

# Loss of *Tsc1* or *Tsc2* Induces Vascular Endothelial Growth Factor Production through Mammalian Target of Rapamycin<sup>1</sup>

Nisreen El-Hashemite, Victoria Walker, Hongbing Zhang, and David J. Kwiatkowski<sup>2</sup>

Brigham and Women's Hospital, Department of Medicine, Hematology Division, Boston, Massachusetts 02115

## Abstract

Mutation in either TSC1 or TSC2 causes the autosomal dominant disorder tuberous sclerosis, in which widespread hamartomas are seen, some of which have a high level of vascularization. Tuberous sclerosis complex (TSC) gene products negatively regulate mammalian target of rapamycin (mTOR) activity. We found that vascular endothelial growth factor (VEGF) is secreted by *Tsc1*- or *Tsc2*-null fibroblasts at high levels compared with wild-type cells. In *Tsc1*<sup>+/-</sup> mice, serum levels of VEGF were increased and appeared to be associated with the extent of tumor development. Rapamycin, a mTOR inhibitor, reduced the production of VEGF by *Tsc1*- and *Tsc2*-null fibroblasts to normal levels. Moreover, short-term treatment of *Tsc1*<sup>+/-</sup> mice with rapamycin at 20 mg/kg led to some changes in tumor morphology and a reduction in serum VEGF levels. These observations have three implications. First, TSC gene products regulate VEGF production through a mTOR signaling pathway. Second, serum VEGF levels may be a useful clinical biomarker to monitor the progression of TSC-associated lesions. Last, rapamycin or related inhibitors of mTOR may have therapeutic benefit in TSC both by direct tumor cell killing and by inhibiting the development of TSC lesions through impairment of VEGF production.

## Introduction

TSC<sup>3</sup> is an autosomal dominant genetic disorder characterized by multiorgan hamartomatosis as a result of mutations in either TSC1 or TSC2 (1). TSC1 and TSC2 form a protein complex (2, 3), and mutations in either gene lead to similar clinical phenotypes. TSC follows the two-hit model of tumor suppressor genes in that TSC lesions often show loss of heterozygosity for the wild-type allele (4, 5).

Some TSC hamartomas, including renal AML and skin angiofibroma, are characterized by numerous abnormal vascular channels (1). AMLs are typically highly vascular, progress requiring clinical intervention in about 10% of TSC patients, and are associated with both renal failure and sudden death due to massive hemorrhage (1). We have described *Tsc* mouse models that have been developed by gene targeting (6, 7). Both *Tsc1*<sup>+/-</sup> and *Tsc2*<sup>+/-</sup> mice develop liver hemangiomas, renal cystadenomas, and, in rare cases, angiosarcomas. The liver hemangiomas are characterized by extensive abnormal vascularization (Fig. 3, A and B), and fatal hemorrhage from these lesions

is a common event (7). Thus, a high level of angiogenesis occurs in both human and mouse *Tsc* lesions.

Angiogenesis is required for tumor growth and metastasis and is triggered by both oncogene activation and tissue hypoxia (8, 9). VEGF is the major angiogenic growth factor generated by malignant cells and induces angiogenesis and vasculogenesis *in vivo* through interaction with the tyrosine kinase receptors VEGF receptor-1 (flt) and VEGF receptor-2 (flk-1/KDR) on endothelial cells (8, 9).

Recent studies have elucidated an important role for the TSC gene products in a conserved signaling pathway that includes mTOR (7, 10–12). Both *Tsc1*-null and *Tsc2*-null cell lines show an increase in the phosphorylation of S6 kinase and 4EBP1 that is inhibited by rapamycin, consistent with constitutive activation of mTOR (7, 10). Here we investigated the hypothesis that loss of *Tsc1* or *Tsc2* leads to secretion of VEGF, which is also dependent on mTOR signaling, and that this event contributes to the vascular nature of *Tsc* lesions.

## Materials and Methods

*Tsc1*-null and *Tsc2*-null fibroblast cell lines prepared from E10.5 embryos were prepared as described previously (7). They were cultured in DMEM with 10% FCS at 5% CO<sub>2</sub>, in parallel with littermate-derived control fibroblast cell lines. A cell proliferation (MTT) assay measuring NADPH and NADH produced by metabolically active cells was performed using the CellTiter96 Aqueous One Solution Cell Proliferation Assay kit (Promega). Generation of *Tsc2*-null cell lines expressing wild-type and mutant forms of the human TSC2 cDNA will be described in detail elsewhere.<sup>4</sup>

Immunoblot analysis was performed on tissue extracts as described previously (7) using anti-VEGF, TSC2, HSP70 (Santa Cruz Biotechnology), and pS6 Ser 235/236 antibodies (Cell Signaling).

Cell growth and VEGF synthesis experiments were performed after plating 4 × 10<sup>4</sup> fibroblasts in 6-well plates. Six h after plating, fresh DMEM with 1% FCS alone or supplemented with 10 nM rapamycin was added, and cells were incubated for 48 h. Media were collected at 24 or 48 h, and VEGF measurements were determined by ELISA for mouse VEGF (Oncogene Research Products). This ELISA method was also used to assess serum VEGF levels in *Tsc1*<sup>+/-</sup> and wild-type mice, both in strain 129/Sv.

Rapamycin was administered to mice by i.p. injection at 20 mg/kg daily for 4 days. It was reconstituted in absolute ethanol at 10 mg/ml and diluted in 5% Tween 80 and 5% Peg-400 before injection.

Necropsy and pathological analysis was performed by careful inspection of the external surface of both kidneys and the liver for involvement by cystadenomas and hemangioma, respectively. The kidneys were sliced at 1 mm in entirety for pathological review. For immunohistochemistry, 5-μm paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in an ethanol/water series. Sections were stained by the peroxidase method (ImmunoCruz kit; Santa Cruz Biotechnology) using primary antibodies against VEGF (Santa Cruz Biotechnology). Negative control immunohistochemical procedures were conducted on adjacent tissue sections, including omission of the primary antibodies and replacement with the same amount of normal IgG. Apoptosis was detected in tissue sections by TUNEL assay using the ApopTag apoptosis kit (Serologicals Corp.).

<sup>4</sup> H. Zhang, J. Lee, D. J. Kwiatkowski, manuscript in preparation.

Received 11/22/02; 1st revised 2/18/03; 2nd revised 4/15/03; accepted 5/7/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by the LAM Foundation, the Rothberg Fund, and NIH National Institute of Neurological Disorders and Stroke.

<sup>2</sup> To whom requests for reprints should be addressed, at Brigham and Women's Hospital, Genetics Laboratory, Hematology Division, 221 Longwood Avenue, Boston, MA 02115. Phone: (617) 278-0384; Fax: (617) 734-2248; E-mail: dk@rics.bwh.harvard.edu.

<sup>3</sup> The abbreviations used are: TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor; AML, angiomyolipoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HIF-1α, hypoxia-inducible factor-1α.

## Results and Discussion

Strong VEGF expression was seen by immunoblot (Fig. 1A) and immunohistochemistry (Fig. 3, C and E) analyses of mouse liver hemangiomas from *Tsc1*<sup>+/-</sup> and *Tsc2*<sup>+/-</sup> mice, in comparison with normal liver (Figs. 1A and 3D). These immunoblot results understate the extent of VEGF production by these tumors because they are infiltrative and impossible to dissect cleanly from adjacent liver. Unrecognized hemangioma involvement may explain the small amount of VEGF detected in “normal” liver from a *Tsc1*<sup>+/-</sup> mouse (Fig. 1A, Lane 1); VEGF was not seen in two other *Tsc1*<sup>+/-</sup> mouse liver samples (data not shown). Similarly, VEGF expression was high in *Tsc1*<sup>+/-</sup> and *Tsc2*<sup>+/-</sup> renal cystadenomas in comparison with normal kidney tissue (Figs. 1B and 3K).

Although neovascularization has been observed in brain, skin, and kidney lesions from TSC patients (13), the mechanism of this phenomenon has been uncertain, as well as whether *Tsc1*- or *Tsc2*-null cells in these tumors secrete VEGF or induce VEGF production by surrounding tissues. Therefore, we examined VEGF production by fibroblast cell lines that were null for either *Tsc1* or *Tsc2* (6, 7). Conditioned media from *Tsc1*<sup>-/-</sup>, *Tsc2*<sup>-/-</sup>, and wild-type fibroblast cultures were collected 24 and 48 h after plating and subjected to ELISA to measure VEGF. VEGF levels were significantly higher in the *Tsc1*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup> cell media in comparison with media from wild-type cells (Fig. 2, A and B). Production of VEGF was significantly reduced in *Tsc2*<sup>-/-</sup> cells transfected to express human TSC2 (Figs. 1C and 2C). In addition, *Tsc2*<sup>-/-</sup> cells transfected with three different disease-associated (4) mutant TSC2 cDNAs continued to show high-level secretion of VEGF (Figs. 1C and 2C). These data indicate that increased production of VEGF was a direct result of loss of the *Tsc2* gene product in these cells.

We then measured serum VEGF levels in *Tsc1*<sup>+/-</sup> and wild-type mice (Table 1A). Serum VEGF levels in littermate control wild-type

mice were between 50 and 91 pg/ml, whereas levels in *Tsc1*<sup>+/-</sup> mice of similar age were between 99 and 251 pg/ml ( $P = 0.02$ ,  $t$  test). Necropsy analysis showed that *Tsc1*<sup>+/-</sup> mice with high serum levels of VEGF had significant tumor involvement of liver and/or kidney. Although the numbers studied do not permit statistical significance, the extent of liver involvement by hemangioma appeared to be associated with the degree of serum VEGF elevation (Table 1A). VEGF levels also tended to be higher in female compared with male *Tsc1*<sup>+/-</sup> mice, consistent with their higher rate and severity of liver hemangioma development (7).

Recently, we have shown that deficiency in either *Tsc1* or *Tsc2* in cultured cells results in unregulated activation of mTOR, which leads to aberrant high-level phosphorylation of S6 kinase and 4EBP1 (Ref. 7; Fig. 1C). This mTOR activation signature is sensitive to rapamycin treatment, a highly specific inhibitor of mTOR (7, 10). We reasoned that VEGF production was likely downstream of mTOR, and therefore we investigated the effect of the mTOR inhibitor rapamycin on VEGF production in *Tsc*-null cells. Rapamycin inhibited VEGF production by both *Tsc1*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup> cells in a dose-dependent manner (Fig. 2, A, B, and D).

We next explored the effects of rapamycin treatment on VEGF production *in vivo*. Serum VEGF levels were measured in three *Tsc1*<sup>+/-</sup> female mice (age, 10 months), before and after treatment with 20 mg/kg rapamycin i.p. daily for 4 days. Serum levels of VEGF were reduced after rapamycin in all three mice, with large reductions in two mice that began with very high VEGF levels (Table 1B). These results indicating the potent effects of rapamycin treatment on VEGF production *in vitro* and *in vivo* suggest that VEGF production is due to the constitutive activation of mTOR in *Tsc1*- or *Tsc2*-null cell lines and tumors. The reduction of VEGF levels *in vivo* could also have been due to tumor cell death induced by rapamycin in the *Tsc1*<sup>+/-</sup> mice.

We therefore examined the effects of rapamycin treatment on the tumors seen in these mice. Although clear morphological changes were not apparent in liver hemangioma sections after short-term rapamycin treatment, VEGF expression was reduced in hemangioma sections in the treated mice (Fig. 3, F and L). Kidney sections from rapamycin-treated mice showed a flattening of the epithelial cells lining the cystadenomas, with hematoxylin-positive necrotic debris in the lumen of the cysts (compare Fig. 3G, an untreated *Tsc1*<sup>+/-</sup> mouse kidney cystadenoma, with Fig. 3H, a rapamycin-treated kidney cystadenoma). A solid adenoma from a rapamycin-treated mouse showed less dramatic cellular changes but also showed regions of apoptotic cells that were TUNEL positive (Fig. 3, I and J). These findings were not seen in control untreated mice. In addition, VEGF expression was reduced in kidney lesions from the rapamycin-treated mice in comparison with untreated mice (Fig. 3, K and L). Thus, these observations suggest that short-term treatment with rapamycin had some tumor cell kill effect. However, liver hemangiomas did not change in appearance, suggesting that rapamycin therapy led to a reduction in VEGF production before tumor cell death.

We next examined whether the inhibition of VEGF production by rapamycin was due to an antiproliferative effect on *Tsc1*<sup>-/-</sup> or *Tsc2*<sup>-/-</sup> cells. Using the MTT cell proliferation assay, we observed that rapamycin treatment inhibited VEGF production during a 24-h treatment interval but had no effect on *Tsc2*<sup>-/-</sup> cell proliferation (Fig. 2, E and F). Longer-term treatment with rapamycin may have had a greater effect on growth in this assay, but the observations indicate that the effect of rapamycin on VEGF is rapid and occurs before an antiproliferative effect.

Intense investigation has led to the recognition that several different factors regulate and induce VEGF production, including

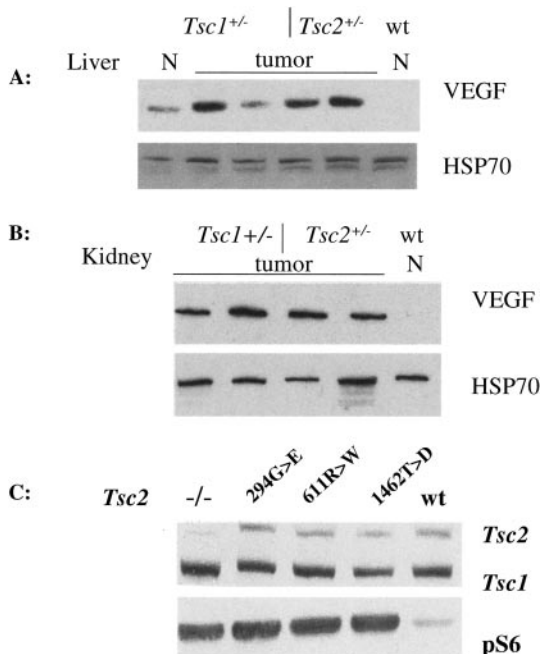


Fig. 1. VEGF expression by *Tsc* mouse tumors and analysis of fibroblast cell lines. A and B, immunoblot analysis of liver (A) and kidney (B) tissues from control and *Tsc1*<sup>+/-</sup> and *Tsc2*<sup>+/-</sup> mice. Note VEGF expression by liver hemangiomas and kidney tumors. C, expression of *Tsc2* (tuberin), *Tsc1* (hamartin), and pS6 by *Tsc2*<sup>-/-</sup> cells and derivative lines transfected to express three different TSC2 mutations or the wild-type (wt) cDNA.

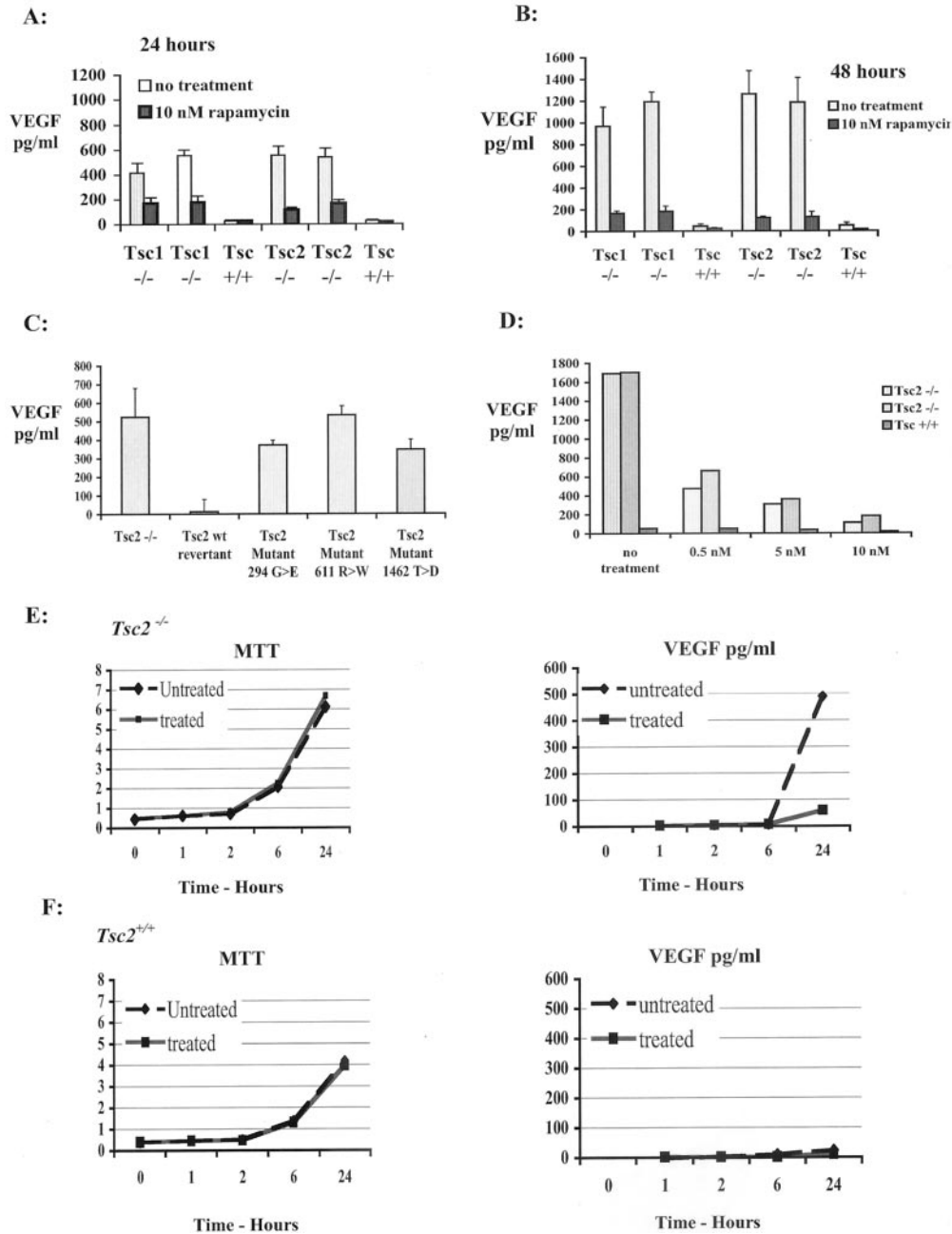


Fig. 2. VEGF secretion by *Tsc*-null and wild-type cell lines. VEGF levels were assessed by ELISA in media at 24 (A) or 48 h (B–D). A and B, fibroblasts that are either *Tsc1*<sup>-/-</sup> or *Tsc2*<sup>-/-</sup> show increased levels of VEGF in their media that are markedly reduced by treatment with rapamycin. C, *Tsc2*<sup>-/-</sup> cells transfected to express the wild-type (*wt*) *TSC2* cDNA but not any of three mutant *TSC2* cDNAs show reduced levels of VEGF in their media. D, rapamycin inhibits VEGF production by *Tsc2*-null cells in a dose-dependent manner. E and F, comparison between a MTT assay and VEGF levels in *Tsc2*<sup>-/-</sup> (E) and control (*F*) cells treated with or without rapamycin shows that over a 24-h period, there was no effect of rapamycin on cell growth, whereas there was a marked effect on VEGF production in the *Tsc2*-null cell line.

cytokines, hormones, and hypoxia (8, 9, 14–16). Hypoxia is one of the major stimulators of VEGF gene expression in neoplastic cells and is thought to act primarily through regulation of the stability of HIF-1 $\alpha$  (8, 9, 14, 15). However, several oncogenes, such as *v-src*, are known to promote VEGF production in the absence of hypoxia by increasing HIF-1 $\alpha$  expression (17). Recent studies have demonstrated that activation of mTOR up-regulates HIF-1 $\alpha$  expression, which results in an increase in VEGF production (18, 19). These previous results, combined with our data, suggest a model in which cells lacking either tuberlin or hamartin activate mTOR, which in turn leads to increased expression of HIF-1 $\alpha$ , resulting in stimu-

lation of VEGF production. Rapamycin treatment, a mTOR inhibitor, reverses this effect.

In summary, our data indicate that a lack of either the TSC1 or the TSC2 gene products leads to VEGF production by both cultured cells and tumors *in vivo*. VEGF levels are rapidly reversed *in vitro* by treatment with rapamycin, before an antiproliferative effect, suggesting that increased VEGF production occurs secondary to activation of mTOR. *In vivo*, short-term rapamycin treatment of *Tsc1*<sup>+/-</sup> mice rapidly reduces VEGF levels before clear evidence of tumor cell death in liver hemangiomas, consistent with an inhibitory effect on VEGF production in *Tsc1*-null tumor cells.

Table 1 Serum levels of VEGF in *Tsc1*<sup>+/-</sup> mice

A. Serum levels of VEGF (pg/ml) and pathological findings in <i>Tsc1</i> <sup>+/-</sup> and wild-type control mice						
Genotype	Sex	Age (mo)	Liver tumor <sup>a</sup>	Kidney tumor <sup>a</sup>	VEGF level	
<i>Tsc1</i> <sup>+/-</sup>	Female	13	Massive multi lobar	1 (1.5 mm)	249	
<i>Tsc1</i> <sup>+/-</sup>	Female	10	Massive multi lobar	None	244	
<i>Tsc1</i> <sup>+/-</sup>	Female	9	nd <sup>b</sup>	nd	154	
<i>Tsc1</i> <sup>+/-</sup>	Female	9	nd	nd	174	
<i>Tsc1</i> <sup>+/-</sup>	Female	9	nd	nd	186	
<i>Tsc1</i> <sup>+/-</sup>	Female	11	nd	nd	251	
<i>Tsc1</i> <sup>+/-</sup>	Male	13	Multilobar	1 (1 mm)	140	
<i>Tsc1</i> <sup>+/-</sup>	Male	13	Multilobar	11 (<1 to >2 mm)	138	
<i>Tsc1</i> <sup>+/-</sup>	Male	12	Massive multilobar	None	152	
<i>Tsc1</i> <sup>+/-</sup>	Male	12	None	2 (0.5–1 mm)	99	
<i>Tsc1</i> <sup>+/+</sup>	Female	9	nd	nd	57	
<i>Tsc1</i> <sup>+/+</sup>	Female	9	nd	nd	67	
<i>Tsc1</i> <sup>+/+</sup>	Female	9	nd	nd	77	
<i>Tsc1</i> <sup>+/+</sup>	Female	10	nd	nd	91	

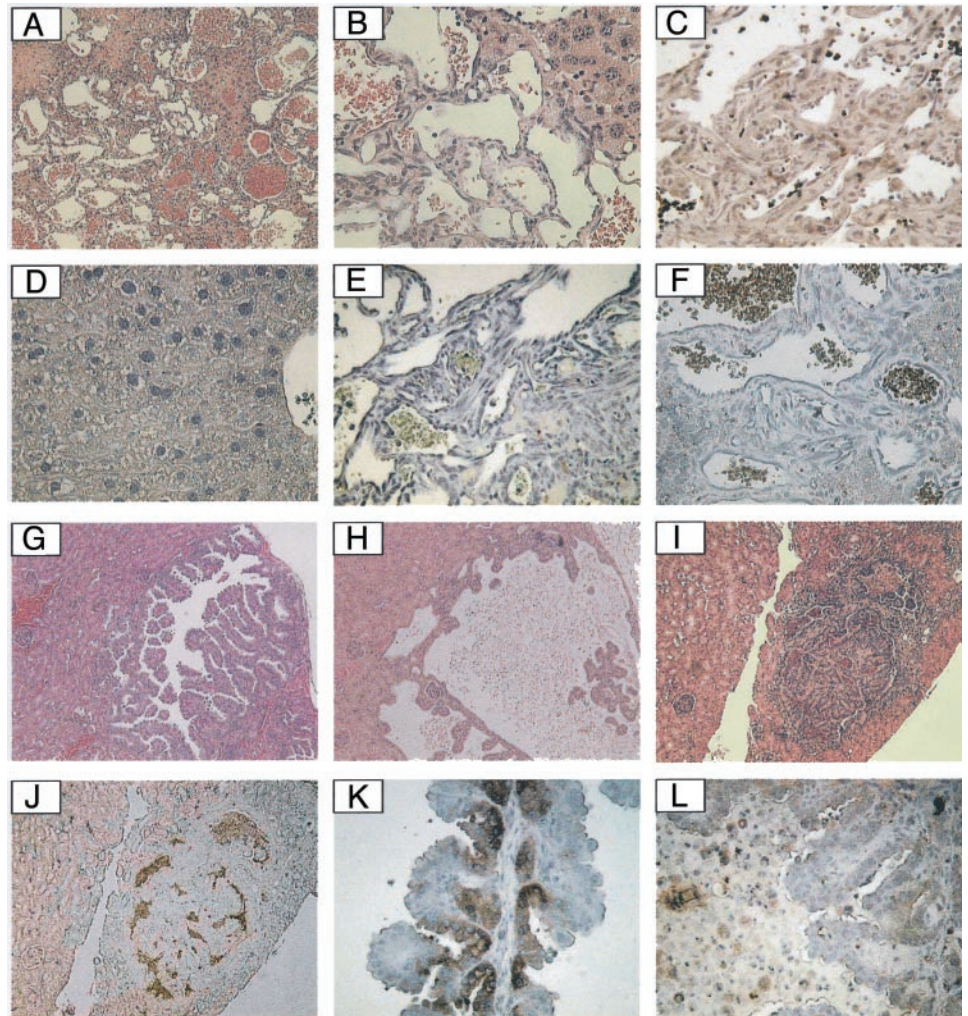
  

B. Serum levels of VEGF in <i>Tsc1</i> <sup>+/-</sup> mice before and after rapamycin treatment						
Genotype	Age (mo)	Liver tumor	Kidney tumor	VEGF level pretreatment	VEGF level posttreatment	
<i>Tsc1</i> <sup>+/-</sup>	10	None	None	148	132	
<i>Tsc1</i> <sup>+/-</sup>	10	Multilobar	None	346	115	
<i>Tsc1</i> <sup>+/-</sup>	10	Massive multilobar	1 (1 mm)	594	103	

<sup>a</sup> Both liver and kidney tumors were assessed by gross pathological inspection.

<sup>b</sup> nd, not done, necropsy analysis not performed.

Fig. 3. Pathology of *Tsc1*<sup>+/-</sup> mice and effects of treatment with rapamycin. *A* and *B*, liver hemangioma from an untreated mouse showing proliferating smooth muscle cells and abnormal vascular channels of variable size. *C–F*, VEGF immunohistochemistry of mouse liver shows strong expression of VEGF in untreated hemangioma (*C*, arrow indicates spindle-shaped cells expressing VEGF), no reactivity in normal liver from a wild-type mouse (*D*), no reactivity in a parallel section (to *C*) of a hemangioma when the primary antibody was omitted (*E*), and reduced VEGF expression in a hemangioma from a *Tsc1*<sup>+/-</sup> mice treated with rapamycin for 4 days (*F*). *G–I*, renal cystadenomas of *Tsc1*<sup>+/-</sup> mice. *G*, untreated mouse tumor shows papillary projections with elongated epithelial lining cells. *H*, rapamycin-treated mouse tumor shows flattened epithelial cells, with fewer papillary projections, and necrotic debris in the cyst lumen. *I*, rapamycin-treated mouse solid adenoma shows a more modest change in cell morphology but with regions of cellular debris. *J*, TUNEL stain highlights regions of apoptosis in the rapamycin-treated solid adenoma. *K* and *L*, VEGF expression in control (*K*) and rapamycin-treated (*L*) kidney tumors. Note reduced expression by the treated tumor.



These data provide further support for the concept that rapamycin may have unique therapeutic benefit for the lesions that occur in TSC patients (7, 20), potentially through both tumor cell death and inhibition of VEGF production. Our observations also suggest that serum VEGF levels could be a clinically useful biomarker to monitor the development and progression of TSC-associated lesions, particularly renal AMLs.

### Acknowledgments

We thank Justin Lee for technical assistance.

### References

- Gomez, M., Sampson, J., and Whitmore, V. *The Tuberous Sclerosis Complex*, 3rd ed. New York: Oxford University Press, 1999.
- Plank, T. L., Yeung, R. S., and Henske, E. P. Hamartin, the product of the tuberous sclerosis 1 (TSC1) gene, interacts with tuberin and appears to be localized to cytoplasmic vesicles. *Cancer Res.*, *58*: 4766–4770, 1998.
- van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van den Ouweland, A., Reuser, A., Sampson, J., Halley, D., and van der Sluijs, P. Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. *Hum. Mol. Genet.*, *7*: 1053–1057, 1998.
- Cheadle, J. P., Reeve, M. P., Sampson, J. R., and Kwiatkowski, D. J. Molecular genetic advances in tuberous sclerosis. *Hum. Genet.*, *107*: 97–114, 2000.
- Niida, Y., Stemmer-Rachamimov, A. O., Logrip, M., Tapon, D., Perez, R., Kwiatkowski, D. J., Sims, K., MacCollin, M., Louis, D. N., and Ramesh, V. Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions. *Am. J. Hum. Genet.*, *69*: 493–503, 2001.
- Onda, H., Lueck, A., Marks, P. W., Warren, H. B., and Kwiatkowski, D. J. *Tsc2*(+/-) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. *J. Clin. Investig.*, *104*: 687–695, 1999.
- Kwiatkowski, D. J., Zhang, H., Bandura, J. L., Heiberger, K. M., Glogauer, M., el-Hashemite, N., and Onda, H. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in *Tsc1* null cells. *Hum. Mol. Genet.*, *11*: 525–534, 2002.
- Kaelin, W. G. How oxygen makes its presence felt. *Genes Dev.*, *16*: 1441–1445, 2002.
- Dvorak, H. F. Vascular permeability factor/vascular endothelial growth factor: a critical cytokin in tumor angiogenesis and a potential target for diagnosis and therapy. *J. Clin. Oncol.*, *20*: 4368–4380, 2002.
- Kwiatkowski, D. J. Tuberous sclerosis complex: from tubers to mTOR. *Ann. Hum. Genet.*, *67*: 1–10, 2003.
- Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.*, *4*: 648–657, 2002.
- Tee, A. R., Fingar, D. C., Manning, B. D., Kwiatkowski, D. J., Cantley, L. C., and Blenis, J. Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc. Natl. Acad. Sci. USA*, *99*: 13571–13576, 2002.
- Arbiser, J. L., Brat, D., Hunter, S., D'Armiento, J., Henske, E. P., Arbiser, Z. K., Bai, X., Goldberg, G., Cohen, C., and Weiss, S. W. Tuberous sclerosis-associated lesions of the kidney, brain, and skin are angiogenic neoplasms. *J. Am. Acad. Dermatol.*, *46*: 376–380, 2002.
- Semenza, G. L. Involvement of hypoxia-inducible factor 1 in human cancer. *Intern. Med.*, *41*: 79–83, 2002.
- Harris, A. L. Hypoxia: a key regulatory factor in tumour growth. *Nat. Rev. Cancer*, *2*: 38–47, 2002.
- LeCouter, J., Lin, R., and Ferrara, N. Endocrine gland-derived VEGF and the emerging hypothesis of organ-specific regulation of angiogenesis. *Nat. Med.*, *8*: 913–917, 2002.
- Jiang, B. H., Agani, F., Passaniti, A., and Semenza, G. L. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Res.*, *57*: 5328–5335, 1997.
- Mayerhofer, M., Valent, P., Sperr, W. R., Griffin, J. D., and Sillaber, C. BCR/ABL induces expression of vascular endothelial growth factor and its transcriptional activator, hypoxia inducible factor-1 $\alpha$ , through a pathway involving phosphoinositide 3-kinase and the mammalian target of rapamycin. *Blood*, *100*: 3767–3775, 2002.
- Hudson, C. C., Liu, M., Chiang, G. G., Ottermess, D. M., Loomis, D. C., Kaper, F., Giaccia, A. J., and Abraham, R. T. Regulation of hypoxia-inducible factor 1 $\alpha$  expression and function by the mammalian target of rapamycin. *Mol. Cell. Biol.*, *22*: 7004–7014, 2002.
- Kenerson, H. L., Aicher, L. D., True, L. D., and Yeung, R. S. Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors. *Cancer Res.*, *62*: 5645–5650, 2002.