Transgenic Expression of Dominant Negative Tuberin through a Strong Constitutive Promoter Results in a Tissue-specific Tuberous Sclerosis Phenotype in the Skin and Brain*

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Tuberous sclerosis (TS) is a common autosomal dominant disorder caused by loss or malfunction of hamartin (tsc1) or tuberin (tsc2). Many lesions in TS do not demonstrate loss of heterozygosity for these genes, implying that dominant negative forms of these genes may account for some hamartomas and neoplasms in TS. To test this hypothesis, we expressed a dominant negative allele of tuberin (ΔRG) behind the cytomegalovirus promoter in NIH3T3 cells and transgenic mice. This allele binds hamartin but has a deletion in the C terminus of tuberin, leading to constitutive activation of rap1 and rabs5/rabaptin. Expression of ΔRG in NIH3T3 cells led to a strong induction of reactive oxygen species, induction of vascular endothelial growth factor, and malignant transformation in vivo. Expression of ΔRG driven by the constitutive cytomegalovirus promoter led to high level expression in all murine tissues examined, including skin, kidney, liver, and brain. Surprisingly, mice expressing the ΔRG transgene developed a fibrovascular collagenoma in the dermis, which closely resembles the Shagreen patch observed in human patients with TS. In addition, numerous small subpial collections of external granule cells in the cerebellum were observed, which may be the murine equivalent of subependymal giant cell astrocytomas or tubers commonly seen in TS patients. Thus, expression of a dominant negative tuberin in multiple tissues can lead to a tissue-specific phenotype resembling some of the findings in human TS. Our data are the first to demonstrate that specific signaling abnormalities underlie specific hamartomas in a model of a human genetic disorder.

The abbreviations used are: TS, tuberous sclerosis; VEGF, vascular endothelial growth factor; LOH, loss of heterozygosity; PDGF, platelet-derived growth factor; DCF, dichlorofluorescein; PBS, phosphate-buffered saline.
Constitutive Expression of Dominant Negative Tuberin

MATERIALS AND METHODS

Plasmids and Cell Lines—The plasmid pCMVΔRG was obtained from Loren Fields (Indiana University, Indianapolis, IN). This plasmid was transfected into NIH3T3 cells using Lipofectin and selected with G418. Resistant colonies were pooled and labeled 3T3ΔRG. pCMVΔRG was linearized with BglII and XbaI, gel-purified, and injected into mouse embryos.

Western Blotting—For signal transduction analysis, starved NIH3T3 and ΔRG NIH3T3 cells, untreated and treated with 50 ng/ml PDGF-BB, were lysed in Nonident P-40 lysis buffer (1% Nonident P-40, 150 mM NaCl, 10% glycerol, 20 mM HEPESS, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM EDTA, 100 μM Na3VO4, and 1% sprotinin). The sample was separated by SDS-polyacrylamide gel electrophoresis in 10% gels and transferred to a nitrocellulose membrane. The membranes were incubated with anti-tyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY), anti-Akt antibody (catalog number 9272, Cell Signaling, Beverly, MA), anti-phospho Akt (Ser-473) antibody (catalog number 9271, Cell Signaling), phospho P70 S6 ribosomal protein (Ser-240/-244, catalog number 2215, Cell Signaling), phospho Akt (Ser-473) antibody (catalog number 9271, Cell Signaling), phospho P70 S6 kinase (Thr-421/Ser-424, catalog number 2215, Cell Signaling), and phospho P70 S6 kinase (Thr-421/Ser-424, catalog number 2215, Cell Signaling) and detected using horseradish peroxidase-conjugated antibody. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Reactive Oxygen Generation—Confluent cells in 100-mm dishes were washed with 6 ml of Hanks’ balanced salt solution and released by using 0.25% trypsin (w/v)/1 mM EDTA followed by the addition of 2 ml of Hanks’ balanced salt solution. After pelleting, the cells were resuspended in 1 ml of Hanks’ balanced salt solution. Dichlorofluorescein diacetate was added to a final concentration of 2 μM and incubated for 1 h in the dark at room temperature. Dichlorofluorescein (DCF) fluorescence was determined by using a FACS from BD Biosciences (excitation wavelength, 488 nm; emission wavelength, 515–545 nm).

Reverse Transcription-PCR for VEGF—Total RNA was isolated using TRI reagent (Sigma). Reverse transcription-PCR was conducted with the Promega accession reverse transcription-PCR kit. Primers used were as follows: actin (728 bp), forward 5′-AAG ATG ACC CAG ATG ATG TTT GAC AC-3′ and reverse 5′-CTG CTT CCT GAT CCA CAT M and incubated for 2 min followed by 40 PCR cycles under standard conditions with an annealing temperature of 60 °C were performed. β-Actin mRNA was used as a reference message to normalize the content of total RNA. VEGF expression was calculated as the relative expression ratio to that of β-actin. All reactions were carried out in triplicate. Quantification was determined by triplicate repeats.

Generation of Transgenic Mice—Transgenic mice were produced by standard mechanisms. Briefly, the ΔRG transgene (see Fig. 5) was purified using a QiAex II extraction kit (Qiagen), resuspended in injection buffer at 2 ng/μl, and microinjected into C57BL/6 fertilized embryos. Embryos were transferred into ICR recipient females, and resultant pups were screened for the presence of the transgene by both PCR (see Fig. 6) and Southern blot (data not shown). Fig. 6 shows the screening methodology and representative results for PCR screening of the transgenic mice. Primers TSC2F (GTCCAGGAGAGACTCAGGT-GCCAGT) and TSC2R (CTGTAAGGTCTGCAACTCGGAGAA) span intron 8 and thus give a wild-type band of 568 bp and a transgene specific band of 281 bp. Three independent founder mice were identified (MCP-1 (Genzyme Technne, Minneapolis, MN) and anti-CCR-2B (see Fig. 8) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (both diluted in PBS, 1:250) (13). Subsequently, they were incubated with biotinylated secondary antibody followed by the streptavidin-peroxidase treatment. The sections were developed with 3,3′-diaminobenzidine solution as chromogen and then counterstained with hematoxylin and dehydrated, cleared, and mounted. Negative controls were prepared by omitting the primary antibodies and by their substitution with corresponding IgG at the dilution used for the specific antibodies in this study.

Histology and Immunohistochemistry—Formalin fixed, paraffin-embedded sections were prepared on poly-L-lysine coated slides. To block the endogenous peroxidase activity, the sections were treated with methanol containing 0.3% hydrogen peroxide for 15 min and then washed in PBS. They were stained using a standard avidin-biotin peroxidase kit (Nichirei Co., Tokyo, Japan) with antibodies against MCP-1 (Genzyme Technne, Minneapolis, MN) and anti-CCR-2B (Fig. 8) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (both diluted in PBS, 1:250) (13). Subsequently, they were incubated with biotinylated secondary antibody followed by the streptavidin-peroxidase treatment. The sections were developed with 3,3′-diaminobenzidine solution as chromogen and then counterstained with hematoxylin and dehydrated, cleared, and mounted. Negative controls were prepared by omitting the primary antibodies and by their substitution with corresponding IgG at the dilution used for the specific antibodies in this study.

Primary Single Muscle Fiber Isolation and Culture—Mice were euthanized by CO2 inhalation. The skin was prepared with 70% ethanol, and the legs were skinned and placed into room temperature Dulbecco’s modified Eagle’s medium. Muscles were dissected out and placed into Dulbecco’s modified Eagle’s medium. Muscles were dissected out and placed into collagenase type I (Worthington) 400 units in 10 ml of Dulbecco’s modified Eagle’s medium and digested at 37 °C for 70 min. Individual live muscle fibers were collected under a dissecting microscope using polychrom glass pipettes and then cultured in Dulbecco’s modified Eagle’s medium + 20% fetal bovine serum supplemented with antibiotics and glutamine.

Satellite Cell Proliferation Assay—Bromodeoxyuridine was added to cultures at a final concentration of 10 μM (for varying length pulses all ending at 72 h of culture). At the end of the pulse, fibers were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature, then washed again in PBS. Fibers were then treated with 4 μ
HCl for 10 min, washed with PBS, then treated with 1% Triton-X in PBS for 4 min and washed again with PBS. Primary anti-bromodeoxyuridine antibody (rat monoclonal, Harlan Sera Labs) was applied in a 1:10 dilution at 4 °C overnight. After PBS washes, the secondary antibody donkey anti-rat (Alexa Fluor 594, Molecular Probes) was incubated for 1 h at room temperature at 1:400 dilution. After PBS washes, the fibers were mounted using VectaShield with 4,6-diamidino-2-phenylindole (Vector Labs, Burlingame CA). Fluorescent, stained satellite cells were counted as a function of unit length of fiber (one 200X field diameter) (14).

Satellite Cell Apoptosis—After 72 h in culture, fibers were fixed in 4% paraformaldehyde as above, washed in PBS, then permeabilized with 0.1M sodium citrate/0.1% Triton-X on ice for 2 min. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling was then performed with antibody to the nick end DNA conjugated to fluorescein isothiocyanate (Roche Applied Science) for 1 h at 37 °C. Fibers were mounted, and stained fluorescent satellite cells were counted as for proliferation studies.

RESULTS

The ΔRG allele of tuberin was introduced into NIH3T3 fibroblasts to assess signaling abnormalities. Because various oncogenes have been shown to induce reactive oxygen, and reactive oxygen has been shown to contribute to cellular transformation (15–17), we assessed the effect of this mutant tuberin allele on generation of reactive oxygen. The ΔRG allele of tuberin caused a significant increase in intracellular hydrogen peroxide as shown by DCF fluorescence (Fig. 1).

We have shown previously that PDGF-BB plays an important role in the pathogenesis of tuberous sclerosis lesions. Thus, we assessed the effect of the ΔRG allele on response to exogenous PDGF-BB. Cells expressing ΔRG or parental NIH3T3 were exposed to PDGF-BB. Cells expressing ΔRG showed an increase in phosphorylation of a 185-kB protein consistent with PDGFRβ (Fig. 2A). In addition, cells expressing ΔRG had increased levels of phosphorylation of akt at baseline and enhanced phosphorylation of akt in response to PDGF-BB (Fig. 2B).

Loss of tuberin has been associated with activation of protein synthesis, namely through the activation of the ribosomal proteins S6 and S6 kinase. Similarly, expression of the ΔRG mutant tuberin led to activation of S6 and S6 kinase (Fig. 3). Expression of the ΔRG allele also caused a strong induction of the angiogenic factor VEGF (Fig. 4).

Three lines of transgenic mice were established (922, 928, and 945). Integration of the mutant allele was confirmed by PCR using primers flanking exons 8 and 9 of tuberin. Genomic DNA gives a band of 567 bp, whereas transgene DNA (cDNA) will give a 281-bp band.

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cases did the aggregates extend outward into the subarachnoid space. No developmental or dysplastic changes resembling cortical tubers were noted in the cortex of cerebral hemispheres. No subependymal dysplastic growths were noted in along the ventricular system. No neoplastic growths were noted within the lateral ventricles of any brains.

The dermis and perimuscular area were remarkable for thickened muscle and a myxoid accumulation of cells in a perimuscular area, which was rich in mast cells (Fig. 7, E–G). These lesions histologically resemble collagenomas (Shagreen patches), which are present in a significant number of patients with tuberous sclerosis. These lesions are present in all of the transgenic mice but not in the wild type littermates. To assess whether the transgene resulted in increased proliferation or apoptosis assays on wild type and transgenic mice. No differences in proliferation or apoptosis were observed, suggesting that these do not represent accumulations of muscle satellite cells (data not shown).

Monocyte chemotactic factor 1 (MCP-1) has been associated with various mesenchymal proliferations, including cirrhosis of the liver. Recently, MCP-1 has been shown to be up-regulated in human lesions of TS. We examined the expression of MCP-1 and its receptor, CCR-2, and found that the expression of both of these genes is elevated in the dermal lesions of the transgenic mice but not in wild type mice (Fig. 8).

**Fig. 6.** PCR screening of typical litter from ΔRG line number 922.

**Fig. 7.** Mice constitutively expressing mutant tuberin develop skin and brain hamartomas. A and B represent low and high power micrographs of cerebellum from normal mice, whereas C and D represent similar sections from age matched ΔRG transgenic mice. Note the clusters of external granule cells in C and D, which have failed to migrate to the appropriate site and are retained in a subpial fashion, whereas normal cerebellum (A and B) does not show persistence of external granule cells. E (low power) represents a fibrovascular proliferation at the junction between the dermis and muscle in ΔRG mice, reminiscent of collagenoma histopathology. F represents a high power view of the hematoxylin and eosin histology of E. This collagenous proliferation, which is not observed in normal mouse skin, is highly rich in mast cells as observed through toluidine blue staining (G).

**Fig. 8.** Transgenic mice express high levels of MCP-1 and its receptor in the skin. A shows a high power view of MCP-1 staining in transgenic skin, whereas Fig. 6B shows MCP-1 staining in wild type skin. Fig. 8, B and C, show low power views of transgenic and wild type skin. Note the thickened muscle layers and additional cellularity in the transgenic mouse (C) compared with the wild type mouse (D). Fig. 6, E and F, represent CCR2 staining of transgenic (Fig. 8E) and wild type (F), respectively. The staining for CCR2 appears predominantly on mast cells (E).

**DISCUSSION**

Tuberous sclerosis is a common autosomal dominant disorder characterized by an increased incidence of benign and malignant tumors as well as developmental hamartomas. In childhood, the major cause of morbidity and mortality are cerebral tubers, which histologically are giant cells with a tendency to calcify and become foci for seizures. Subependymal giant cell astrocytomas appear in a periventricular distribution and may cause ventricular obstruction through continued growth.

Lesions in TS can be divided into those that usually show LOH for tsc1/tsc2 and those that do not. Angiomyolipomas and lymphangiomyomatosis, clonal neoplasms with features of smooth muscle, fat, and melanocytes, commonly exhibit LOH (10, 18). On the other hand, cerebral tubers usually do not exhibit LOH (19). We propose two potential explanations for the development of lesions in TS in the absence of LOH. First, we propose that certain mutations in tsc2 can act as dominant negatives and acquire novel functions through the activation of aberrant signaling pathways (12). A consequence of this is heightened sensitivity of certain cells to physiologic levels of growth factors. We and others have previously demonstrated that TS model cells are highly sensitive to PDGF (20, 21). A second mechanism that we have observed is LOH of other tumor suppressor genes, and we have previously observed LOH of p16ink4a in a tumor arising in a mouse heterozygous for tuberin (22). Interestingly, although this tumor had lost p16ink4a, it retained a wild type tsc2 gene (22).

Recently, several other potential targets of tuberin have been discovered. Several groups have found that tuberin serves as an inhibitor of phosphoinositol-3 kinase/akt signaling, and overexpression of tuberin leads to decreased phosphorylation of

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\(^2\) T. Darling, personal communication.
akt (23–25). In addition, overexpression of tuberin has been shown to inhibit signaling through pathways that inhibit protein processing, such as target of rapamycin, ribosomal protein S6, and S6 kinase (Fig. 3) (25–27). These proteins are involved in the translation of proteins involved in cell growth, such as VEGF (Fig. 4) (28). However, systemic long term use of rapamycin in humans may not be an optimal therapy for TS, as rapamycin is a potent immunosuppressant and may lead to severe infections and neoplasms because of loss of immune surveillance (29). Other targets of tuberin have been proposed. In an in vivo study of kerat cells containing a tetracycline inducible tuberin, gene chip array has found several genes induced, including thyroid-specific antioxidant and glutathione peroxidase, indicating that tuberin may protect cells against oxidative stress, and cells lacking tuberin or may have increased reactive oxygen species (30). We have previously shown that reactive oxygen species have been shown to contribute to mitogenesis and tumorigenesis (31).

In normal cerebellar development, external granular cells at the cerebellar surface are a proliferative population that eventually migrates inward to populate the internal granular cell layer. After this process early in life, the external granular layer is largely depleted with only scattered remnant granular cells. In the transgenic animals in this study, we found numerous microscopic aggregates of mature granular cells in the subpial region suggesting that their normal migration inward may have been disrupted. This particular phenotype is not encountered in the cerebellum of tuberous sclerosis patients. However, the cortical tubers and subependymal nodules that characterize this disease in the cerebral hemispheres are architecturally abnormal collections of neuronal and glial elements that are most likely caused by disordered migration and development. Subpial cerebellar aggregates of granular cells may be the murine manifestation of this disordered developmental program. No other central nervous system stigmata of tuberous sclerosis were noted in transgenic mice, and no neurologic symptoms could be ascribed to the cerebellar changes. We have demonstrated that mutant tuberin can cause increased levels of intracellular reactive oxygen. Reactive oxygen has been implicated in the induction of fibrogenic cytokines, such as MCP-1, and increased expression of MCP-1 and its receptor CCR2 have been observed in fibrotic processes associated with reactive oxygen, such as bleomycin-induced scleroderma, idiopathic scleroderma, and hepatic fibrosis/cirrhosis (32–35). Lesional skin from transgenic mice demonstrate elevated levels of MCP-1 compared with wild type littermate skin, suggesting that MCP-1 may play a pathogenic role in TS lesions. Interestingly, PDGF is a known stimulant of MCP-1, and the introduction of the ARG allele of tuberin results in increased responsiveness to PDGF (35). This increased sensitivity could underlie in part the increased production of MCP-1 in our transgenic mice. Inhibition of MCP-1/CCR2-2 interactions may be beneficial in the treatment of tuberous sclerosis, and mice deficient in either of these genes have a decreased fibrotic response to various experimental stimuli (32, 36).

Prior studies have addressed signaling abnormalities in the absence of tsc2. However, none of these studies have addressed signaling abnormalities seen in the presence of a dominant negative tuberin. In this study, we show that a dominant negative tuberin can recapitulate many of the signaling abnormalities observed because of loss of tuberin. In vivo expression of mutant tuberin results in a unique phenotype not previously observed in nonhuman tuberous sclerosis models. Finally, we show that constitutive expression of a dominant negative gene leads to a tissue-specific phenotype resembling the human disease. Our model may be valuable in assessing contributions to tissue specificity and may prove useful in pharmacologic studies to prevent or treat tuberous sclerosis.

REFERENCES

1. Shepherd, C. W., Gomez, M. R., Lie, J. T., and Crowson, C. S. (1991) Mayo Clin. Proc. 66, 792–796
2. Sampson, J. R., and Harris, P. C. (1994) Hum. Mol. Genet. 3, 1477–1480
3. van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, O. A., Hailey, D. Young, J., Buckland, M., Jeremiah, K., Woodward, K., Nahmias, J. Fox, M., Ekong, E., Osborne, J., Wolfe, J., Povey, S., Blag, R. E., Cheadle, J. P., Jones, A. C., Tachakital, M., Ravine, D., and Kwiatkowski, D. J. (1997) Science 277, 940–948
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