not express K6 in either experimental group (Figure 5I and J). In accordance with these data, the number of proliferating epithelial cells, examined by 5-bromodeoxyuridine (BrdU) labeling, was unchanged in untreated skin (data not shown) and in skin papillomas of TSP-2-deficient mice (Figure 6B) as compared with wild-type mice (Figure 6A). Quantitative analysis of the rate of tumor cell proliferation in size-matched papillomas of both genotypes did not detect any significant differences between TSP-2-deficient and wild-type mice ($P = 0.38$; Figure 6E).

To evaluate whether the enhanced tumorigenesis in TSP-2-deficient mice might have been mediated, at least in part, by the modulation of inflammatory stromal responses, we performed a series of histochemical and

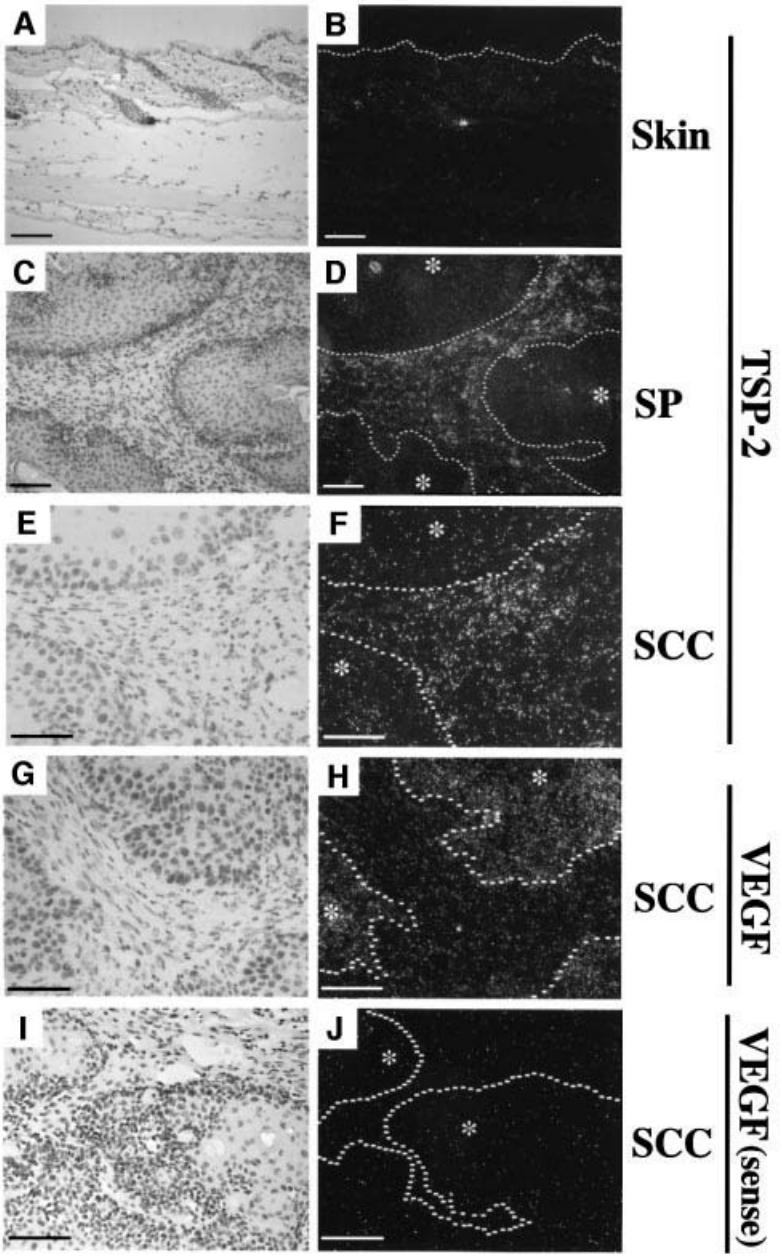

Fig. 4. Upregulation of stromal TSP-2 expression during skin carcinogenesis. *In situ* hybridization demonstrates low level TSP-2 mRNA expression in the dermis of untreated skin (**A** and **B**) and marked upregulation in the stroma of a representative small papilloma (**C** and **D**) and squamous cell carcinoma (**E** and **F**), whereas little or no TSP-2 mRNA expression was detected in tumor cells (asterisks). In contrast, VEGF mRNA was expressed predominantly by tumor cells in SCCs (**G** and **H**). Hybridization with a VEGF sense control probe demonstrates low background signal (**I** and **J**). Bright-field (A, C, E, G and I) and dark-field (B, D, E, H and J) micrographs. The dotted lines delineate the border between epithelium and mesenchymal stroma. Bar = 100 μm.

immunohistochemical stains of normal skin, papillomas and SCC, obtained from both genotypes. Whereas only rare inflammatory cells were observed in the untreated skin of both genotypes, increased numbers of CD11b/Mac-1-positive inflammatory cells, including macrophages and granulocytes, were detected in the stroma of papillomas (Figure 5M and N) and SCC. No major differences in the abundance of CD11b/Mac-1-positive cells were observed between wild-type (Figure 5M) and TSP-2-deficient (Figure 5N) mice. Similarly, chloroacetate esterase stains did not reveal any major differences in the number of mast cells in papillomas of both genotypes (Figure 5O and P). Moreover, no differences in the abundance of natural killer cells or T lymphocytes were found in the stroma of wild-type and TSP-2-deficient tumors (data not shown).

Decreased tumor cell apoptosis in TSP-2-deficient mice

Tumor sections were labeled with the TUNEL technique, and the number of apoptotic cells was determined per mm of epidermal–dermal basement membrane. Large numbers of apoptotic cells were detected in the suprabasal layers of papillomas in wild-type mice (Figure 6C), whereas apoptotic cells were less numerous in TSP-2-deficient

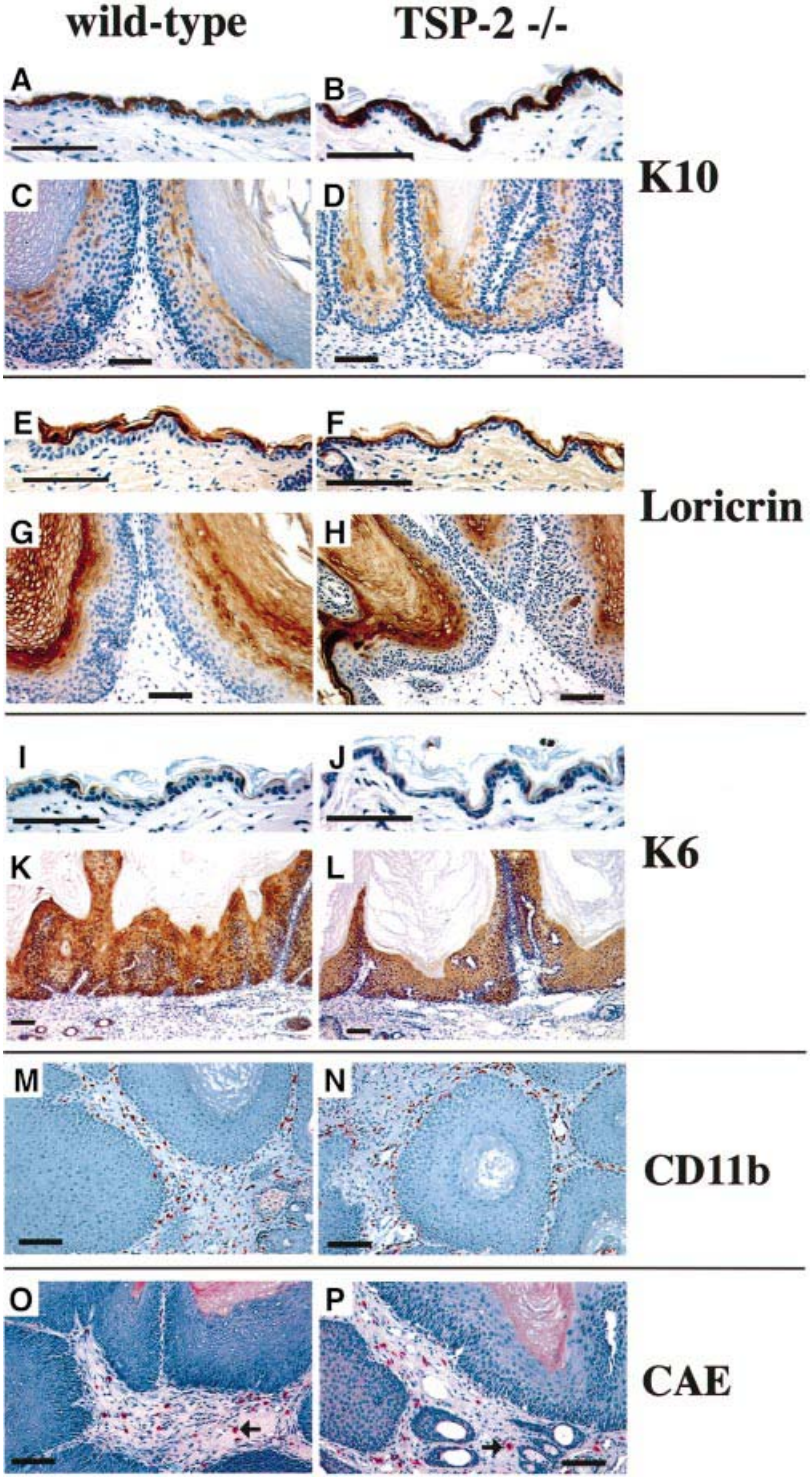

Fig. 5. Comparable epidermal differentiation in TSP-2-deficient and wild-type skin and tumors. (A–D) Comparable K10 expression was detected in suprabasal epidermis of untreated skin (**A** and **B**) and in suprabasal layers of papillomas (**C** and **D**) in both genotypes. (E–H) Identical loricrin expression in the granular layer of untreated epidermis (**E** and **F**) and in papillomas of wild-type (**G**) and TSP-2-deficient (**H**) mice. (I–L) Expression of the proliferation-associated keratin K6 was absent in untreated skin of both wild-type (**I**) and TSP-2-deficient (**J**) mice and was upregulated to a similar extent throughout the epithelial layer of wild-type (**K**) and TSP-2-deficient (**L**) papillomas. No major differences in the abundance of CD11b/Mac-1-positive cells were observed between wild-type (**M**) and TSP-2-deficient (**N**) mice. Chloroacetate esterase stains did not reveal major differences in the number of mast cells in wild-type (**O**) and TSP-2 deficient (**P**) papillomas. Arrows indicate mast cells. Bars = 100 μm.

papillomas (Figure 6D). Computer-assisted image analysis revealed a significant (>2.7-fold; P <0.001) decrease in the number of apoptotic cells in TSP-2-deficient tumors, as compared with wild-type tumors (Figure 6E).

Discussion

In this study, we demonstrate an important biological role for the endogenous angiogenesis inhibitor TSP-2 in the

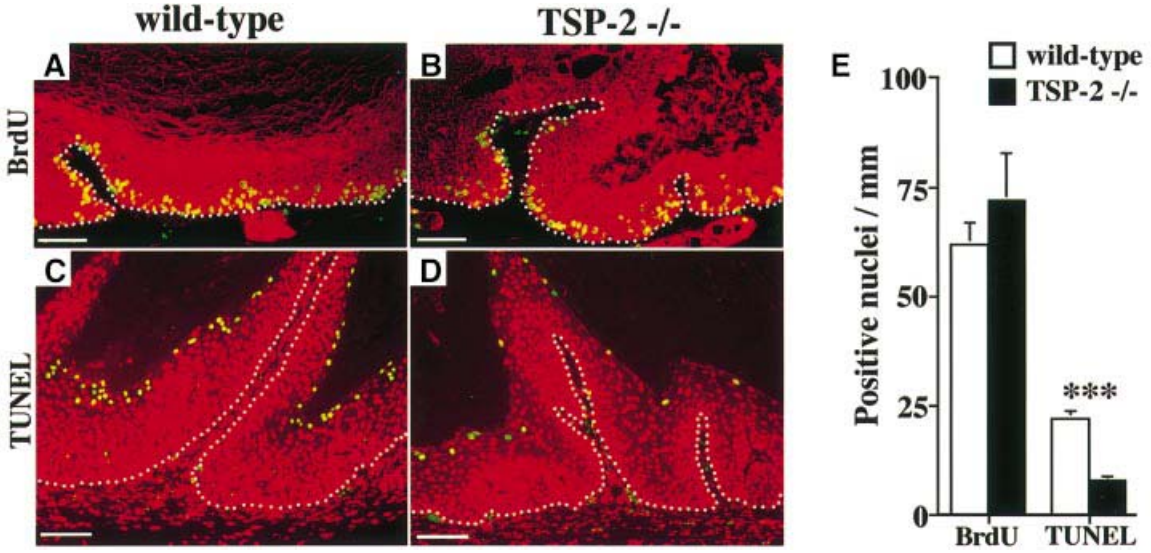

Fig. 6. Comparable cell proliferation but decreased apoptosis in TSP-2-deficient papillomas. BrdU incorporation analysis of large papillomas isolated from wild-type (**A**) and TSP-2-deficient (**B**) mice demonstrates comparable numbers of proliferating cells (yellow). Sections were counterstained for keratin K14 (red) to depict epithelial cells. The dotted lines delineate the border between epithelium and mesenchymal stroma. (C and D) TUNEL staining (green) revealed reduced numbers of apoptotic tumor cells (yellow) in TSP-2-deficient papillomas (**D**), as compared with wild-type papillomas (**C**). Propidium iodide was used as a nuclear counterstain (red). Bar = 100 μm. (**E**) Computer-assisted image analysis revealed a significant decrease in the number of apoptotic cells per mm of basement membrane ($P < 0.001$) in TSP-2-deficient papillomas, as compared with wild-type tumors. No significant differences ($P = 0.38$) were found in the number of BrdU-labeled, proliferating cells. Data are expressed as mean values ± SEM ($n = 3$). ***$P < 0.001$.

control of multistage carcinogenesis. Mice deficient in TSP-2 and wild-type controls were subjected to a standard two-step chemical carcinogenesis regimen, which has been well characterized with respect to the genetic and epigenetic changes that occur during tumor development (DiGiovanni, 1992). TSP-2-deficient mice showed significantly accelerated tumor development and a dramatic increase in the number and size of skin tumors, as compared with wild-type mice. These findings reveal that TSP-2 deficiency results in enhanced tumor promotion. However, loss of TSP-2 did not act as a classical promoter since no tumors developed in TSP-2-deficient mice treated with a single subcarcinogenic application of DMBA. Moreover, the absence of TSP-2 alone was not sufficient as an initiating event since treatment with PMA alone for 20 weeks failed to induce skin tumors. The extent of papilloma formation in wild-type 129/Sv mice was comparable to previously reported results in the 129/Sv genetic background (Moore *et al*., 1999). SCC developed earlier and were found in increased numbers in TSP-2-deficient mice, as compared with wild-type mice. However, taking the higher number of papillomas in TSP-2-deficient mice into account, the ratio of malignant conversion to SCC was comparable to that observed in wild-type mice. These findings suggest that TSP-2 deficiency did not result in major additional genetic events. Instead, the increased number of SCC in TSP-2-deficient mice most probably reflects the accelerated tumor development in early stages of skin carcinogenesis since comparable latency periods between papilloma and SCC formation were observed in both genotypes. Taken together, our findings demonstrate that loss of TSP-2 expression results in an early growth advantage of initiated/promoted epidermal cells but not in enhanced malignant conversion rates.

Angiogenesis has been shown to be a rate-limiting step in multistage carcinogenesis (Bergers *et al*., 1999). Using computer-assisted image analysis of skin and tumor sections stained for the endothelial junction molecule CD31 (Dejana *et al*., 1995), we found that angiogenesis was induced at an early stage in benign tumors of both genotypes. The early angiogenic response appears to be characteristic for the development of exophytic pre-malignant lesions (Bolontrade *et al*., 1998), whereas the angiogenic switch has been demonstrated as a late-in-progression event in an experimental model of pancreatic tumorigenesis (Hanahan *et al*., 1996). Importantly, the extent of tumor vascularization was significantly higher in TSP-2-deficient mice than in wild-type mice, with a prominent increase in the number of large angiogenic blood vessels. These results are in agreement with previous findings that TSP-2 deficiency resulted in more vascularized granulation tissue in full-thickness skin wounds (Kyriakides *et al*., 1999b) and in enhanced vascularity of cutaneous foreign body reactions (Kyriakides *et al*., 1999a). Our results demonstrate that the enhanced vascularization was not due to effects of TSP-2 deficiency on VEGF expression, since no major differences of VEGF protein levels were found between lysates obtained from untreated skin and skin tumors of wild-type and TSP-2-deficient mice. Similarly, we have found previously that overexpression of TSP-2 in SCC xenotransplants did not affect tumor cell VEGF expression (Streit *et al*., 1999a). The strong correlation between the extent of angiogenesis and the development of papillomas in our study therefore indicates that the control of angiogenesis by TSP-2 functions as a rate-limiting step in the genesis and promotion of pre-malignant skin lesions.

The exact molecular mechanisms by which TSP-2 inhibits tumor angiogenesis remain to be established. It

has been suggested that the anti-angiogenic effects of the related molecule, TSP-1, are mediated partly through interaction of the CSVTCG sequence, contained within the properdin-like type I repeats, with the CD36 receptor on endothelial cells (Tolsma *et al*., 1993; Volpert *et al*., 1995; Dawson *et al*., 1997; Jimenez *et al*., 2000). Although the importance of this interaction for the biological activity of TSP-2 remains to be determined, it is of interest that TSP-2 also contains two CSVTCG domains within the type I repeats. In addition, analysis of proteolytic fragments of the TSP-1 molecule revealed anti-angiogenic activity of peptides derived from the procollagen-like domain, in particular the peptide NGVQYRN (Tolsma *et al*., 1993). However, this sequence is absent from the procollagen-like domain of TSP-2, which shows little structural similarity to TSP-1. We are currently investigating whether other active sites reside within this domain of the TSP-2 molecule. Whereas TSP-1 has been shown to activate latent transforming growth factor-β (TGF-β) via the unique sequence KRFK found between the first and the second type 1 repeats of TSP-1 (amino acids 412–415) (Schultz-Cherry *et al*., 1995), the KRFK sequence is absent from the TSP-2 molecule, and recombinant TSP-2 does not activate TGF-β (Schultz-Cherry *et al*., 1995). Moreover, preliminary data show that active TGF-β levels are comparable in tissues of TSP-2-deficient and wild-type mice (P.Bornstein, unpublished results), indicating that lack of TSP-2 enhanced angiogenesis and tumor formation independently from any effects on TGF-β activation. It has been suggested previously that acquisition of an angiogenic phenotype may be associated with increased activation of matrix metalloproteinases (MMPs) (Bergers *et al*., 2000; Fang *et al*., 2000). The recent findings that TSP-2 interacts directly with MMP-2 and that TSP-2-deficient fibroblasts show enhanced MMP-2 activity *in vitro* (Bein and Simons, 2000; Yang *et al*., 2000) suggest modulation of MMP activity as an additional mechanism of action by which TSP-2 may inhibit tumor angiogenesis.

The switch to the angiogenic phenotype has been thought to be controlled by a change in the balance of positive and negative regulators of endothelial cell growth (Hanahan and Folkman, 1996). Consistent with this hypothesis, we found upregulation of the major tumor angiogenesis factor VEGF during the progressive stages of tumor development, in accordance with previously reported results (Larcher *et al*., 1996, 1998). We also found downregulation of TSP-1, an inhibitor of tumor growth and angiogenesis (Volpert *et al*., 1998; Streit *et al*., 1999b), in agreement with the previously reported inverse correlation of TSP-1 expression and malignant tumor progression (Zabrenetzky *et al*., 1994; Campbell *et al*., 1998). Surprisingly, however, we detected a strong induction of TSP-2 expression during early papilloma formation, which remained at high levels throughout further tumor progression. *In situ* hybridization analyses revealed that the cellular expression pattern of TSP-2 was inversely related to that of VEGF, with upregulation of TSP-2 mRNA expression exclusively in the tumor stroma. In contrast, VEGF was detected predominantly in epithelial tumor cells. These findings provide an explanation for the increased angiogenesis during early tumorigenesis in TSP-2-deficient mice, at a time when TSP-2 expression was highly upregulated in wild-type mice. These results also indicate that upregulation of an endogenous inhibitor of angiogenesis may counteract the pro-angiogenic effects of VEGF during skin carcinogenesis, and they identify induction of stromal TSP-2 expression as a novel host defense mechanism against tumor development and progression. The molecular mechanisms leading to stromal upregulation of TSP-2 expression during tumorigenesis remain to be investigated. It is conceivable that factors produced by tumor cells or by invading inflammatory cells might induce TSP-2 expression in the tumor stroma. We currently employ cell-based reporter gene assays to identify potential factors with modulatory activity on the TSP-2 promoter in stromal cells. Whereas it remains to be established whether the inhibitory effects of stromal TSP-2 induction on tumor development apply to the majority of malignancies or only to select types of carcinogenesis, the relevance of these findings to human disease is supported by the recently reported inverse correlation between tumor expression of TSP-2 and increased vascularity and metastasis in VEGF-expressing colon (Tokunaga *et al*., 1999) and lung (Oshika *et al*., 1998) cancers.

Mouse skin carcinogenesis is associated with major alterations of epidermal differentiation and proliferation (Toftgard *et al*., 1985). When we compared the expression of keratin K10, an early marker for terminal keratinocyte differentiation (Roop *et al*., 1988; Huitfeldt *et al*., 1991), and of the late terminal differentiation marker loricrin (Mehrel *et al*., 1990), we found that the pattern of epidermal and tumor differentiation in TSP-2-deficient mice was indistinguishable from that in wild-type controls. These results demonstrate that loss of TSP-2 did not induce additional alterations in terminal differentiation that might have contributed to enhanced epithelial tumorigenesis (Greenhalgh *et al*., 1993). Moreover, the expression of the proliferation-associated keratin K6 (Weiss *et al*., 1984) was comparable in skin tumors of wild-type and TSP-2-deficient mice. Combined with the identical tumor proliferation rates, these findings indicate that loss of TSP-2 did not modulate the hyperproliferative epidermal response. In contrast, TSP-2 deficiency resulted in a significant reduction in tumor cell apoptosis, as compared with wild-type mice. Our findings are in accordance with the previously reported induction of tumor cell apoptosis by therapeutic inhibition of angiogenesis (Holmgren *et al*., 1995; Bergers *et al*., 1999; Streit *et al*., 1999a) and support the concept that tumor angiogenesis may act as a paracrine regulator of tumor apoptosis (Hanahan and Folkman, 1996). Previously, it has been shown that inflammatory mast cells can upregulate angiogenesis during epithelial carcinogenesis (Coussens *et al*., 1999) and that the inflammatory response to tumor promotion plays an important role in chronic skin carcinogenesis (Moore *et al*., 1999). We did not detect any major differences in the abundance of mast cells and other inflammatory cells in wild-type versus TSP-2-deficient papillomas and SCC, suggesting that the enhanced tumorigenesis and angiogenesis in TSP-2-deficient mice was not mediated by modulation of the inflammatory response. In summary, our results identify an important role for a naturally occurring angiogenesis inhibitor in the control of multistep skin carcinogenesis

and suggest that TSP-2-mediated inhibition of angiogenesis contributes significantly to the natural stromal defense against tumor development and progression.

Materials and methods

Chemical skin carcinogenesis protocol

For tumor initiation, 25 μg of DMBA (Sigma, St Louis, MO), dissolved in 200 μl of acetone, were applied topically to the shaved back skin of 8-week-old female TSP-2-deficient (n = 25) and wild-type (n = 24) 129/SvJ mice, followed by weekly topical application of 5 μg of the tumor promoter PMA (Sigma) over 20 weeks. In addition, five wild-type and five TSP-2-deficient mice were treated either with PMA alone or with a single application of DMBA. The construction of the targeting vector and the generation of TSP-2-deficient mice on a homogenous 129/SvJ background have been described in detail elsewhere (Kyriakides *et al*., 1998a). Raised lesions of a minimum diameter of 1 mm that had been present for at least 1 week were recorded as tumors. Mice were sacrificed after 35 weeks, or earlier if tumors reached 10 mm in diameter or became ulcerated. The rate of malignant conversion was calculated for each group of mice as the ratio of total carcinomas per total papillomas, expressed as a percentage. The two-sided unpaired Student's t-test was used to analyze differences between the two genotypes in the number of tumors per mouse. All animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

RNase protection assays and western blot analyses

A single-stranded 350 bp antisense mTSP-2 (Streit *et al*., 1999a), a 140 bp antisense mTSP-1 and a 420 bp antisense VEGF (Detmar *et al*., 1998) riboprobe were prepared by *in vitro* transcription, using the Riboprobe II kit (Promega, Madison, WI) in the presence of [α-^{32}P]UTP. A β-actin (Promega) riboprobe was used as a loading control. Total RNA was isolated from adult mouse skin and from tumors as previously described (Detmar *et al*., 1998), using the RNeasy kit (Qiagen, Chatsworth, CA). Samples of RNA (10 μg each) were hybridized with ^{32}P-labeled riboprobes and digested with RNase using the RPA II kit (Ambion, Austin, TX) according to the manufacturer's protocol. As controls, two aliquots of 10 μg of yeast RNA were hybridized with the same amount of labeled TSP-2 riboprobe, with or without addition of RNase. The hybridization products that were protected from nuclease digestion were separated on a 6% polyacrylamide–8 M urea gel. RNA Century Marker Template (Ambion) was used to generate transcripts of known size, which served as molecular weight markers. Levels of TSP-2 mRNA expression were quantitated by densitometry and were normalized to the expression levels of the housekeeping gene β-actin. For western blot analyses, skin and tumor samples were snap-frozen in liquid nitrogen and were homogenized in lysis buffer (2% SDS, 50 mM Tris pH 7.4, 20 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin and 50 μg/ml aprotinin). Samples (30 μg of protein each) were analyzed by denaturating SDS–PAGE and were immunoblotted with an antibody against mouse VEGF (R&D Systems Inc., Minneapolis, MN). Immunoreactive proteins were visualized using a chemiluminescence detection system (ECL; Amersham, Arlington Heights, IL).

Histology, immunohistochemistry and in situ hybridization

One half of each tumor or skin sample was fixed for 1 h in 10% formalin and was processed and embedded in paraffin. The other half was embedded in OCT compound (Sakura Finetek, Torrance, CA) and was snap-frozen in liquid nitrogen. Chloroacetate esterase histochemistry was performed on paraffin sections as described previously to reveal the presence of the chymotrypsin-like serine esterase activity of mast cells (Leder, 1979; Caughey *et al*., 1988). Immunohistochemical staining was performed on 6 μm paraffin sections or cryostat sections as previously described (Streit *et al*., 1999a), using rabbit polyclonal antibodies against mouse keratin K6, K10 and loricrin (Babco, Richmond, CA), or monoclonal rat antibodies against mouse CD11b/Mac-1, NK1.1, Thy-1.2 (CD90.2) and CD31 (Pharmingen, San Diego, CA). *In situ* hybridization was performed on 6 μm paraffin sections as described (Detmar *et al*., 1998). An RNA probe to mouse TSP-2 was transcribed from a pBluescript II KS+ vector containing a 350 bp fragment of the coding region that is specific for mouse TSP-2 but not TSP-1. Antisense and sense single-stranded ^{35}S-labeled RNA probes for VEGF were prepared from a 393 bp PCR rat VEGF cDNA fragment, cloned into pGEM-3Zf(+). The VEGF riboprobe used recognizes all isoforms of mouse VEGF mRNA (Streit *et al*., 2000).

Computer-assisted, morphometric analysis of blood vessels

Representative CD31-stained frozen sections, obtained from biopsies of normal untreated skin (n = 3), small papillomas (1–3 mm in diameter; n = 7), large papillomas (>5 mm in diameter; n = 6) and SCC (n = 2) were analyzed for each genotype, using a Nikon E-600 microscope. Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), and morphometric analyses were performed using the IP-LAB software (Scanalytics, Billerica, MA) as described (Streit *et al*., 1999a). Three different 60× fields in each section were examined and the number of dermal vessels per mm^2, the average vessel size and the relative area occupied by blood vessels were determined as described (Streit *et al*., 1999a). Statistical analysis was performed using the two-sided unpaired Student's t-test.

Cell proliferation and apoptosis assays

For labeling of proliferating cells, mice received intraperitoneal injections of 40 mM BrdU (Sigma) in 200 μl of 0.9% NaCl at 2 h before sacrifice. Large papillomas of similar size were evaluated in TSP-2-deficient (n = 3) and wild-type (n = 3) mice. Cryostat sections (6 μm) were incubated with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to BrdU (Pharmingen) and with a rabbit antibody to mouse keratin K14, followed by incubation with a secondary antibody labeled with Texas red (Jackson Immuno Research Laboratories, West Grove, PA). Proliferating tumor cells were counted in an area overlying a total length of 14 mm of basement membrane in TSP-2-deficient mice and of 18 mm in wild-type mice. Apoptotic cells were identified by TUNEL staining using the fluorescein-FragEL DNA fragmentation kit (Oncogene, Cambridge, MA) according to the manufacturer's instructions. Nuclei were stained with propidium iodide in phosphate-buffered saline (2 μg/ml). Labeled tumor cells were counted in an area overlying a total length of 52 mm of basement membrane in TSP-2-deficient mice and of 54 mm in wild-type mice. Results are expressed as the mean ± SEM of BrdU- or TUNEL-positive nuclei per mm of basement membrane. The two-sided unpaired Student's t-test was used to analyze differences in proliferation and apoptosis rates.

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