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**Research Article**

Thrombospondin (TSP) is a trimeric glycoprotein which is synthesized and incorporated into the extracellular matrix by a wide variety of cells. TSP is involved in a number of cellular processes which govern tumor cell behavior including mitogenesis, attachment, migration, and differentiation. To directly assess the role of TSP in tumor cell growth and spread, a human squamous carcinoma cell line, with high TSP production and an invasive phenotype, was transfected with a TSP cDNA antisense expression vector. Five unique transfected clones were obtained with reduced TSP production. Expression of the transfected antisense sequence in these clones was verified by a ribonuclease protection assay. These clones demonstrated reduced growth rates in vitro when compared with a vector transfected control. After subcutaneous inoculation into athymic mice, the antisense clones formed either no tumors or tumors that were slow growing and highly differentiated. This contrasted with the vector-transfected clone which produced poorly differentiated, rapidly growing, invasive tumors. Our results argue in favor of a direct role for TSP in determining the malignant phenotype of certain human tumors.



# Antisense-mediated Reduction in Thrombospondin Reverses the Malignant Phenotype of a Human Squamous Carcinoma

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## Abstract

**Thrombospondin (TSP) is a trimeric glycoprotein which is synthesized and incorporated into the extracellular matrix by a wide variety of cells. TSP is involved in a number of cellular processes which govern tumor cell behavior including mitogenesis, attachment, migration, and differentiation. To directly assess the role of TSP in tumor cell growth and spread, a human squamous carcinoma cell line, with high TSP production and an invasive phenotype, was transfected with a TSP cDNA antisense expression vector. Five unique transfected clones were obtained with reduced TSP production. Expression of the transfected antisense sequence in these clones was verified by a ribonuclease protection assay. These clones demonstrated reduced growth rates in vitro when compared with a vector transfected control. After subcutaneous inoculation into athymic mice, the antisense clones formed either no tumors or tumors that were slow growing and highly differentiated. This contrasted with the vector-transfected clone which produced poorly differentiated, rapidly growing, invasive tumors. Our results argue in favor of a direct role for TSP in determining the malignant phenotype of certain human tumors. (*J. Clin. Invest.* 1991. 87:1883–1888.) Key words: thrombospondin • extracellular matrix • antisense RNA • tumor invasion**

## Introduction

The extracellular matrix (ECM)[1] provides a semisolid support upon which cells attach and adhere. Thrombospondin (TSP), a homotrimeric protein of $M_r$ 420 kD, is synthesized by a wide variety of normal and transformed cells and incorporated into the ECM (1–4). Functionally, TSP acts as a growth supportive matrix component. TSP has been shown to facilitate cell attachment and serve as an adhesion factor for many cell types (5–9). Several converging lines of evidence point to a role for TSP in controlling cellular proliferation. First, TSP mRNA is induced as an immediate early response to growth factor stimulation (10). Second, exogenous TSP potentiates the mitogenic response of epidermal growth factor (EGF) on smooth muscle cells (SMC), and this effect is inhibited by agents which prevent the incorporation of TSP into the matrix (11). Third, TSP is found in areas of cellular proliferation in developing mouse embryos (12). Fourth, TSP is made in significant quantities by proliferating undifferentiated human epidermal keratinocytes (13), and its production falls in cells induced to differentiate by a variety of means including exposure to high external $Ca^{2+}$ concentrations, exposure to interferon-$\gamma$ or depletion of growth supplements from the culture medium (7, 14).

In recent studies we have found an inverse correlation between TSP production and degree of in vitro differentiation among several squamous carcinoma cell lines (6). Because the degree of epithelial tumor cell differentiation is also inversely correlated with malignant behavior, we investigated the possibility that the malignant phenotype of squamous carcinoma cells could be altered by stably lowering TSP levels using antisense RNA. The antisense RNA technique leads to a reduction in the expression of a target gene at the protein level through the formation of a specific double-stranded RNA hybrid which interferes with normal mRNA transport and translation (15). This technique has been used successfully to reduce c-*fos* expression and inhibit 3T3 cell proliferation (16), to determine the role of the c-*myc* protooncogene in cell cycle progression and differentiation (17, 18), and more recently, to investigate the role of the c-*raf*-1 protooncogene product in conferring radiation sensitivity to a line of human squamous carcinoma cells (19). In the present study we have constitutively expressed TSP antisense transcripts in a human squamous carcinoma cell line which normally produces high levels of TSP and forms aggressive tumors in nude mice. The antisense clones were analyzed for integration and expression of transfected sequences, TSP production, and in vitro and in vivo growth characteristics. Clones with a reduction in TSP grew at a slower rate in vitro and were less tumorigenic in athymic mice, when compared with parental or vector control lines.

## Methods

*Vector constructs.* The TSP antisense expression plasmid was constructed by isolating the 4.5 kb Xho I fragment containing the entire TSP cDNA from the F2 plasmid (20) and ligating it into the Xho I site of the MC CMV expression vector (Fig. 1, gift of M. Clarke, University of Michigan). This construction placed the cDNA downstream from and under the transcriptional control of the cytomegalovirus immediate early response promoter and HTLV-1 translational enhancer (21). The antisense orientation of this new construction was confirmed by restriction endonuclease digestion. The MC CMV expression vector contains the gene-encoding neomycin resistance which enables selection for the presence of this plasmid in mammalian cells using the antibiotic G418 (Geneticin; Sigma Chemical Co., St. Louis, MO).

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1. *Abbreviations used in this paper:* 11B, UM-SCC-11B squamous carcinoma cells; ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinase; TSP, thrombospondin.

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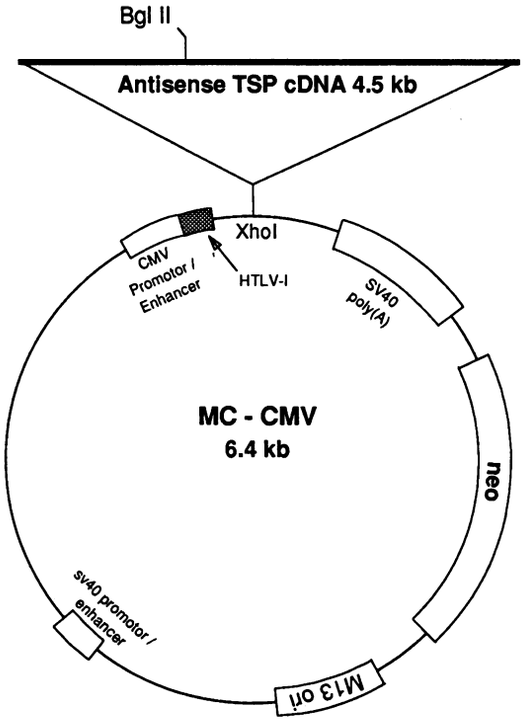


*Figure 1.* Schematic representation of the MC CMV expression plasmid (gift of M. Clarke, University of Michigan) containing the entire TSP cDNA in a 4.5-kb Xho I fragment. This construction places the TSP cDNA in an antisense orientation downstream of the cytomegalovirus immediate early response promoter and the HTLV-1 translational enhancer (21). Downstream of the TSP sequence, SV40 splicing and polyadenylation signals are found for message processing. The gene coding for neomycin resistance is also contained in this vector. Before electroporation the antisense expression plasmid was linearized at a unique Sal I site within the vector backbone.

*Cell culture.* The human squamous carcinoma cell line UM-SCC-11B (11B) previously described (5, 6) with high TSP production was used for these experiments. The cells were grown in DME supplemented with nonessential amino acids, 2 mM glutamine, 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C with 5% $CO_2$ and subcultured by trypsinization.

*Transfections.* $2 \times 10^7$ 11B cells derived as a subclone of the parental line were washed and suspended in 0.5 ml PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$). 10 μg of linearized antisense expression plasmid or vector-only DNA was added to the cells which were then electroporated using a Gene Pulser (Bio-Rad Laboratories, Inc., Richmond CA) set at 1.1 kV and 25 μF. After electroporation the cells were suspended in 20 ml of supplemented DME. 48 h after electroporation the cells were subcultured at a 1:10 dilution, and then 24 h later selection was begun in G418 at 400 μg/ml (Geneticin; Sigma Chemical Co.). G418 resistant clones were individually transferred using filter paper soaked in trypsin and subcultured separately in six-well dishes.

*Quantitative immunoprecipitations.* Individual transfected clones were grown to 80% confluency in 10-cm dishes and labeled for 6 h with 2 ml cysteine and methionine-free MEM supplemented with 1% BSA and 100 μCi/ml of $^{35}S$ methionine-cysteine (*trans* label; ICN Radiochemicals, Irvine, CA). The medium was collected and an equal number of TCA precipitable counts were incubated with rabbit polyclonal anti-TSP antibody overnight at 4°C in the presence of protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin, and buffer containing 15 mM NaCl, 10 mM $NaH_2PO_4$, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 20 mM EDTA. The specificity of the anti-TSP antibody has been described previously (12). Antigen-antibody complexes were precipitated with protein A–Sepharose beads. The pellets were washed and boiled in a sample buffer containing 8 M urea, 50 mM Tris HCl, and 1% SDS, and the eluted proteins resolved on a 7.5% SDS polyacrylamide gel under reducing conditions. After electrophoresis, gels were fixed in methanol and acetic acid, treated with Entensify (New England Nuclear, Boston, MA), dried, and exposed to Kodak XAR film at −80°C. TSP production was quantitated using the Betascope 603 Blot Analyzer (Betagen, Waltham, MA).

*Southern analysis.* 12 μg of DNA from each clone and the vector transfected control line were cut to completion using Bgl II under conditions suggested by the manufacturer (Boehringer-Mannheim Biochemicals, Mannheim, Germany). Digested DNA was precipitated with linear acrylamide, 0.1 vol 3 M sodium acetate, pH 5.2, and 2 vol of 95% ethanol, electrophoresed on a 0.6% TBE gel, and transferred to a Nytran filter (Schleicher & Schuell, Keene, NH). The filter was hybridized with a PCR generated $^{32}P$-labeled fragment of the TSP cDNA at 42°C. The filter was washed three times at room temperature in 2× SCC, 0.1% SDS, and once at 65°C in 2× SSC, 0.2% SDS. After blotting dry, the filter was exposed to Kodak XAR film at −80°C with an intensifying screen.

*Ribonuclease protection assay.* Antisense transcript expression was verified using the SP6 riboprobe protocol as previously described (22). A 328 bp Bam H1–Ava 1 fragment from the TSP cDNA was cloned in the sense orientation into the SP64 vector (Promega Biotec, Madison, WI) (Fig. 2 *b*). The resultant plasmid was linearized at a unique Bgl I site. A 1.8-kb sense transcript was synthesized using SP6 RNA Polymerase (Bethesda Research Laboratories, Gaithersburg, MD) and $^{32}P$-GTP (400 μCi/mmol; Amersham Corp., Arlington Heights, IL). This transcript was allowed to hybridize with equal amounts (10 μg) of RNA from each antisense clone and the vector control line at 51°C for 16 h in 10 μl of 80% formamaide, 0.4 M NaCl, 40 mM Pipes, pH 6.4, and 1 mM EDTA (23). Single-stranded overhangs were removed using

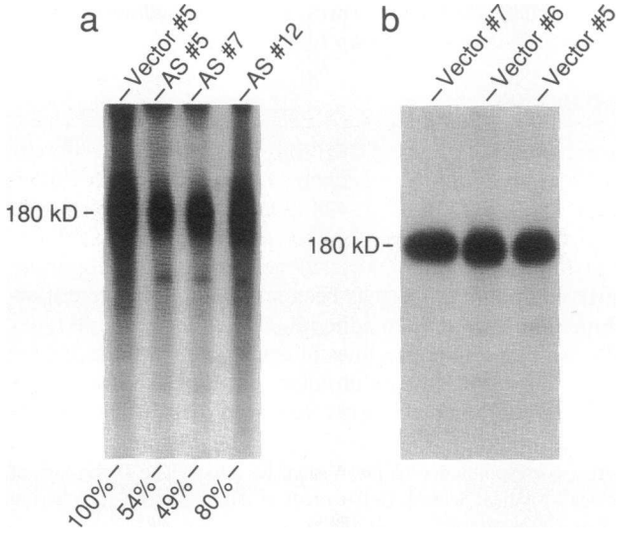


*Figure 2.* Quantitative immunoprecipitation of the media from the antisense clones and the vector-transfected control with rabbit polyclonal TSP antibody (12). Cells were metabolically labeled with $^{35}S$ methionine and $^{35}S$ cysteine, the media fraction was collected and an equivalent number of counts immunoprecipitated. (*a*) The antisense clones showed a marked decrease in the production of TSP when compared with the vector transfected control No. 5: antisense clone Nos. 5 and 7, 54 and 49%, and No. 12, 80%. (*b*) Three unique vector-transfected controls produce approximately equivalent amounts of TSP with < 5% variation between unique clones. As noted, TSP migrates at 180 kD.

RNase A (40 mg/ml) and RNase T1 (2 μg/ml; Sigma Chemical Co.). The entire sample was resolved on a 6% polyacrylamide gel. After electrophoresis the gel was dried and exposed to Kodak XAR film at −80°C with an intensifying screen.

*In vitro and in vivo growth characteristics.* In vitro growth rates were determined by plating cells at $7.5 \times 10^4$ cells/dish in 35-mm culture dishes. Triplicate dishes of each clone were harvested and counted 1–5 d after plating. In vivo growth was assessed by injecting Swiss athymic mice with $1.0 \times 10^7$ cells in the hind limb. Animals were examined every other day for signs of tumor growth and tumor size was assessed with a caliper. Animals were sacrificed at 6 wk or when tumors reached a geometric mean diameter of 15–20 mm and sections were taken of the tumor mass and surrounding tissue. After hemotoxylin and eosin staining, sections were examined blindly for degree of invasiveness and differentiation.

## Results

After transfection of the 11B squamous carcinoma cell line 12 G418 resistant clones were obtained. To ascertain the uniqueness of these clones, we examined the integration site of the transfected plasmid with Southern analysis of genomic DNA cut with Bgl II, which has only a single site in the transfected constructs. Thus, the size of the hybridizing fragment is dependent on the distance between the site of random integration of the transfected DNA and the nearest Bgl II recognition sequence in the flanking genomic DNA. A Southern blot containing the Bgl II–digested DNA was probed with a $^{32}$P-labeled fragment of the TSP cDNA. This fragment contains a portion of the cDNA which is 3′ to the Bgl II recognition site found in the transfected sequence. The group of 12 transfectants contained three uniquely transfected clones. As shown in Fig. 3 *a*, the three clones uniquely integrated the TSP antisense expression plasmid: antisense clone No. 5 has a unique 2.75-kb band, antisense clone No. 7 has a 9.0-kb band, and antisense clone No. 12 has an 8.5-kb band. Besides these unique bands, a 13.5- and a 3.2-kb band from the endogenous TSP gene are detected in each clone and the vector transfected control. The faint 8.0-kb band detected in antisense No. 7 is the result of incomplete digestion.

We next examined each unique clone for the expression of antisense TSP transcripts. An SP6-generated uniformly labeled RNA sense probe was used for this purpose. As seen in Fig. 3 *b*, antisense transcripts were identified in all three clones but were absent in the vector-transfected control line.

Immunoprecipitation with a TSP polyclonal antibody was performed to determine TSP production in the transfected clones (12). The media fraction from cultures of the antisense clones and one unique vector control line were immunoprecipitated using equivalent numbers of TCA-precipitable counts. The supernatants from the initial precipitations were reimmunoprecipitated to ensure quantitative removal of all TSP. Compared with each vector control and the nontransfected parental lines, each of the antisense clones produced less TSP. The level of TSP produced by antisense clone No. 7 was 49% of the amount produced by the vector control No. 5. Antisense clone No. 5 produced 54% and antisense clone No. 12 80% relative to the vector control No. 5 (Fig. 2 *a*). To assess the degree to which TSP production is affected by clonal variation, we analyzed the amount of TSP production in three unique vector control transfected lines by metabolic labeling and subsequent immunoprecipitation. The result of this analysis indicates there is minimal variability (< 5% by scanning densitometry) in TSP production among the transfected clones (Fig. 2 *b*).

In vitro growth characteristics are presented in Fig. 4. All antisense clones showed reduced growth rates when compared with the vector-transfected control. To determine tumorigenicity, we inoculated athymic mice with antisense clones. To strengthen any interpretation resulting from in vivo data, we generated three additional antisense lines in order to have a sufficient number of independent clones assayed for tumorige-

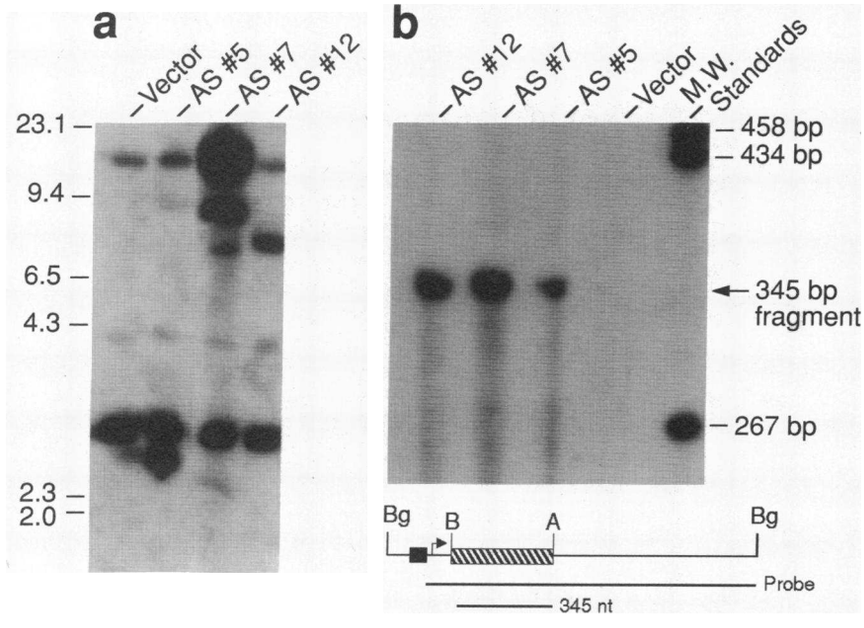


*Figure 3.* (*a*) Southern blot showing unique integration of TSP antisense cDNA. 12 μg of genomic DNA from each clone was digested with Bgl II and resolved on a 0.6% agarose gel and transferred onto nylon. The blot was probed with a $^{32}$P-labeled fragment of the TSP cDNA. All three antisense clones have a unique band in addition to the endogenous TSP 13.5- and 3.2-kb bands: antisense clone No. 5, a 2.75-kb band; antisense clone No. 7, a 9.0-kb band; antisense clone No. 12, an 8.5-kb band. (*b*) Antisense transcript expression was demonstrated using the SP6 Riboprobe protocol (21). As shown in the schematic, a Bam HI–Ava I fragment of the TSP cDNA was cloned in the sense orientation into the SP64 vector (Promega Biotec). A 1.8-kb sense probe was synthesized and hybridized to 10 μg of RNA from the antisense clones and vector control line. Single-stranded overhangs were removed using RNase A and RNase T1. As is seen, all three antisense clones have an antisense transcript which was absent in the vector-transfected control. The protected TSP fragment is 328 nt but migrates at 345 nt due to adventitious homology between flanking regions in the SP64 vector and TSP cDNA.

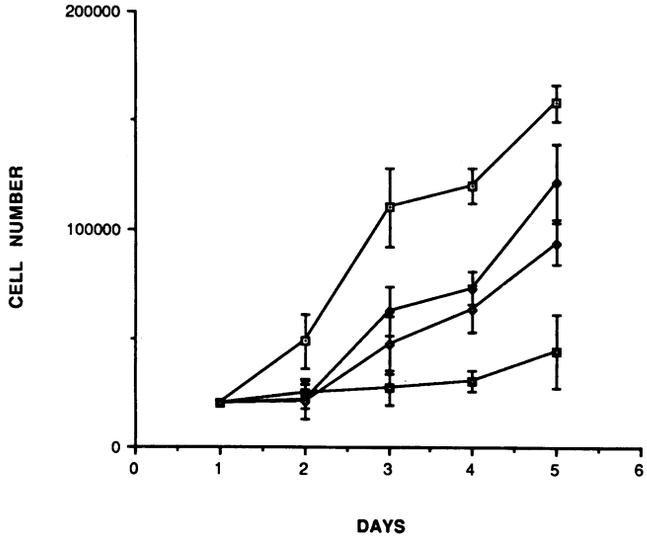


*Figure 4.* In vitro growth curves were generated by plating cells at 7.5 $\times$ $10^4$ cells/dish in 35-mm culture dishes. Triplicate cultures were harvested for counting 1–5 d after plating. Standard deviations are indicated. Antisense clone Nos. 5 (♦), 7 (■), and 12 (●) grew slower than the vector-transfected control line (□).

nicity. Characteristics of all clones are summarized in Table I. Similar molecular and biochemical analyses on the additional clones were carried out to verify uniqueness and production of antisense transcript (data not shown). Clones 14 and 10 had a 95% reduction in TSP, whereas clone 8 had a 60% reduction. The vector-transfected control line produced tumors in five out of five animals after a latency of 1–2 wk. This was similar to the parental line which produced tumors in five out of five animals in 1–2 wk. Mice injected with antisense clone Nos. 7, 8, 10, and 14 failed to produce tumors through the duration of the study (45 d). Two out of five animals injected with antisense clone No. 5 failed to produce tumors, two animals produced tumors only after a 3-wk latency, while one animal injected with this clone produced a tumor after 10 d. It is noted that none of the

*Table I. Tumorigenic Phenotype of Antisense Clones*

| Clone No. | TSP production | AS transcript | Tumor formation | Latency |
|---|---|---|---|---|
| | % | | | *wk* |
| 11B parental | 100 | – | 5/5 | 1–2 |
| Vector control | 100 | – | 5/5 | 1–2 |
| AS No. 5 | 54 | + | 3/5 | 3 |
| AS No. 7 | 49 | + | 0/5 | * |
| AS No. 8 | 40 | + | 0/6 | * |
| AS No. 10 | 5 | + | 0/6 | * |
| AS No. 12 | 80 | + | 3/5 | 2–3 |
| As No. 14 | 5 | + | 0/6 | * |

* >45 d. Tumorigenic phenotype of 11B parental, vector control, and antisense clones. Antisense clone Nos. 7, 8, 10, and 14 failed to form tumors after a latency of 45 d. Antisense clone Nos. 5 and 12 formed tumors in three out of five animals with a longer latency than the parental or vector control.

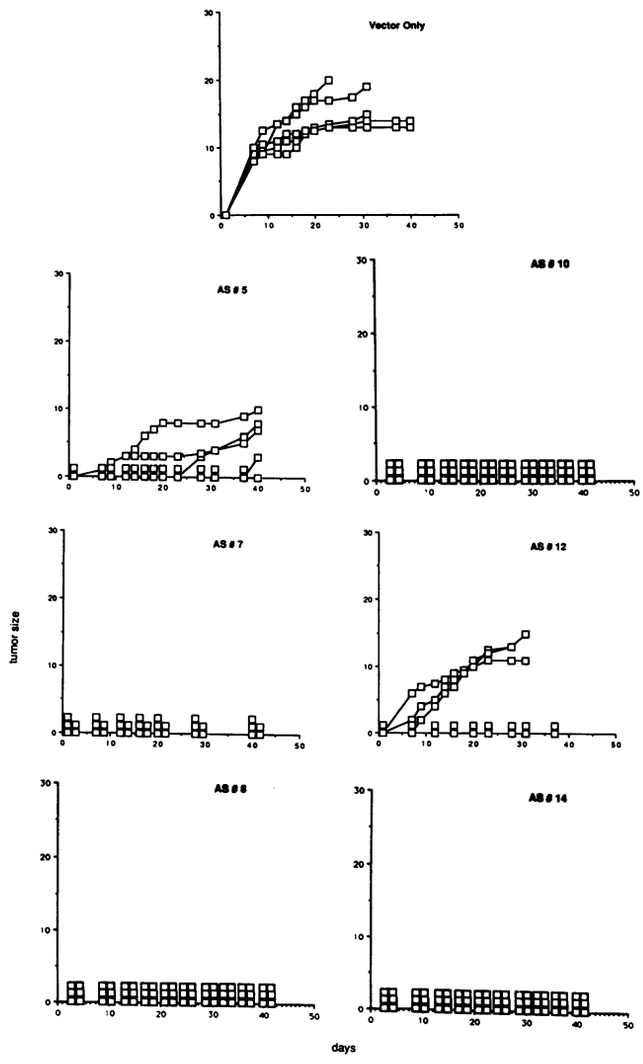


*Figure 5.* In vivo growth of antisense clones and the vector-transfected control was assessed by injecting Swiss athymic mice with 1.0 $\times$ $10^7$ cells in the hind limb. Animals were examined every other day for signs of tumor growth and the size was assessed by a caliper. The vector-transfected controls developed tumors in five out of five animals within 1 wk of inoculation. No tumors developed in antisense clone Nos. 7, 8, 10, or 14, whereas three animals developed tumors in antisense clones 5 and 12 after a longer latency.

tumors in this group reached a size equivalent to even the smallest tumors of the vector control group. Two out of the five animals injected with antisense clone No. 12 failed to produce tumors, whereas three animals injected with this clone produced tumors with a latency greater than the vector control animals. (Fig. 5)

Grossly the tumors from the vector controls were hemorrhagic without a clear tumor/normal tissue border (Fig. 6 *a*). Histologically, they were poorly differentiated with many mitotic figures and direct extension into the surrounding tissue (Fig. 6 *b*). This was in direct contrast to the tumors arising from the antisense clones which grossly were well demarcated from normal tissue, lacked a hemorrhagic appearance (Fig. 6 *c*), and histologically were more differentiated, showing development of keratin whorls and pseudoencapsulation (Fig. 6 *d*).

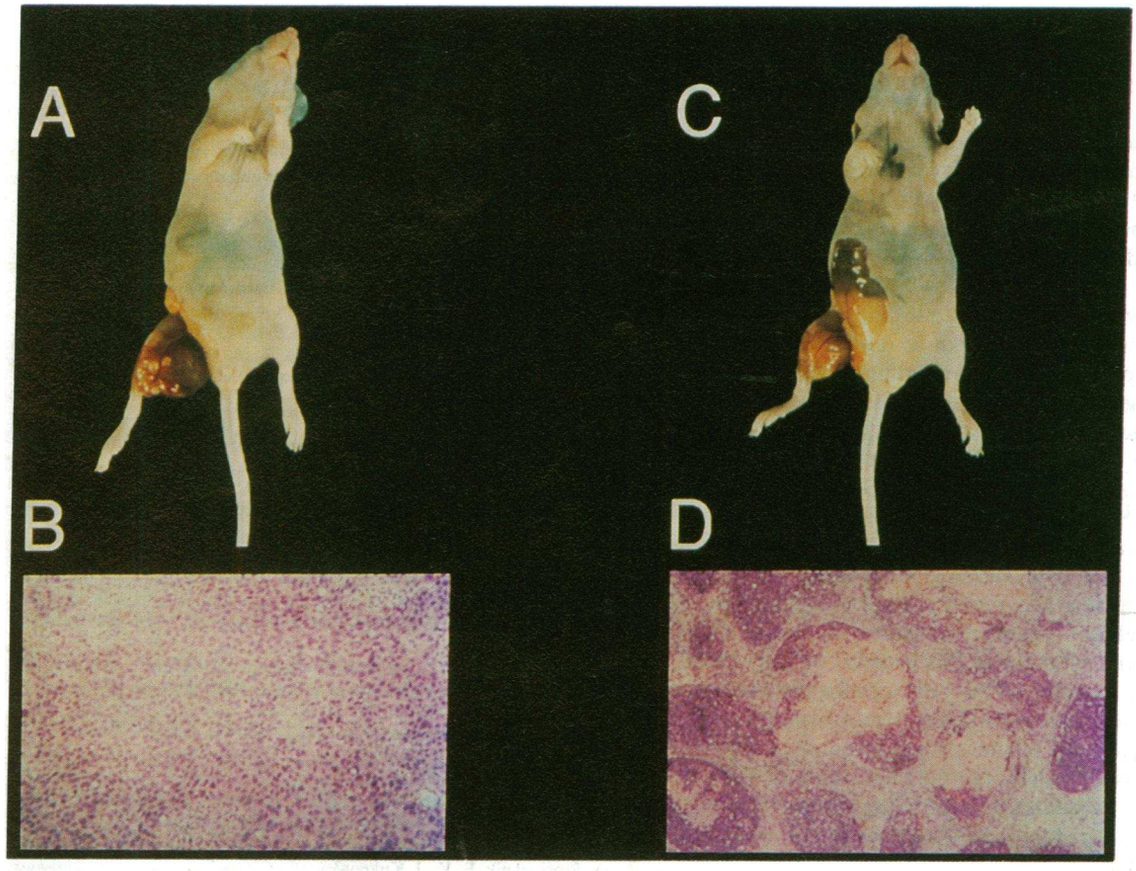


*Figure 6.* (*a–d*) Gross dissection of tumors from a vector-transfected control (*a*) and antisense clone No. 12 (*c*). The vector tumors were larger, hemorrhagic, and lacked a clear tumor/normal tissue boundary. In contrast, tumors from the antisense clones were smaller, nonhemorrhagic, and had a clear tumor border separate from normal surrounding muscle. Light microscopic views of tumor tissue revealing an undifferentiated phenotype with high mitotic number in the vector-transfected controls (*b*) and a differentiated phenotype in the antisense clone No. 12 (*d*) as evidenced by the production of keratin whorls, low mitotic number, and pseudoencapsulation.

## Discussion

Previous studies have established a relationship between TSP production and proliferation in squamous epithelial cells. Histochemically, TSP is localized at sites of rapid proliferation (12). In squamous epithelium, this includes the basal cell layer and hair follicles (14). In vitro, undifferentiated, rapidly-proliferating epidermal keratinocytes synthesize large amounts of TSP and incorporate it into the ECM. When the cells are induced to differentiate, TSP production is reduced concomitantly with inhibition of proliferation (7, 14). In addition, stimulation of proliferation in quiescent keratinocytes by addition of epidermal growth factor (EGF) or somatomedin C is associated with a rapid induction of TSP synthesis (11). Among squamous carcinoma cell lines, there is a direct relationship between proliferation and TSP production (5). Although these data clearly shown that TSP production is associated with proliferation in squamous epithelial cells, cause and effect has not been determined.

To address this critical issue, we have, in the present study, utilized antisense-mediated inhibition of TSP synthesis. This approach is a powerful tool for modulating synthesis of specific proteins and has been used in the past to investigate the role of oncogenes such as c-*fos*, c-*myc*, and c-*raf*-1 in the control of cell proliferation/differentiation (17–19, 24). More recently, it has been used to modulate the production of tissue inhibitor of metalloproteinase (TIMP) to study its role in tumor metastasis (25). Our present work extends this approach to studying a structural component of the ECM. In this study we were able to markedly reduce TSP production in squamous carcinoma cells and assess the effect of this alteration on the in vitro and in vivo growth. Five unique antisense transfected clones were obtained with reduced production of TSP. The degree to which clonal variation affects TSP production in individual cell lines was determined by assessing TSP levels in three unique vector control lines. Clonal variation in TSP production was $< 5\%$ and could not account for the dramatic reduction observed in each antisense clone. The in vitro growth rates were assessed for three of the antisense clones and were shown to correlate with the level of production of TSP in these clones. In vitro growth analysis was not performed on antisense clones 8, 10, and 14 as a consequence of these lines being lost to contamination in tissue culture. In nude mice clones 7, 8, 10, and 14 failed to produce tumors through the duration of the study. Antisense clone No. 12 produced tumors in three out of five animals. These tumors differed from the vector controls by having both a longer latency and slower growth rate. Antisense clone No. 5 had a production of TSP greater than antisense clone Nos. 7, 8, 10, and 14 but less than antisense No. 12. This clone's in vitro growth rate and tumorigenic potential were similarly interme-

diate. Three of the five mice inoculated formed tumors, the latency was longer than antisense clone No. 12 and the tumors failed to reach the geometric mean diameter of the vector controls.

Tumors from the vector control animals were grossly hemorrhagic, had increased mitotic activity and a poorly differentiated phenotype. In contrast, tumors from antisense clone Nos. 12 and 5 showed a highly differentiated phenotype as evidenced by pseudoencapsulation, low mitotic activity, and keratin whorls.

These data are the first to demonstrate that production of a structural component of the ECM can be modulated by antisense inhibition of protein synthesis. In addition, they provide evidence that doing so alters in vitro and in vivo cell growth behavior. This suggests that TSP plays a functional role in control of cellular proliferation. A similar growth supportive role for TSP has been suggested in mesenchymal cells as discussed earlier (11). Thus TSP may serve to modulate growth of cells of both mesodermal and ectodermal origin. The mechanism by which this occurs is unknown, although it has been shown that endogenously-synthesized TSP serves as a potent adhesion factor for both normal and malignant squamous epithelial cells (5–9). By altering the synthesis of this protein, we may have interfered with the capacity of the cells to correctly interact with their matrix and altered their ability to receive and process growth controlling signals. Whereas this is speculation at present, this study provides insight into the importance of TSP in tumorigenesis and emphasizes how any comprehensive theory of cancer biology must address the unique nature of the ECM and its components.

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