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Animal Model

Subependymal Astrocytic Hamartomas in the Eker Rat Model of Tuberous Sclerosis

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Tuberous sclerosis (TSC) is an autosomal dominant syndrome that is linked to two genetic loci: *TSC1* (9q34) and *TSC2* (16p13). Brain manifestations such as cortical tubers and subependymal hamartoma/giant cell astrocytomas are major causes of TSC-related morbidity. In this study, we describe the central nervous system involvement in a unique rodent model of tuberous sclerosis. The Eker rat carries a spontaneous germline mutation of the *TSC2* gene and is predisposed to multiple neoplasia. In a series of 45 adult Eker carriers ($TSC2^{+/-}$), three types of focal intracranial lesions were found, of which the subependymal and subcortical hamartomas were most prevalent (65%). There exist remarkable phenotypic similarities between the Eker rat and human subependymal lesions. Our study indicates that the predominant cellular phenotype of the subependymal hamartomas is astroglial and suggests that the neuronal contribution within these lesions is, in part, the result of pre-existing myelinated axons. The hamartomas did not show evidence of loss of the wild-type *TSC2* allele; it remains to be determined whether *TSC2* inactivation is necessary for their pathogenesis. This genetically-defined rodent model may be useful in elucidating the molecular and developmental basis of the subependymal giant cell astrocytoma in humans. *(Am J Pathol 1997, 151:1477–1486)*

Tuberous sclerosis (TSC) is a complex, multiorgan disease characterized by the development of benign tumors (hamartomas) and malformations (hamartias).[1] This autosomal dominant disorder affects ~1 person per 6000 to 10,000 with at least 50% of cases representing new mutations. Since the initial description of the neurological involvement in a patient with TSC by Bourneville in 1880, accounts of lesions arising in all anatomical sites have been reported with the rare exceptions of the spinal cord, peripheral nerves, pineal gland, thymus, and skeletal muscle.[1] The disease frequently presents during infancy but the phenotype is highly variable. Most commonly, affected individuals develop signs and symptoms of neurological dysfunction (epilepsy, mental retardation, motor deficits, behavioral derangements). Clinical studies have provided correlative data that linked the development of epilepsy with cortical tubers,[2] but little is known about the pathoanatomical correlates underlying psychiatric and behavioral symptoms. Another common intracranial manifestation of TSC is the presence of subependymal hamartomas. These nodular excrescences often protrude into the ventricles as smooth, rounded elevations resembling "candle gutterings". In contrast to cortical tubers, subependymal lesions have a tendency to grow and expand resulting in subependymal giant cell astrocytomas (SEGAs). These lesions may cause obstruction of the cerebrospinal fluid circulation leading to signs and symptoms of increased intracranial pressure and/or seizures. Current therapeutic modalities are limited to symptomatic relief and/or treatment of complications. The overall survival of TSC-affected individuals is diminished as a result of the central nervous system (CNS) and renal manifestations.[3]

Genetic analyses of families with TSC have identified two loci that account for the majority of kindreds.[4] *TSC1* maps to human chromosome 9q34 and *TSC2* to chromosome 16p13.3. Recent effort by the European Consortium has resulted in the positional cloning of the *TSC2* gene.[5] The latter is found to be a novel coding sequence consisting of 41 exons and encodes an ~190-kd protein with specific GAP (GTPase activating protein) activities toward the Ras family of monomeric GTPases.[6,7] Whereas the biochemical pathway of tuberin, the *TSC2* gene product, remains to be elucidated, its GAP activity is postu-

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Table 1. Antibodies to Glial and Neuronal Proteins

Clone	Antigen/Epitope	Ig Type	Dilution	Source
Mouse monoclonal antibodies*				
SMI-22†	GFAP	IgG2b	1:2000	Sternberger Monoclonals Incorporated, Baltimore, Maryland
SMI-31	NF-HM P+	IgG1	1:2000	Sternberger Monoclonals Incorporated
SMI-33	NF-H/M P−/Pind	IgM§	1:2000	Sternberger Monoclonals Incorporated
SMI-311‡	NF P−	IgG1/IgM§	1:2000	Sternberger Monoclonals Incorporated
SMI-94	MBP¶	IgG1	1:2000	Sternberger Monoclonals Incorporated
CL-300	Calbindin-D28k	IgG1	1:200	Sigma Immunochemicals, St. Louis, Missouri
TuJ1	βIII	Ig2a	1:500	A. Frankfurter, Department of Biology, University of Virginia
Rabbit polyclonal antiserum				
	anti-GFAP		1:800	Dako, Santa Barbara, California

Abbreviations: NF-H/M P+, phosphorylated epitope in extensively phosphorylated high molecular mass (H) subunit of neurofilament protein (~200 kd) and, to a lesser extent, in middle molecular mass (M) (~150 kd) subunit of neurofilament protein; NF-H/M P−/Pind, nonphosphorylated, probably phosphorylation-independent, epitope in phosphorylated and nonphosphorylated neurofilament H and, to a lesser extent, in neurofilament M; NF P−, nonphosphorylated neurofilament protein, not otherwise specified; MBP, myelin basic protein.

*All SMI mAbs react with rat brain tissue.[40] The immunogens for SMI-31, SMI-33, and SMI-311 are rat species derived; †Cocktail of clones Mab1B4All ("cocktail of Bigner-Eng antibodies to GFAP"). The mixture of mAbs provides a more comprehensive detection of astrocytes; ‡Cocktail of mAbs against nonphosphorylated NFs serving as a generic neuronal marker; §Reacts with goat anti-mouse IgG and Clono PAP (SMI) via light chains common to IgG and IgM; ¶Peptide fragment corresponding to sequence 70–89 of human MBP shared by the rat.

lated to be important in regulating cell growth and proliferation. Molecular analyses have identified loss of heterozygosity at the *TSC2* locus in various lesions isolated from TSC patients.[8,9] It is inferred that both alleles of the *TSC2* gene are inactivated during the pathogenesis of the hamartomas and therefore functions as a tumor suppressor gene. This is analogous to the genes involved in other phakomatoses (eg, NF1, NF2 in neurofibromatosis or VHL in von Hippel Lindau syndrome). Attempts at *in vivo* and *in vitro* biological and biochemical studies of this complex human disease are made difficult in the absence of a suitable animal model. In this study, we report the frequent occurrence of subependymal lesions and other CNS pathology that are unique to the Eker rat carrying a *TSC2* germline mutation. The striking parallel in genotype and phenotype between the human and rat highlights the potential usefulness of this genetically defined model in the investigation of the molecular neuropathogenesis of tuberous sclerosis.

The Eker rat has been described several decades ago as a model for hereditary renal cell carcinoma.[10] In this autosomal dominant syndrome, carriers develop multiple and bilateral kidney tumors with near-complete penetrance. Other manifestations of the affected rat include renal cysts and neoplasia of the spleen (hemangiosarcoma) and uterus (leiomyoma). To date, no neurological abnormality has been documented. In the study of the genetic mechanism of these unique cancer-prone rats, we and others discovered that the *TSC2* gene is mutated in the germline due to an intronic retrotransposition of a rat intracisternal-A particle (IAP) element.[11–13] The molecular consequence of this mutation gives rise to the transcription of aberrant mRNAs that encode for putative gene products that are deleted in their carboxy termini containing the GAP homology domain. As in human, the analysis of the spontaneous Eker renal and uterine tumors demonstrated loss of heterozygosity (LOH) in the majority of cases.[14] Predictably, these tumors do not express the normal 190-kd protein. Direct experimental evidence of the tumor suppressor function of tuberin came from studies in which the wild-type *TSC2* gene was introduced into the Eker derived tumor cells. Expression of the normal *TSC2* protein effectively inhibited cell proliferation *in vitro* and tumorigenicity *in vivo*.[15]

Given that the Eker rats carry a mutation of the *TSC2* gene, we further explored the possibility of phenotypic resemblance with the clinical disorder. On detailed histological analysis of aging Eker carriers, we found evidence of CNS lesions that are homologous to the SEGA and subcortical hamartomas described in tuberous sclerosis. This report summarizes the pathological features of these CNS lesions in the Eker rat.

Materials and Methods

Animals

The Eker rats of the Fischer 344 strain were bred and genotyped as previously described.[11] Forty-three carriers and four noncarriers greater than 18 months of age were killed by CO_2 inhalation. Complete autopsies were performed with focus on the central nervous system. After fixation in 10% neutral buffered formaldehyde, five coronal consecutive sections of the brain were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Immunohistochemistry

The phenotypic profile of tumefactive lesions was examined using a panel of antibodies to neuronal and glial antigens (Table 1). Paraffin sections were dehydrated in xylene and graded alcohols and washed in Tris-buffered saline, pH 7.6. Endogenous peroxidase was blocked by

incubation of slides in 1.2% hydrogen peroxide in cold methanol for 30 minutes. Sections were subsequently stained according to the unlabeled antibody peroxidase-antiperoxidase method as previously described.[16] All primary antibodies were incubated for 1 hour at room temperature. No antigen retrieval method was used. The sections were stained with 3,3′-diaminobenzidine as the substrate and then lightly counterstained with Mayer's hematoxylin and mounted. For comparison, adjacent sections were reacted in parallel according to the avidin-biotin complex (ABC) method using rabbit IgG and mouse IgG Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations for the rabbit anti-glial fibrillary acidic protein (GFAP) antiserum and mouse monoclonal antibodies, respectively. Both peroxidase-antiperoxidase and ABC methods yielded comparable results. Negative controls included nonspecific rabbit IgG and mouse ascites fluid in the absence of the primary antibodies and did not demonstrate cross-reactivity.

Loss of Heterozygosity

Materials from CNS and pituitary lesions were obtained by microdissection from 5 to 10 5-μm thick paraffin sections. The tissues were deparaffinized, rehydrated in the presence of Chelex 100 (Bio-Rad, Hercules, CA), and treated with proteinase K to isolate DNA, according to previously described procedure.[17] Two sets of polymerase chain reaction (PCR) primers were designed to identify specific *TSC2* alleles in the Eker rat.[14] Semi-nested PCR amplification was used to produce a 215-bp fragment of the wild-type allele. DNA samples were first amplified with primers RY152 (5′-GTTTAGGAGCAGGCTCG TGC) and RY75 (5′-GCTGACGTATGAGTGTCCTC) followed by another 35 cycles using RY152 and RY74 (5′-GGGAGCACACAAGCAGGCAA). The Eker mutant allele produced no PCR products due to the IAP insertion. In cases in which semi-nested PCR failed to amplify the expected size fragment, allele nonspecific primers RY201 (5′-GCCATACAGAGTCAACCACG)and RY203 (5′-CAAGTCCTTCCTGCACATTGG) were used to monitor DNA quality and quantity. Each experiment was repeated twice to assure reproducibility.

Results

Forty-three matured rats from Ek/+ × +/+ mating were genotyped as Ek/+ carriers and underwent complete necropsy at ages between 18 and 24 months. It should be noted that none of these rats displayed any recognizable neurological or behavioral disturbance. All animals harbored renal tumors ranging from 3 mm to 3 cm in size, which confirmed their carrier status. Gross examination of the brains did not reveal any overt structural abnormality expect for hydrocephalus in one case. No cortical tubers were detected. However, subependymal hamartomas (SEH) in 19 (44%), subcortical hamartomas (SCH) in 9 (21%), and other changes consistent with SEH in 2 were present on histological evaluation (Table 2). In addition, 14 (33%) meningiomas and 25 (58%) pituitary adenomas were evident. No significant correlation was found between the presence of SEH/SCH and the occurrence of pituitary lesions or meningioma. Of the 25 carriers with SEH or SCH, 14 were associated with pituitary tumors and 8 had meningioma. Among the 25 rats with pituitary lesions, 9 also developed meningioma. Only four Eker carriers had all three types of CNS lesions. In total, 63% (27 of 43) of the Eker rats developed brain lesions bearing resemblance to those found in tuberous sclerosis. Examination of four age-matched noncarrier littermates failed to identify any such lesions ($P = 0.03$).

Subependymal Lesions

Examination of coronal sections at the levels of the corpus striatum and posterior thalamus revealed discrete, nodular parenchymal hamartomas. The origin of these circumscribed lesions was traceable to the subependymal (paraventricular) region of the lateral ventricle most commonly in the anterior horn (Figure 1a). The majority of paraventricular tumors occupied portions of the caudate nucleus and deep periventricular white matter as well as extending to the mesial frontal gyri. On the whole, these tumors grew in an expansive, as opposed to diffuse, infiltrative/invasive fashion thus remarkably sparing the histoarchitecture of surrounding basal ganglia. Bilateral lesions were found in 21% (4 of 19) of cases. Unlike the more florid candle guttering exophytic growth pattern described in human SEH, the Eker rat lesions were generally small (<2 mm) and did not protrude as prominently into the ventricles. We found, however, 4 of 19 hamartomas that projected *in toto* into the lateral ventricles.

These lesions were composed in varying proportions of large cells with abundant eosinophilic or amphophilic cytoplasm, large nuclei and occasionally conspicuous nucleoli, and intermingled with elongated, highly fibrillated cell processes (Figure 1b). Scattered Rosenthal fibers were present within the tumors (not shown). Frequently, vacuolar change was noted around cell bodies. The identity of the large "balloon" cells, particularly those with prominent nucleoli, was difficult to ascertain on morphological grounds. Although most cells were convincingly astroglial, a small number of cells had a ganglioid morphology, which raised the issue of divergent neuronal differentiation. The phenotype of these cells was additionally analyzed by immunohistochemistry (see below).

As with the SEGA in tuberous sclerosis, the Eker lesions were characterized by an anomalous local growth and disturbance in neuroepithelial cell architecture. Multifocal intratumoral calcifications were present in 26% (5 of 19) of the hamartomas, particularly in those with a predominant intraventricular growth. There was no evidence of malignant growth in the context of overt anaplasia, invasion, or dissemination within the neuraxis. Twenty percent of the paraventricular tumors contained in their periphery dense clusters of immature neuroepithelial cells (Figure 2F). These were not found in lesions situated

Table 2. Intracranial Manifestations in the Eker Rats

Case	Renal tumors	Intracranial manifestations				
				Brain		
		Meningioma	Pituitary	Lesion	Location	Others
Carriers (Ek/+)						
1 M	+	−	−	SCH	Parietal	
2 M	+	+	+; C	−		
3 M	+	−	+; C	−		
4 F	+	−	−	−		
5 M	+	−	+; C	SEH	Striatum	
6 F	+	+	+; A	SEH	Striatum	
7 M	+	−	+; C	−		Hydrocephalus
8 M	+	+	−	−		
9 F	+	−	−	SCH	Occipital, parietal	
10 F	+	−	−	SEH	Frontal septal	
11 M	+	−	+	SEH	Frontal septal	
12 M	+	−	−	−		
13 M	+	−	−	−		
14 M	+	−	−	−		
15 M	+	−	+; C	Early changes*	Frontal	
16 M	+	+	+; C	−		
17 F	+	−	−	SEH	Frontal septal	
18 M	+	−	+; C	SEH	Frontal septal	
19 M	+	+	+; C	SEH	Lateral ventricle	
20 F	+	+	−	SEH	Frontal	
21 F	+	−	−	SEH	Frontal	
22 F	+	−	+; C	−		
23 M		−	+; C	−		Hydrocephalus
24 F	+	−	−	SCH	Frontal	
25 M		+	−	SEH	Parietal	
26 F	+	+	+; C/A	−		
27 F	+	+	−	SEH, SCH	Parietal	
28 F	+	−	+; C	SCH	Parietal	
29 F	+	+	−	−		
30 M	+	−	+; C	SCH	Frontal	
31 F	+	−	−	−		
32 M	+	−	−	SCH	Frontal	
33 F	+	−	−	SEH	Parietal	
34 F	+	+	+; C	SEH	Frontal, rhinencephalon	
35 M	+	−	+; C	SEH	Frontal, rhinencephalon	
36 M	+	−	+; C/A	Early changes*	Frontal	
37 M	+	−	+; C/A	SEH, SCH	Frontal, parietal	Prominent germinal matrix aggregates†
38 M	+	−	+; C	SEH	Frontal	
39 M	+	+	+; C	−		
40 M	+	+	+; C	SCH	Parietal	
41 M	+	+	+; C	SEH	Septal, frontal parietal	Prominent germinal matrix aggregates†
42 M	+	−	+; C	SEH	Septal, frontal	
43 F	+	−	+; A	SEH, SCH	Septal, frontal, Rhinencephalon	Prominent germinal matrix aggregates†
Noncarriers (+/+)						
1 M	−	−	−	−		
2 M	−	−	−	−		
3 F	−	−	−	−		
4 F	−	−	−	−		

Abbreviations: C, chromophobe; A, acidophilic; *early changes: cytoarchitectural disorganization, astrocytosis, and vacuolization; †invariably ipsilateral to the hamartomas; +, present; −, absent.

away from the subependymal matrix (ie, SCH, see below).

Subcortical Lesions

Somewhat less common, smaller lesions resembling the SEH were found at some distance away from the ventricles. They were either in the hemispheric white matter or in the corticomedullary junction of the frontoparietal convexity (Figure 1c). One tumor was present in the body of the corpus callosum, whereas another was found in the deep periventricular white matter about the inferior horn involving portions of the adjoining forniceal bundle. The growth patterns of these extraventricular hamartomas, in so far as they were discrete, nodular, and noninvasive, were identical to the SEHs. Differences between the

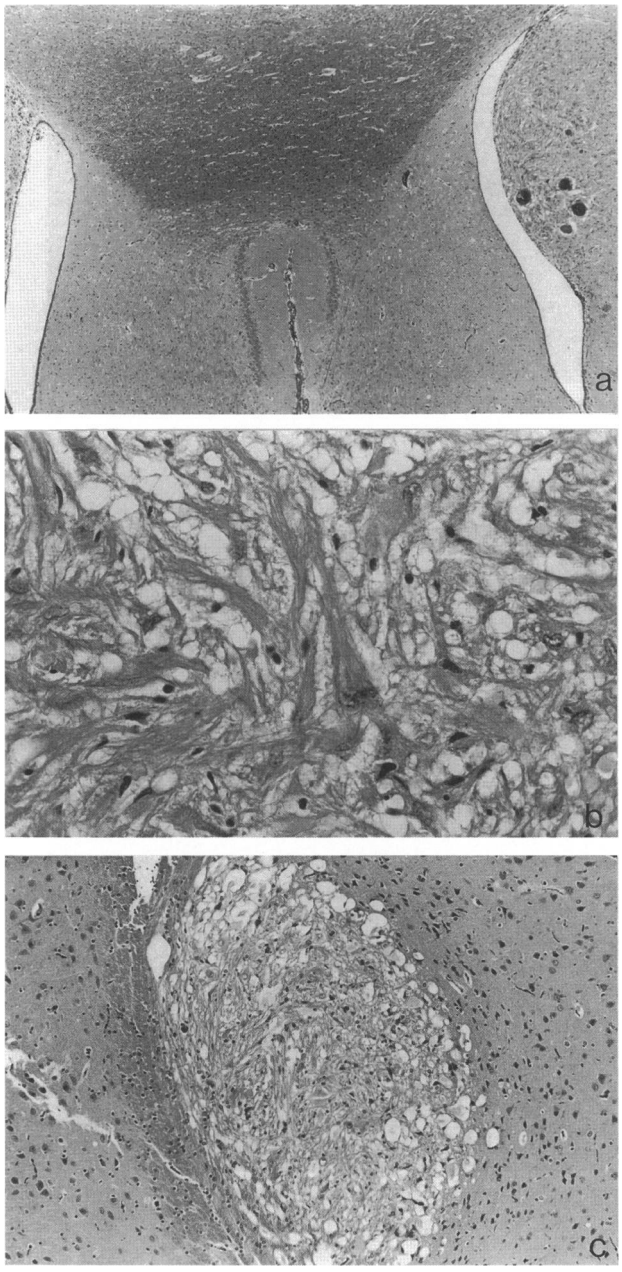


Figure 1. a: Low-power view of a subependymal hamartoma located adjacent to the lateral ventricle near the frontal horn. Note the expansive growth pattern and the presence of intralesional calcifications. Original magnification, ×16. **b:** High-power view of a typical SEH depicting the admixture of spindle-shaped cells and giant eosinophilic cells with large lobulated or multiple nuclei, intermingled with dense fibrillary processes. Original magnification, ×250. **c:** Example of a subcortical hamartoma located in the corticomedullary junction. The morphological features of these lesions resemble those of the SEH. Note the characteristic vacuolation around large, presumably gliotypic cells. Original magnification, ×120. Hematoxylin and eosin.

paraventricular and subcortical lesions included the smaller size of SCH, the absence of Rosenthal fibers, and rare intratumoral calcifications.

Other Findings

The presence of pituitary tumors in more than 50% of Eker carriers is highly significant. Although adult wild-type rats can develop such tumors, the observed incidence is far below 50%. The majority of these lesions were chromophobic (20 of 25), whereas two were acidophilic and three were of mixed histology. Detailed immunophenotypic characterization of these lesions is the focus of ongoing investigation. Also of interest is the predisposition to meningiomas that was arising from the inferofrontal/orbital parietal leptomeninges. These benign transitional tumors comprised meningothelial, psammomatous, and fibrous components.

In the cerebral neocortex, no overt cytoarchitectural disturbances were noted. No classical cortical tubers were identified. However, as described above, occasional SCHs were seen in the corticomedullary junction. With the exception of rare, maloriented (heterotaxic) pyramidal neurons, the overall architecture of the Ammon's horn was preserved.

Immunohistochemistry

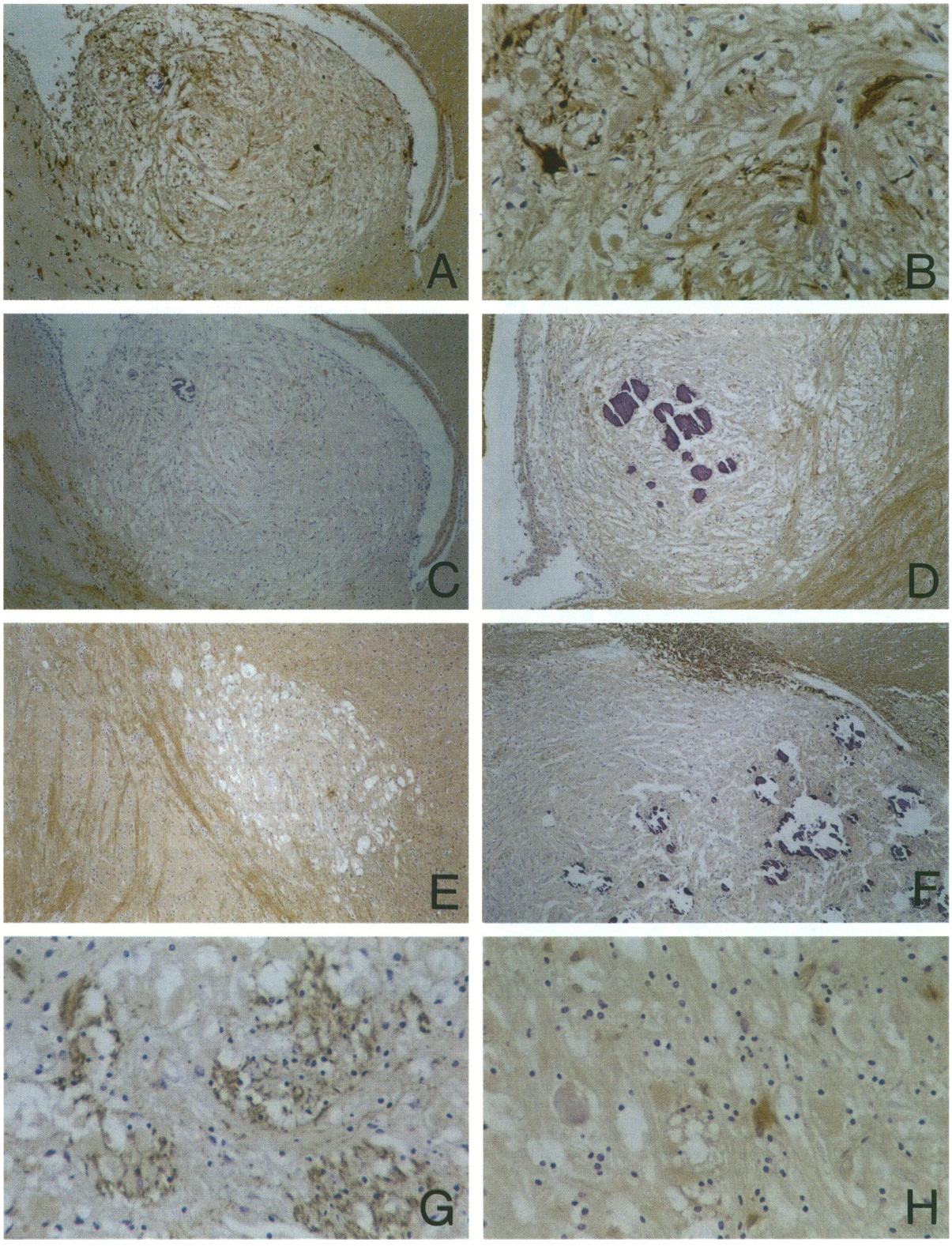
The phenotype of the cells within the subependymal (SEH) and subcortical (SCH) lesions was characterized using a panel of antibodies to neuronal and glial markers expressed in the rat (Table 1).

Glial Fibrillary Acidic Protein

The majority of cells in the SEHs and SCHs were immunoreactive with antibodies to GFAP attesting to a predominantly glial/astrocytic phenotype. Robust GFAP immunoreactivity was present in some of the "giant" astrocytes and their multipolar, stellate processes (Figure 2, A and B). Reactivity was more widespread with the SMI-22 mAb as compared with the polyclonal antiserum to GFAP. In some areas of the hamartomas, little or no anti-GFAP reactivity was detected, including some giant cells. Widespread GFAP staining was noted in reactive astrocytes in the adjoining caudate nucleus and hemispheric white matter (Figure 2A).

Neurofilament Protein

The bulk of the hamartomas, including the large astroglial or ganglioid-appearing cells, did not stain with anti-neurofilament monoclonal antibodies, SMI-33 and SMI-311 (Figure 2C). However, SMI-31 immunoreactivity was evident in pre-existing axons traversing the hamartomas, particularly in those embedded with the substance of the caudatum or cerebral hemispheric white matter (Figure 2D). This raises the possibility that the fibrillary matrix in most hamartomas is contributed, at least in part, by native axons either originating in the striae of the caudate nucleus or hemispheric white matter. Interestingly, these axonal projections are also present in the subependymal lesions exhibiting a prominent intraventricular growth pattern. SMI-31 (anti-phosphoneurofilament) localization was seen in rare, bulbous masses in the periphery of the SEH in 2 of 15 cases (not shown). In the neocortex, there was no significant difference in the distribution and com-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partmentalization of the neurofilament epitopes between the Eker carriers and noncarriers.

Class III β-Tubulin (βIII)

Monoclonal antibody TuJ1 (anti-βIII) is a panneuronal marker yielding strong immunoreactivity in normal cell bodies, dendrites, and axons. Mature mesenchymal, glial, and ependymal cells are nonreactive. In SEH/SCH, only pre-existing axons traversing the substance of the Eker hamartomas were immunoreactive for βIII (Figure 2E). In contrast, the large eosinophilic cells and their processes were consistently negative. Staining for βIII was also present in the morphologically immature cells of the subependymal matrix. In some lesions, these cells were noted in increased numbers in the periphery of SEHs (Figure 2F).

Myelin Basic Protein

In the mature rat brain, monoclonal antibody SMI-94 stains myelinated axons exclusively. Colocalization of myelin based protein in the intralesional fibers that are also phosphoneurofilament (SMI-31)-positive and βIII-positive provides additional evidence that these represent pre-existing mature axons originating from neighboring brain parenchyma (Figure 2G).

Calbindin-D28k

This protein is normally distributed in subpopulations of cerebral and cerebellar neurons (striatal, hippocampal, hypothalamic, and Purkinje neurons). Only rare entrapped striatal interneurons and small glial-appearing cells situated in the periphery of the few (2 of 15) subependymal hamartomas were calbindin-D28k positive (Figure 2H).

Loss of Heterozygosity

To determine whether the CNS lesions undergo two-hit inactivation of *TSC2* gene similar to those reported for tumors/hamartomas of other sites in the Eker rat and patients with tuberous sclerosis, five SEHs and one SCH

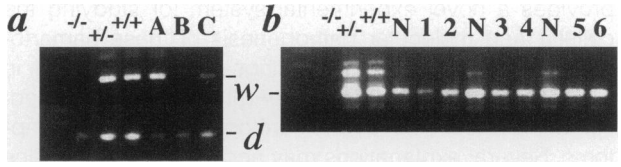

Figure 3. a: Pituitary adenomas and **b:** SEH were microdissected from paraffin sections and tested for LOH using allele-specific PCR amplification. Although pituitary lesion (**B**) has lost the wild-type *TSC2* allele (**w**), none of the 6 SEH showed LOH. PCR products were separated in 0.8% agarose gel, stained with ethidium bromide and photographed. −/−, +/−, +/−, +/+, controls. **N**, adjacent tissue. **d**, product of nonEker specific *TSC2* allelic amplification.

from three animals were microdissected from paraffin sections for allelic analysis. By choosing appropriate primer sequences that flanked the Eker 6.5-kb insertion, we can distinguish whether the DNA sample contained a wild-type *TSC2* allele. Previous analysis of renal and uterine tumors of the Eker rat has demonstrated that in every case of LOH, the wild-type allele was lost while retaining the mutant Eker allele. Compared with adjacent normal brain parenchyma, semi-nested PCR amplification of all CNS lesions tested scored positive for the 215-bp fragment implying that the normal *TSC2* allele in these lesions has not been lost (Figure 3b). We cannot rule out with certainty that the source of the wild-type allele may be arising from normal cells lying adjacent to or within the lesion of interest. In contrast, one of three pituitary adenomas sample (B) showed evidence of LOH (Figure 3a). Compared with PCR products of RY202 and RY203, which does not distinguish between wild-type and Eker *TSC2* alleles, the quantity of the wild-type allele-specific semi-nested product of tumor 921B was significantly reduced but not absent. We interpreted this as being the result of normal tissue contamination within the primary tumor.

Discussion

A neuropathological hallmark of tuberous sclerosis is the development of cortical tubers and subependymal hamartomas. In this study, we demonstrated that analogous subependymal lesions were found in 63% of the Eker rats carrying a *TSC2* mutation. This animal model

Figure 2. Immunohistochemical findings of subependymal hamartomas in the adult Eker rat, using glial and neuronal markers. **A:** Low-power view of a SEH immunostained with mAb SMI-22 (comprising a mixture of clones of antibodies to glial fibrillary acidic protein). Widespread GFAP immunoreactivity is present in fibrillated astrocytic cells constituting the bulk of the lesion. Note GFAP-labeled reactive astrocytes in the subjacent caudate nucleus. Original magnification, ×100. **B:** Higher magnification of **A** showing filamentous GFAP localization in astrocytic cell bodies and processes. Original magnification, ×400. **C:** Low-power view of the same subependymal hamartoma depicted in **A**, immunostained with mAb SMI-311 (comprising a mixture of clones of antibodies to nonphosphorylated epitopes of neurofilament protein). Staining is absent in the hamartomatous lesion as compared with the immunoreactive neuropil in the contiguous caudate nucleus. Note a single intralesional calcification. Original magnification, ×100. **D:** Low-power view of the SEH immunostained with mAb TuJ1 specific for the class III β-tubulin isotype. Although the bulk of the hamartoma is nonreactive, there is staining of fascicles of nerve fibers traversing the substance of the lesion from its periphery. These axonal fibers probably originate in the caudoputamen and merge imperceptibly with normal brain neuropil. Similar pattern of staining was seen using mAb SMI-31 (not shown). Original magnification, ×100. **E:** Low-power view of a subcortical hamartoma immunostained with mAb TuJ1. The hamartoma is negative in contrast to βIII-positive axonal projections and neuropil. **F:** Low-power view of a large subependymal hamartoma immunostained with mAb TuJ1. The tumor-proper is essentially negative, however, βIII staining is present in a cluster of immature neuroepithelial cells (**top**), representing elements of the contiguous subventricular germinal matrix, as well as in the neuropil of the caudatum. This localization suggests that βIII is transiently expressed also in glial precusors during their growth phase in the germinal neuroepithelium. A similar distribution of immunoreactivity is observed in normal controls. Original magnification, ×100. **G:** High-power view of a SEH immunostained with mAb SMI-94 (specific for a conserved epitope of myelin basic protein). Overt myelin basic protein staining in intralesional nerve fascicles suggests that these represent pre-existing myelinated axons traversing portions of the hamartoma. Original magnification, ×400. **H:** High-power view of a SEH immunostained with mAb CL-300 to calbindin-D28k. Rare, morphologically glial-appearing cells exhibit calbindin-like immunoreactivity, but the vast majority of the so-called large eosinophilic cells are calbindin-negative. Original magnification, ×400. Peroxidase-antiperoxidase with light hematoxylin counterstain.

provides a novel experimental system for studying the cellular and molecular pathogenesis of these hamartomas. The fact that these findings were overlooked in previous descriptions of the Eker rat is probably a reflection of the absence of overt neurological signs and symptoms. Several explanations may account for this. Studies in human TSC suggested that the size and location of cortical tubers correlated with the onset and severity of the neurological and psychiatric symptoms, whereas subependymal tumors are more likely to cause symptoms related to CSF blockage as they slowly expand during postnatal life.[1] The lesions in the Eker rat are more akin to the subependymal hamartomas and are minute in size even when found at a mature age. However, the presence of seizure activity in the Eker carriers has not been ruled out because we have found a low incidence of unexplained premature deaths in animals with small renal tumors only. Convulsion-related mortality remains a plausible explanation.

The extraordinarily high incidence of all three distinct intracranial tumor types (subependymal hamartomas, pituitary adenomas, and meningiomas) found in the Eker rat carriers strongly suggests that the disruption of the *TSC2* gene is the underlying cause of these lesions. Examination of the age-matched *TSC2* wild-type littermates failed to identify these characteristic lesions. Extensive documentation of rat pathology has reported sex-averaged incidence of spontaneous pituitary tumors of 16%, and meningioma of 0.06% in Fischer 344 rats.[18] Lesions resembling the SEH have not been reported in any other strain. The mechanism by which *TSC2* gene mutation leads to the initiation of these tumors is not understood. Experiments using the Eker rat renal tumors have demonstrated the role of tuberin in suppressing growth and tumorigenicity, thus providing direct evidence that *TSC2* functions as a tumor suppressor gene.[15] This is also consistent with the loss of heterozygosity analyses of tumors in human TSC and the Eker rat.[8,9,14] However, the underlying signaling pathway altered by the inactivation of tuberin has not been elucidated. Studies of *TSC2* function have demonstrated *in vitro* GAP activity of tuberin for Rap1, and these two proteins overlap significantly in their subcellular localization.[6,19] One hypothesis suggests that the loss of tuberin could result in the constitutive activation of Rap1 leading to mitogenesis. This would be analogous to the action of NF1 in the pathogenesis of neurofibromatosis.[20] Indeed, experiments have shown that Rap1 can induce DNA synthesis in Swiss 3T3 cells and activate B-Raf serine/threonine kinase *in vitro*.[21] In contrast, Rap1 has also been implicated in negatively regulating cell growth of Ras-transformed NIH 3T3 cells.[22] Additional investigations are needed to define the exact nature of Rap1-tuberin interaction. Notwithstanding this, a recent study has uncovered another GTPase that serves as a substrate for tuberin GAP activity.[7] The *TSC2* protein can function as a Rab5GAP in modulating vesicular transport. It will be important to define the physiological relevance of Rab5GAP and Rap1GAP activities in governing *TSC2*-related tumor suppression.

The pathological features of the human subependymal hamartomatous lesions are closely recapitulated in the Eker rat. Moreover, the rat SCHs resemble the white matter hamartomas described in TSC infants.[23] The remarkable histological and immunohistochemical similarities of the CNS hamartomas between humans and rodents highlight the potential usefulness of the Eker rat as a novel experimental model to study the pathogenesis of these lesions. Our study confirms a predominant astroglial phenotype of the cerebral hamartomas and provides new information about the contributions of pre-existing myelinated axons to the composition of these lesions. Controversy remains as to the nature of the large eosinophilic cells in SEH and SEGA, but recent studies have postulated a dual glioneuronal pattern of differentiation.[24–27] The immunophenotype of similar large cells in the Eker SEHs demonstrates unequivocal reactivity for GFAP but, on the whole, lacks expression of specific markers of neuronal differentiation. The latter consist of a panel of well-characterized monoclonal antibodies directed against class III β-tubulin, distinct phosphorylated (SMI-31) and nonphosphorylated (SMI-33, SMI-311) epitopes in neurofilament H, and to a lesser extent, neurofilament M subunits. The large eosinophilic cells in all lesions were consistently negative for class III β-tubulin and, to a large extent, to all three anti-neurofilament protein antibodies as well. Certain differences between human and rat may be species-related, or be attributed, in part, to the use and/or interpretation of results obtained by different immunoreagents. Neuronal microtubule proteins, such as βIII and microtubule-associated protein 2 (MAP2) and tau, are expressed in the U-251 MG human glioblastoma cell line[28] but not in the C-6 rat glioma cell line.[29] Also, the reported occurrence of neurofilament-like immunohistochemical localization in a minority of large cells of SEGAs could be the result of variable antibody specificities. Unexpected immunoreactivities or cross-reactivities of certain anti-neurofilament antibodies in neoplastic astrocytes constitute an additional caveat.[30] Other neuronal proteins, such as MAP2 and calbindin-D28k, are indeed associated with normal neurons, but they may also be present in reactive or neoplastic astrocytes.[28,31,32] This becomes relevant in a fundamentally dysontogenetic process, such as SEH/SCH, in which neuroectodermal cells may not maintain their normal antigenic profile. Although, mAbs SMI-31, SMI-33, SMI-311, and TuJ1 would detect a number of neuronal cytoskeletal epitopes, there are undoubtedly many other lineage-associated (not necessarily specific) proteins expressed by neurons. Thus, it remains possible that certain large cells within the hamartomatous lesions may still be neuronal, but their neuronal differentiation has not be detected with the antibodies used in this study. In any case, cells within SEGA that stain for neuronal markers are uncommon,[27] and our findings in the Eker rat are in keeping with the predominant glial component found in human subependymal lesions. Furthermore, this study establishes that the hamartomatous lesions in the rat contain significant contributions from pre-existing myelinated axons. Fascicles of *mature* myelinated fibers, confirmed on the basis of immunoreactivity for myelin basic protein, phos-

phoneurofilaments, and βIII tubulin, become entangled with glial processes within the lesions. This may explain, in part, the paucity of GFAP staining in some fibrillary areas of these lesions in the Eker rat.

Other histological features that are common to both the human and rat lesions include the presence of calcifications and Rosenthal fibers. The latter have been described in human SEGAs, as well.[33] Focal calbindin-D28k staining was detected in rare, small glial-like cells and entrapped striatal neurons in only 10% of the rat hamartomas; the large eosinophilic cells were distinctly negative. Calbindin-D28k is a calcium-binding protein that exhibits a diverse but neuronal-specific distribution.[34,35] It is expressed in a subset of cerebellar medulloblastomas originating from the ventricular matrix neuroepithelium.[36,37] In the rat, this protein has been shown also to be present in interneurons of the corpus striatum[34] and may be expressed in astrocytes *in vitro* under the influence of tumor necrosis factor[32] but not in C-6 glioma cells transplanted into rat brains.[37] Focal calbindin-D28k staining has also been reported in SEGAs of humans.[27] It should be noted that changes consistent with cortical tuber were not found in this *TSC2*-mutant model. This suggests that the pathogenesis of tubers may differ significantly from that of SEGA. Species differences in the target cell number and the timing of neurogenesis may contribute to this phenotypic variance.

Indirect evidence points to a developmental basis for the localized formation of cortical tubers and SEH. Temporally, neurological signs and symptoms typically manifest during the first year of life, and such seizure activities correlate temporally and spatially with the presence of cortical lesions.[2] Spatially, SEH are located in zones of the germinal matrix where progenitor cells reside during neurogenesis. In the Eker rat, we have identified occasional dense clusters of immature neuroepithelial cells in the periphery of the SEHs. Interestingly, these cells exhibit βIII immunoreactivity similar to what has been previously described in the ventricular matrix at the roof of the fourth ventricle in humans.[35,36] These cells are, for the most part, glial precursors in the adult rat brain, and the presence of βIII indicates a transient expression of the protein during glial differentiation. Additionally, the aggregates of primitive cells in the SEHs are probably residual elements of the adjacent subependymal germinal matrix neuroepithelium rather than foci of anaplastic transformation. Two main features support this contention: 1) no immature cells are found in tumors that are situated away from the subependymal matrix and 2) whereas the primitive cells and tumor cells are contiguous in some SEHs, they are present in separate regions of the lesions. The observation that the increased aggregates of subependymal matrix cells are consistently ipsilateral to the tumors suggests an influence of the SEH-associated glial proliferation on the expansion of adjacent germinal matrix neuroepithelial cells. Questions remain as to the identification of the target cell in SEH and the molecular mechanism by which the genetic defect (ie, *TSC2* mutation) alters the normal developmental program and function of such a cell. Until now, such investigations have been limited by the lack of an *in vivo* animal model. The experimental shortcomings imposed by the use of human postmortem tissue can now be overcome by the availability of a genetically-defined animal model of tuberous sclerosis.

The apparent lack of evidence for loss of heterozygosity in the Eker SEH is in agreement with the rarity of LOH found in human TSC-brain lesions.[38] This is in contrast to the results of the rat pituitary adenomas that showed LOH in one of three tumors suggesting that tuberin inactivation may be an important step in pituitary tumorigenesis. One of several hypotheses may explain the observed differences in LOH frequency between the CNS and nonCNS hamartomas. The presence of contaminating adjacent normal tissues in the analysis would negate any LOH present in the tumor cells. Although this may be the case for small lesions requiring microdissection in our rat specimens, this seems to be an unlikely explanation for the gross lesions derived from humans. A second hypothesis, which we favor, is to suggest that tubers and subependymal lesions are composed of an admixture of cells in which the primary target cells undergo two-hit inactivation and consequently expand to encompass normal bystander cells or lead to a secondary reactive component that makes up the remaining lesion. In this model, one would predict two populations of cells with contrasting patterns of tuberin expression. Alternatively, the mechanism of initiation of CNS malformations may be different from extracranial lesions that showed LOH. For example, the CNS target cells may be sensitive to the effects of *TSC2* haploinsufficiency and may not require the complete loss of tuberin activity. However, the focal and heterogeneous nature of the CNS lesions in patients with germline *TSC2* mutation would be more difficult to explain on the basis of haploinsufficiency. The lack of detectable *TSC2* transcripts in giant cells and neurons from two patients reported by Crino et al[39] would support the two-hit hypothesis, but inadequate genetic information of the affected individuals precludes our making conclusions about the molecular events at the *TSC2* loci during tuber development.

Taking advantage of the Eker rat model, future studies will determine: 1) the primary cellular target of tuberin dysfunction during the genesis of subependymal giant cell astrocytoma and subcortical hamartoma in tuberous sclerosis; 2) the requirement for inactivation of both *TSC2* alleles in these lesions; and 3) the role of tuberin in cerebral development. Importantly, the influence of tuberin on cellular proliferation, differentiation, and migration can be addressed by examining the properties of neuroepithelial progenitor cells derived from the Eker homozygous mutants.

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