

Suppression of Thrombospondin-1 Expression During Uveal Melanoma Progression and Its Potential Therapeutic Utility

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Objectives: To determine whether expression of thrombospondin-1 (TSP1), an endogenous inhibitor of angiogenesis, is downregulated during progression of uveal melanoma and whether administration of TSP1 and/or its antiangiogenic peptides attenuate tumor growth.

Methods: Tyrosinase-SV40 T-antigens (Tyr Tag) transgenic mice were used for evaluation of TSP1 expression during tumor progression using immunohistological methods. The therapeutic potential of TSP1 on tumor progression was evaluated either by crossing Tyr Tag mice with a line of transgenic mice overexpressing TSP1 in the eye or by administration of TSP1-mimetic peptide with known antiangiogenic, antitumor activity. Tumor areas were measured in histological sections using Optima software (Media Cybernetics, Inc).

Results: The Tyr Tag tumors from 3-week-old mice showed significant TSP1 expression, which was dramatically downregulated in tumors from 12-week-old mice. Furthermore, the development and progression of tumor was significantly delayed in Tyr Tag TSP1 transgenic mice or Tyr Tag mice receiving TSP1-mimetic peptide (100 mg/kg/d).

Conclusions: Expression of TSP1 was decreased with the angiogenic switch during progression of uveal melanoma, and TSP1 and/or its antiangiogenic peptides were effective in attenuation of tumor growth.

Clinical Relevance: Modulation of TSP1 expression and/or activity may be beneficial in treating uveal melanoma.

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UVEAL MELANOMA IS THE most common primary intraocular malignant tumor in humans, and it occurs predominantly in a nonhereditary, sporadic manner.^{1,2} The current treatments for uveal melanoma are enucleation, radiotherapy, transpupillary thermotherapy, laser photocoagulation, intravenous chemotherapy, immunotherapy, local tumor resection, or a combination of these treatments. Although some patients are successfully treated, approximately half of all patients ultimately develop metastases and die within a year. Angiogenesis, the formation of new blood vessels from preexisting capillaries, is associated with progression of many solid tumors. Although the important role of angiogenesis in progression and metastasis of uveal melanoma has been recently recognized, the molecular and cellular mechanisms involved require investigation.³⁻⁶

Angiogenesis is a very tightly regulated process and normally does not occur except during embryonic development and repair processes. This tight regulation is achieved by a balanced pro-

duction of a variety of promoters and inhibitors of angiogenesis.⁷ The abrogation of this balance, under various pathological conditions such as cancer, promotes the growth of new blood vessels. Although many investigations have historically focused on identification of factors that promote angiogenesis, now more attention is also given to factors that inhibit angiogenesis. Thrombospondin-1 (TSP1) is one of the first potent endogenous inhibitors of angiogenesis, and its decreased expression with the angiogenic switch contributes to progression of many solid tumors.⁸ This is accomplished at least in part through mutations that inactivate the protein 53 gene (*p53*).⁹

The list of inhibitors of angiogenesis has been growing, and more studies have been focusing on potential expression and activity of these factors. Reexpression of TSP1 attenuates the growth and metastasis of a variety of solid tumors.¹⁰ Thrombospondin-1 inhibits angiogenesis *in vitro* and *in vivo* by downregulating B-cell lymphoma 2 protein expression and activation of caspases, driving apoptosis of endothelial cells, the major cells that line the inside of the

blood vessels.¹¹ We have previously shown that TSP1 and its antiangiogenic fragment are present in vitreous and aqueous humor samples prepared from normal human, rat, mouse, and bovine eyes.¹² Furthermore, TSP1 levels are dramatically decreased in ocular samples prepared from diabetic rats. Thus, TSP1 expression plays a significant role in ocular vascular homeostasis, and its altered production may contribute to the pathogenesis of eye diseases with a neovascular component.

We have shown that expression of TSP1 plays a significant role during retinal vascular development such that in its absence developing retinal vasculature fails to undergo proper pruning and remodeling, resulting in increased retinal vascular density.¹³ We also showed that overexpression of TSP1 in the mouse eye prior to postnatal retinal vascularization results in attenuation of retinal neovascularization during oxygen-induced ischemic retinopathy.¹⁴ Thus, manipulation of TSP1 expression may provide a novel target for inhibition of ocular neovascularization. However, to our knowledge, the expression of TSP1 and its altered production during progression of uveal melanoma have not been previously evaluated.

Uveal melanoma most often arises in the choroid and becomes vascularized, presumably via angiogenic mechanisms whose identities remain elusive. Important roles for increased vascular endothelial growth factor expression^{6,15,16} and downregulation of pigment epithelium-derived factor¹⁷ have been proposed in the progression and metastasis of uveal melanoma. In addition, inhibition of vascular endothelial growth factor activity¹⁶ and/or overexpression of pigment epithelium-derived factor¹⁸ have shown therapeutic benefit in preclinical models. However, the underlying mechanisms that drive tumor progression remain poorly defined. We hypothesized that downregulation of TSP1 expression occurs during progression of uveal melanoma, contributing to its pathogenesis. Herein, we demonstrate that the downregulation of TSP1 expression occurs during progression of uveal melanoma using a murine transgenic pigmented ocular tumor model.¹⁹ Although in these mice the tumors develop from the retinal pigment epithelium, their histology, growth, and response to treatment closely resemble that of human choroidal melanoma. This model has proved to be a useful tool in the study of endogenous primary pigmented tumors limited to the eye, and we believe it to be the most useful murine model available for the study of human choroidal melanoma. We also show that increased expression of TSP1 in the eye or administration of TSP1-mimetic peptide attenuated tumor progression and growth in this model. Thus, TSP1 may be an important target for the treatment of uveal melanoma.

METHODS

ANIMALS

All research using mouse models of uveal melanoma was carried out in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. It was also approved by the Institutional Animal Care Committee of the University of Wisconsin School of Medicine and Public Health.

GENERATION OF TYROSINASE-SV40 T-ANTIGENS AND TSP1 TRANSGENIC MICE

The tyrosinase-SV40 T-antigens (Tyr Tag) or TSP1-overexpressing transgenic mice were generated and maintained as previously described.^{14,19} The Tyr Tag mice that overexpress TSP1 in the eyes were generated by crossing the Tyr Tag mice with TSP1 transgenic mice (all on C57BL/6J background), which express TSP1 driven by α A-crystalline promoter. In Tyr Tag mice, tumor development can be histologically detected by 3 weeks of age and is physically visible by 8 to 12 weeks of age. The tumor generally is quite large by 12 weeks, such that it destroys the eye structure. Animals were killed as soon as any sign of ocular discomfort was noted.

TREATMENT OF TYR TAG MICE WITH TSP1-MIMETIC PEPTIDE

The antiangiogenic activity of TSP1 is mapped to peptides from type 1 repeats and procollagen homology domain.²⁰ An overlapping peptide that expands these regions has shown good efficacy for inhibition of angiogenesis in various tumor models²¹ and was the basis for development of ABT-510 and, most recently, newer-generation ABT-898.²² The amino acid sequence of ABT-898 is *N*-acetyl-glycine-valine-D-alloisoleucine-serine-glutamine-isoleucine-arginine-proline-ethylamide; it was synthesized at the University of Wisconsin Biotechnology Center Peptide Synthesis Core Facility, Madison. The purity and sequence of the peptide were confirmed using standard methods. The peptide was dissolved in 5% dextran solution and used for intraperitoneal injections. Three-week-old Tyr Tag transgenic mice received TSP1-mimetic antiangiogenic peptide or vehicle for 5 weeks at 100 mg/kg/d, 5 days a week (≥ 10 mice per group). The histopathological evaluations were performed with eyes obtained from 3- and 8-week-old Tyr Tag transgenic mice receiving TSP1-mimetic antiangiogenic peptide or vehicle.

TUMOR SIZE DETERMINATION

The mice were killed on the last day of experimentation. Their eyes were then enucleated and placed in a 10% neutral-buffered formalin solution. Four serial 5- μ m-thick sections were cut from each of the superior, middle, and inferior areas of the globe in the manner previously described²³⁻²⁵ and stained with hematoxylin-eosin. All 4 of the sections from each globe area were examined under a microscope and the section with the largest area of tumor was used for measurement. The outline of the tumor was traced on a microscopically digitized image and the tumor area was measured using Optimus software version 6.5 (Media Cybernetics, Inc). Three measurements from each tumor representation were averaged to obtain the mean tumor measurement. These methods have been described elsewhere.²³⁻²⁵ The mitotic figure count and capillary density evaluations were recorded on hematoxylin-eosin- and platelet endothelial cell adhesion molecule 1-stained sections using light and fluorescence microscopy (original magnification $\times 400$), respectively. The mitotic figures and capillary densities were determined at the base of the tumors in the most active area in a minimum of 3 consecutive high-powered fields, and the mean numbers of mitotic figures and capillaries per high-powered field were determined in eyes from 5 mice.

IMMUNOHISTOCHEMICAL STAINING OF FROZEN EYE SECTIONS

Mouse eyes were enucleated and embedded in optimal cutting temperature compound at -80°C . Sections (9 μ m) were cut

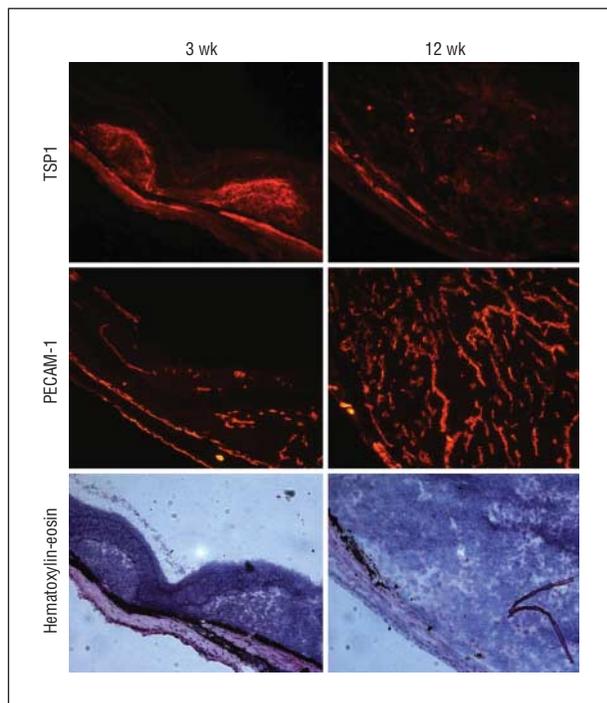


Figure 1. Thrombospondin-1 (TSP1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) staining of frozen eye sections and histological examination of tumors in tyrosinase-SV40 T-antigens (Tyr Tag) transgenic mice. Expression of TSP1 in Tyr Tag tumors from 3-week-old and 12-week-old mice was examined by immunohistochemistry of frozen eye sections. Strong TSP1 staining was observed in eyes from 3-week-old mice, especially near the site of tumors. However, a significant decrease in TSP1 staining was observed in eyes from 12-week-old mice, where tumor size was increased significantly. The vascularity of tumors was similarly evaluated using anti-PECAM-1, a vascular marker. Although a few blood vessels were observed in eye sections from 3-week-old Tyr Tag mice, a significant number of blood vessels were visible in sections from 12-week-old mice. Hematoxylin-eosin staining shows the tumor histological findings at 3 and 12 weeks. These images are representative of images evaluated in eyes from at least 10 mice (original magnification $\times 200$).

on a cryostat, placed on glass slides, and allowed to dry for 2 hours. For fluorescence microscopy, sections were fixed in cold acetone (4°C) on ice for 10 minutes, followed by 3 washes with phosphate-buffered saline (PBS) for 5 minutes each. Sections were incubated in blocker (1% bovine serum albumin, 0.2% skim milk, and 0.3% Triton X-100 in PBS) for 15 minutes at room temperature. Sections were then incubated with rabbit polyclonal antibodies to human TSP1 (Neo Markers) or murine platelet endothelial cell adhesion molecule 1²⁶ (prepared in our laboratory and diluted 1:250 in blocking solution) overnight at 4°C in a humid environment. After 3 washes in PBS for 5 minutes each, sections were incubated with secondary antibody Alexa 594 goat-antirabbit (Invitrogen Corp; 1:500 dilution prepared in blocking solution). Sections were washed 3 times in PBS, covered with PBS/glycerol (2 vol/1 vol), and mounted with a coverslip. Retina sections were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Carl Zeiss, Inc).

STATISTICAL ANALYSIS

All data were summarized as mean (SE). The effects of TSP1-mimetic peptide administration and ocular TSP1 overexpression on tumor areas in Tyr Tag and Tyr Tag TSP1 transgenic mice, respectively, were assessed using 1-way analysis of vari-

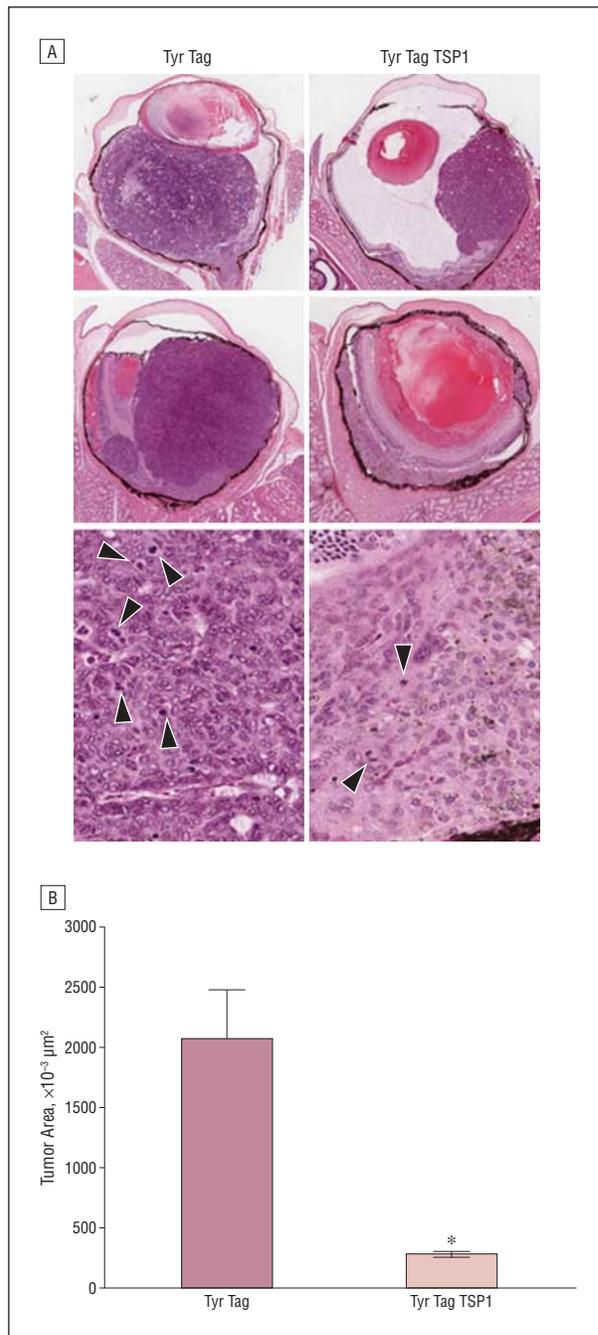


Figure 2. Suppression of tumor growth in tyrosinase-SV40 T-antigens (Tyr Tag) mice overexpressing thrombospondin-1 (TSP1). The Tyr Tag transgenic mice that overexpress TSP1 in the eyes were generated by crossing Tyr Tag mice with a line of transgenic mice that express a high level of TSP1 in the eyes. A slower progression of tumors was observed in Tyr Tag TSP1 mice compared with parental Tyr Tag mice. A, Histological evaluation of tumors in 8-week-old mice showed a significant decrease in tumor size in Tyr Tag TSP1 mice compared with Tyr Tag mice (original magnification $\times 40$ in the upper and middle rows, $\times 400$ in the lowest row). The tumor areas were evaluated as described in "Methods." Although the tumor cells looked similar in the 2 groups, tumors in Tyr Tag TSP1 mice showed fewer mitotic figures (arrowheads) compared with tumors in Tyr Tag mice. B, The mean (SE) tumor volume in Tyr Tag TSP1 mice is approximately 7.3-fold lower than that in Tyr Tag mice (mean [SE], 2.8×10^5 [0.2×10^5] vs 20.8×10^5 [4.0×10^5] μm^2 , respectively; $n=10$). $*P=.001$.

ance. The tumor area was transformed to the logarithmic scale before calculating the mean area. Differences were considered statistically significant at $P < .05$.

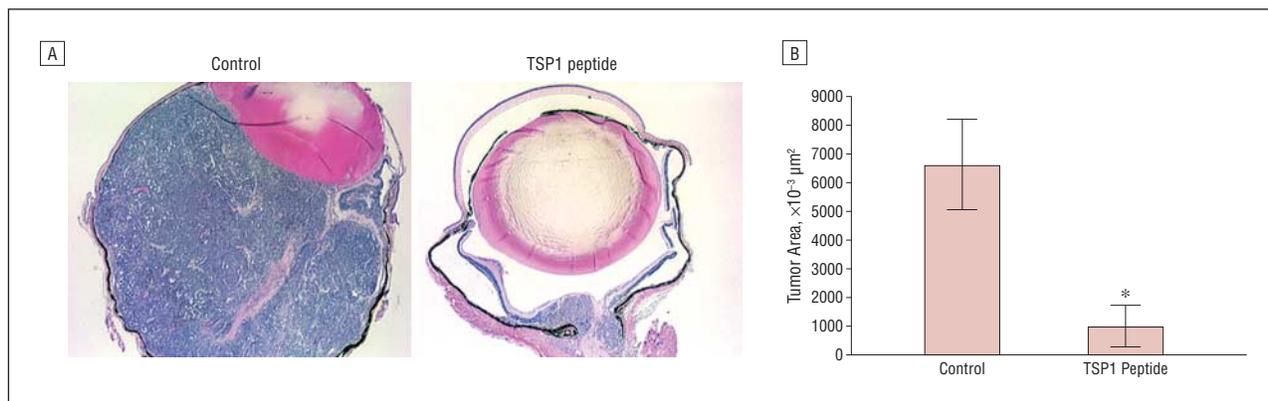


Figure 3. Inhibition of tumor growth in tyrosinase-SV40 T-antigens (Tyr Tag) mice receiving thrombospondin-1 (TSP1)-mimetic antiangiogenic peptide. The 3-week-old Tyr Tag mice received TSP1-mimetic antiangiogenic peptide or vehicle for 5 weeks (100 mg/kg/d, 5 d/wk), and tumor volumes were evaluated as described in "Methods." A, Histological sections showed a significant decrease in tumor size in mice receiving peptide compared with those receiving vehicle (original magnification $\times 40$). B, The mean (SE) tumor areas were approximately 10-fold smaller in mice receiving TSP1-mimetic peptide than in mice receiving vehicle ($n=10$). * $P=.003$.

RESULTS

DOWNREGULATION OF TSP1 EXPRESSION AND INCREASED ANGIOGENESIS DURING TUMOR PROGRESSION

Expression of TSP1 during tumor progression was monitored by immunohistological staining. At 3 weeks, the tumor was small and TSP1 staining was strong, suggesting increased expression at early stages of tumor development (**Figure 1**). At 12 weeks, the tumor grew significantly larger and TSP1 expression was almost undetectable. This is consistent with previously reported negative staining of TSP1 in uveal melanoma samples.³ However, platelet endothelial cell adhesion molecule 1 staining in the tumors showed few blood vessels at 3 weeks of age, although the number of blood vessels significantly increased by 12 weeks of age in Tyr Tag mice (Figure 1).

ATTENUATION OF TUMOR GROWTH IN TYR TAG MICE OVEREXPRESSING TSP1 IN THE EYES

To provide additional evidence for the important role of TSP1 in modulation of tumor progression and its potential use as an antitumor agent, we determined the effect of TSP1 overexpression. We generated the Tyr Tag TSP1 transgenic mice. Tumor progression was assessed by histological examination of eyes at different postnatal days. Tumor development and progression were significantly delayed in Tyr Tag TSP1 transgenic mice compared with Tyr Tag mice (**Figure 2**). The sizes of mouse ocular tumors were measured at 8 weeks for comparison. Average areas of ocular tumor in the Tyr Tag mice expressing TSP1 were approximately 7.3-fold smaller than the areas of tumors in the parental Tyr Tag mice (mean [SE], 2.8×10^5 [0.2×10^5] vs 20.8×10^5 [4.0×10^5] μm^2 , respectively). Although the tumor cells appeared similar in the 2 groups, Tyr Tag TSP1 mice compared with parental Tyr Tag mice showed fewer mitotic figures (mean [SE], 8.80 [0.86] vs 22.83 [2.56] mitotic figures; $P=.001$;

$n=5$) as well as smaller capillary densities (mean [SE], 27 [4] vs 55 [7] capillaries, respectively; $P=.008$; $n=5$).

ATTENUATION OF TUMOR GROWTH IN TYR TAG MICE RECEIVING TSP1-MIMETIC ANTIANGIOGENIC PEPTIDE

To demonstrate TSP1's therapeutic potential, we synthesized TSP1-mimetic antiangiogenic peptide. The Tyr Tag mice were injected intraperitoneally after initiation of tumors (at 3 weeks old) for 5 weeks. The tumor size in treated mice was significantly smaller than that in control mice (**Figure 3**). The results were quite similar to those observed in Tyr Tag TSP1 transgenic mice. The mean tumor areas in TSP1-treated mice decreased by approximately 10-fold compared with tumors in mice receiving vehicle alone (7×10^5 vs 67×10^5 μm^2 , respectively). Thus, TSP1-mimetic peptide was effective in blocking the progression of tumor in this model.

COMMENT

This study demonstrates, to our knowledge for the first time, that there is a decrease in expression of TSP1 during uveal melanoma progression in Tyr Tag mice and shows a significant correlation between reduced TSP1 expression and increased tumor vascularity and size. Moreover, overexpression of TSP1 in the eye or administration of TSP1-mimetic peptide with antiangiogenic activity inhibited tumor growth in Tyr Tag mice. Thus, modulation of TSP1 expression or its antiangiogenic mimetic peptide may provide a novel approach for treatment of uveal melanoma and inhibition of tumor growth.

Angiogenesis plays an important role in tumor growth, invasion, and eventually metastasis. Although most emphasis has been placed on identifying factors that promote angiogenesis, the alteration in expression of agents that normally inhibit angiogenesis has gained significant interest and is shown to be critical in the progression of many solid tumors. Antiangiogenic strategies, including the use of TSP1 and its peptides, have been proven to be a promising approach for clinical therapy of a va-

riety of solid tumors.¹⁰ However, to our knowledge, changes in TSP1 expression during uveal melanoma progression as well as its potential therapeutic utility have not been previously evaluated. Although the role of tumor angiogenesis in the pathogenesis of uveal melanoma has been recognized for quite some time, antiangiogenic therapies have only recently been attempted to prevent tumor growth.²⁷ Anti-vascular endothelial growth factor and pigment epithelial-derived factor are effective in halting tumor growth.^{16,18}

Expression of TSP1 in uveal melanomas was examined previously and shown to be attenuated in most human uveal melanomas.³ This is consistent with our results, where very limited staining of TSP1 was observed in mature tumors of Tyr Tag mice. Together, these results support the notion that changes in TSP1 expression occur during progression of uveal melanoma and that administration of TSP1 and/or its antiangiogenic mimetic peptides can effectively halt tumor progression and metastasis. These conclusions are consistent with upregulation of TSP1 by the tumor suppressor gene *p53* and its downregulation by oncoproteins such as *Myc* and *Ras*, whose alterations have been linked to the pathogenesis of uveal melanoma.²⁸⁻³⁰

Thrombospondin-1 may impact uveal melanoma growth in Tyr Tag mice by a direct effect on neoplastic cells. It can induce direct tumor cell apoptosis via the CD36/caspase pathway in some leukemic cells,³¹ and a similar mechanism may operate in other tumors such as breast carcinoma.³² Although suppression of tumor growth in transgenic mice that overexpress TSP1 suggests a direct role for TSP1 on tumor cells, the direct effect of TSP1 on uveal melanoma cells needs further investigation. Alternatively, TSP1 may also have an indirect antitumor effect by inhibiting angiogenesis. Thrombospondin-1 inhibits angiogenesis through direct effects on endothelial cell migration and survival and through effects on vascular endothelial growth factor bioavailability. Together, our results suggest that TSP1 could be a novel therapeutic target for the treatment of uveal melanoma; this requires further validation in human uveal melanoma cells and other in vivo melanoma models.

The molecular and cellular mechanisms that contribute to the pathogenesis of uveal melanoma have been the subject of numerous studies. A number of studies have attempted to address the potential contribution of mutations in *p53*, a gene mutated in more than half of human tumors, to the pathogenesis of uveal melanoma. A genetic link between *p53* mutation and uveal melanoma has been previously reported.³³ Other studies of *p53* in uveal melanoma have focused on its role in apoptosis and/or enhanced proliferation of tumor cells. The decreased *p53* expression in a low percentage of uveal melanomas is reported to be associated with increased proliferation.³⁴ In addition, other studies have failed to detect *p53* protein in uveal melanomas²⁸ or have reported very low expression,²⁹ despite the report of infrequent loss of heterozygosity at the *p53* locus in uveal melanomas.³¹ Thus, a role for *p53* downstream pathways has been proposed in the pathogenesis of uveal melanomas. However, the effect of these changes on TSP1 expression and tumor vascularization needs further investigation. The

presence of microcirculation patterns in uveal melanomas associated with lack of *p53* expression³² further supports a role for decreased expression of *p53* and TSP1 in promoting angiogenesis and tumor growth.

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Author Contributions: Dr Sheibani had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Correction

Omission of Journal Club Designations and Acknowledgment. In the Clinical Sciences article titled "Postoperative Visual Acuity in Patients With Fuchs Dystrophy Undergoing Descemet Membrane-Stripping Automated Endothelial Keratoplasty: Correlation With the Severity of Histologic Changes" by Happ et al, published in the January issue of the *Archives* (2012;130[1]:33-38), the Journal Club designations and a related Acknowledgment were omitted. At the end of the "Acknowledgments" on page 38, an entry titled "Online-Only Material" should have been included and read as follows: "Online-Only Material: This article is featured in the *Archives* Journal Club. Go to <http://www.archophthalmol.com> to download teaching PowerPoint slides." In addition, "JOURNAL CLUB" should have appeared above the title on page 33, and the Journal Club logo should have appeared in the second column of text on that page, along with a slug that read: "Journal Club slides available at www.archophthalmol.com."