KNOCKOUT OF A TUBEROUS SCLEROSIS GENE HIGHLIGHTS ROLE OF GLIA IN EPILEPTOGENESIS

Astrocyte-Specific *Tsc1* Conditional Knockout Mice Exhibit Abnormal Neuronal Organization and Seizures

Uhlmann EJ, Wong M, Baldwin RL, Bajenaru ML, Onda H, Kwiatkowski DJ, Yamada K, Gutmann DH

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In persons affected with tuberous sclerosis complex (TSC), a wide range of neurologic abnormalities develop, including aberrant neuronal migration and seizures. In an effort to model TSC-associated central nervous system abnormalities in mice, we generated two independent lines of astrocyte-specific *Tsc1* conditional knockout mice by using the Cre-LoxP system. Astrocyte-specific *Tsc1*-null mice exhibit electroencephalographically proven seizures after the first month of age and begin to die at 3 to 4 months. *Tsc1*-null mice show significant increases in astrocyte numbers throughout the brain by age 3 weeks and abnormal neuronal organization in the hippocampus between 3 and 5 weeks. Moreover, cultured *Tsc1*-null astrocytes behave similar to wild-type astrocytes during log phase growth but demonstrate increased saturation density associated with reduced $p27^{Kip1}$ expression. Collectively, our results demonstrate that astrocyte-specific disruption of *Tsc1* in mice provides a context-dependent growth advantage for astrocytes that results in abnormalities in neuronal organization and epilepsy.

COMMENTARY

The tuberous sclerosis complex (TSC) is an autosomal dominant disorder, resulting from mutations in one of two genes, *TSC1* or *TSC2* (1,2), which occurs with an estimated incidence of 1:8000–10,000 live births. Although TSC affects multiple body organ systems, including the heart, kidney, skin, and eye, the most disabling manifestations of TSC reflect abnormalities in brain function. For example, epilepsy that is often refractory to medical management despite antiepileptic drug polytherapy occurs in more than 70% to 80% of TSC patients (3). Similarly, infantile spasms, a devastating epilepsy syndrome often associated with profound mental retardation and poor neurologic prognosis, occurs in an estimated 20% to 30% of babies with TSC.

Neurologic and neuropsychiatric manifestations of TSC are intimately related to brain lesions, called tubers, which are present in more than 80% of patients with TSC. Tubers are developmental abnormalities of cerebral cortical cytoarchitecture. They are characterized histologically by disorganized cortical lamination, astrocytic proliferation, and the presence of giant cells (GCs), which exhibit aberrant morphologies and cytomegaly. Much of our understanding of epilepsy in TSC has resulted from clinical studies such as positron emission tomography (PET) scanning (4) and direct molecular analysis of human tuber specimens resected during epilepsy surgery (5). These approaches provide important insights into TSC, but they do not permit understanding of how disorganized cerebral cortical cytoarchitecture in TSC progresses during brain development or how tubers are related to epileptogenesis. Indeed, a clear limitation in the study of TSC has been the paucity of animal TSC models. A rat strain (the Eker rat) serves as one model system to study the pathology of TSC. A spontaneous germline mutation in the rat homologue (*Tsc2*) of the human *TSC2* gene results in a truncated isoform of tuberin. Consequently, the Eker rat strain is predisposed to multiple hamartomas involving the kidneys and subependymal nodules in the brain; however, lesions that are histologically similar to tubers are detected very rarely in this strain. Mice heterozygous for targeted mutations in either *Tsc1* or *Tsc2* exhibit only renal tumors. Homozygous mice *Tsc1* or *Tsc2* mutations die by mid-embryogenesis, and therefore, the effects of the *Tsc1*- or *Tsc2*-null state on brain development cannot be fully assessed.

Recently Uhlmann et al. generated a mouse strain in which the *Tsc1* gene has been conditionally inactivated ("conditional knockout," [cKO]) in astrocytes during brain development. The resulting phenotype includes cytomegaly, astrocytic proliferation, laminar disorganization, and spontaneous seizures. These *Tsc1* cKO mice provide a fascinating model system to study how cellular abnormalities develop in TSC and, potentially, to define the mechanisms of epileptogenesis in the TSC brain. The strain

was generated by engineering nuclear-targeted Cre-recombinase expression under the control of the human glial fibrillary acidic protein (GFAP) promoter and then crossing this strain with mice containing a *Tsc1* allele with two inserted LoxP sites surrounding exons 17 and 18. The cross yields a selective inactivation of *Tsc1* solely in cells that express the GFAP promoter (e.g., in astrocytes, but not in neurons or oligodendrocytes). Transcription of the *Tsc1*-GFAP-Cre transgene is initiated by approximately embryonic day 14. Thus a significant number of astrocytes will be affected by the loss of *Tsc1*, whereas adjacent neurons will express normal amounts of the *Tsc1*-encoded protein hamartin. Absent expression of hamartin was confirmed in cultured astrocytes from these mice by immunoblotting with antihamartin antibodies. Expression of tuberin, the encoded protein of the *Tsc2* gene, is not affected by the *Tsc1* knockout.

At birth, the *Tsc1* cKO mice appear normal, but by age 2 months, these mice are less active and assume a retracted posture. At this time, the mice begin to exhibit seizures consisting of a brief period of tonic stiffening of the trunk followed by rhythmic head bouncing and forelimb clonus. The interictal EEG reveals a burst-suppression pattern with frequent spikes. Seizure onset was bilaterally synchronous in the frontocentral region, as measured with epidural electrodes, although in some animals, seizure onset was first detected in the hippocampal depth electrodes. Histologic examination of the *Tsc1* cKO brains demonstrated increased numbers of GFAP-immunolabeled astrocytes in the neocortex, hippocampus, and subcortical structures and was first detectable by age 3 weeks. By age 6 weeks, a five- to sixfold increase in astrocyte number was found, compared with those in wild-type mice. Many of these astrocytes exhibited cytomegaly. Enhanced expression of proliferating cell nuclear antigen (PCNA) was observed in the astrocytes between ages 3 and 6 weeks, suggesting that one effect of *Tsc1* inactivation was progressive astrocytic proliferation. Interestingly, no tumors were identified. Alterations in hippocampal cytoarchitecture were clearly evident by age 3 weeks. Specifically, disorganization of the pyramidal layers was seen in the cornu ammonis sectors, with malpositioning of pyramidal neurons. Moreover, the width between the two blades of the dentate gyrus was widened, and abnormal collections of neurons were found within the hilus of the dentate gyrus. Surprisingly, the laminar architecture of the cerebral cortex was preserved in these mice.

Astrocytes were grown in culture to study the increases in astrocytic proliferation occurring from the loss of hamartin. No differences in proliferation were observed during the log phases of cell growth. However, whereas wild-type astrocytes grow in a monolayer, astrocytes from the *Tsc1* cKO mice formed small foci that protruded from the monolayer. Expression of nestin was increased in these astrocytes, and based on previous studies in *Tsc2*-null fibroblasts, Uhlmann and colleagues also found a reduction in $p27^{Kip1}$ expression in *Tsc1* cKO astrocytes.

The *Tsc1* cKO mouse provides a useful model to study several important phenotypic features of TSC including aberrant cell proliferation, astrocytosis, cytomegaly, dyslamination, and epilepsy. One concern regarding this new strain is that the mice do not exhibit cortical tubers. However, tubers likely form from a complete loss of gene function within a restricted population of cells superimposed on a backdrop of adjacent cells that are heterozygous for *TSC1* or *TSC2* mutations. The *Tsc1* cKO is an engineered germline mutation that affects all astrocytes in the brain, and thus it may be theoretically more appropriate to view the entire brain of a *Tsc1* cKO mouse as modeling the cellular features of tubers. The removal of hamartin from astrocytes throughout the brain affects astrocytic development from mid-corticogenesis through maturity and now can be assayed as one potential window to view tuber formation.

One fascinating observation to be made from the study of Uhlmann et al. is that astrocytic proliferation appears to increase with advancing age, suggesting that the effects of *Tsc1* mutations are dynamic, rather than restricted to specific phases of cortical development. Further study of the role that the growth-modulating protein $p27^{Kip1}$ plays in TSC seems warranted by the in vitro results. The *Tsc1* cKO mice also permit investigation of how loss of hamartin leads to abnormalities of cell size and cytomegaly, a feature found in *Tsc1* and *Tsc2* gene mutants in *Drosophila* as well (6,7). Additionally, the appearance of seizures is delayed until age 2 months and is concordant with increased astrocyte numbers, implying that the process of epileptogenesis may relate to astrocytic proliferation and, therefore, may be studied in a time-dependent fashion. The model also provides a compelling system to study how progressive changes in gene and protein expression may antedate the onset of the first seizure—with obvious and significant ramifications for understanding epileptogenesis in its most broad mechanistic sense.

Finally, clear evidence exists for abnormalities in neural organization within the hippocampus of these mice, raising the intriguing question as to how selective loss of hamartin in astrocytes may disrupt *neural* development. The only real limitations of the model are that no way is known to study the potential roles that adjacent normal cortex might play in tuber formation or in epileptogenesis, as occurs in human TSC patients in whom tubers are focal malformations surrounded by relatively normal cortex. Furthermore, unlike those in many TSC patients, seizures in the *Tsc1* cKO are not evident at birth, and no ictal manifestation models infantile spasms. Thus, the model does not completely recapitulate all neurologic features of human TSC. Future experiments to compare gene and protein expression directly in the *Tsc1* cKO mouse and in tubers resected from patients with identified *TSC1* mutations will provide, for

the first time, a glimpse of which gene-expression alterations in human tissue may be direct downstream effects of gene mutations, as opposed to those that may be more distant, indirect effects.

by Peter B. Crino, M.D., Ph.D.

References

1. van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, van den Ouweland A, Halley D, Young J, Burley M, Jeremiah S, Woodward K, Nahmias J, Fox M, Ekong R, Osborne J, Wolfe J, Povey S, Snell RG, Cheadle JP, Jones AC, Tachataki M, Ravine D, Sampson JR, Reeve MP, Richardson P, Wilmer F, Munro C, Hawkins TL, Sepp T, Ali JBM, Ward S, Green AJ, Yates JRW, Kwiatkowska J, Henske EP, Short MP, Haines JH, Jozwiak S, Kwiatkowski DJ. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science 1997;277: 805–808.
2. Consortium ECTS. Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell 1993;75:1305–1315.
3. Romanelli P, Najjar S, Weiner HL, Devinsky O. Epilepsy surgery in tuberous sclerosis: multistage procedures with bilateral or multilobar foci. J Child Neurol 2002;17:689–692.
4. Chugani DC, Chugani HT, Muzik O, Shah JR, Shah AK, Canady A, Mangner TJ, Chakraborty PK. Imaging epileptogenic tubers in children with tuberous sclerosis complex using alpha-[^{11}C]methyl-L-tryptophan positron emission tomography. Ann Neurol 1998;44:858–866.
5. White R, Hua Y, Lynch DR, Scheitauer B, Crino PB. Differential transcription of neurotransmitter receptor subunits and uptake sites in giant cells and dysplastic neurons in cortical tubers. Ann Neurol 2001;49:67–78.
6. Ito N, Rubin GM. gigas, a *Drosophila* homolog of tuberous sclerosis gene product-2, regulates the cell cycle. Cell 1999;96:529–536.
7. Gao X, Pan D. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev 2001;15:1383–1392.