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One-Hit Wonders and 2-Hit Tubers: A Second-Hit to TSC2 Causes Tuber-Like Cells in Spheroids

Genetically Engineered Human Cortical Spheroid Models of Tuberous Sclerosis

Blair JD, Hockemeyer D, Bateup HS. *Nat Med*. 2018;24(10):1568-1578. doi:10.1038/s41591-018-0139-y. Epub 2018 Aug 20. PubMed PMID: 30127391; PubMed Central PMCID: PMC6261470.

Tuberous sclerosis complex (TSC) is a multisystem developmental disorder caused by mutations in the *TSC1* or *TSC2* genes, whose protein products are negative regulators of mechanistic target of rapamycin complex 1 signaling. Hallmark pathologies of TSC are cortical tubers—regions of dysmorphic, disorganized neurons, and glia in the cortex that are linked to epileptogenesis. To determine the developmental origin of tuber cells, we established human cellular models of TSC by CRISPR–Cas9-mediated gene editing of *TSC1* or *TSC2* in human pluripotent stem cells (hPSCs). Using heterozygous *TSC2* hPSCs with a conditional mutation in the functional allele, we show that mosaic biallelic inactivation during neural progenitor expansion is necessary for the formation of dysplastic cells and increased glia production in 3-dimensional cortical spheroids. Our findings provide support for the second-hit model of cortical tuber formation and suggest that variable developmental timing of somatic mutations could contribute to the heterogeneity in the neurological presentation of TSC.

Commentary

Tuberous sclerosis complex (TSC) is a disease that affects multiple organ systems, with disruption of the central nervous system causing the most severe symptoms. The disease is caused by mutations in TSC1 or TSC2 genes, leading to constitutive activity of mechanistic target of rapamycin complex 1 (mTORC1), which is shown to alter many cellular processes including cell growth and proliferation. A hallmark feature of TSC is the presence of cortical tubers, which are regions of disrupted neocortex that contain dysplastic cells and loss of cortical lamination. Cortical dysplasias are a leading cause of intractable epilepsy. In line with this, cortical tubers are often the site of epileptic foci, with approximately 90% of patients with TSC presenting with epilepsy that begins early in life. One limitation of current rodent models of TSC is the inability to observe cortical tubers, underscoring a potential advantage of studying this disease with human-derived cell lines.

"Second-hit" models have been proposed to explain variability in the expression of many diseases, including both genetic and acquired epilepsies. In the current article, the authors propose that cortical tubers result from somatic mutations in patients with heterozygous germline mutations resulting in complete loss of function of TSC1/TSC2 complex and hyperactivation of mTORC1 in a subset of cortical progenitor cells. To test this notion, Blair and colleagues used CRISPR/Cas9 to generate a constitutive TSC2 knock out in one allele of human embryonic stem cells (hESCs) and a Cre-inducible conditional knock out engineered in the second allele. This allowed the authors to control exactly when the second hit occurs by simply exposing 2-dimensional (2D) and 3-dimensional (3D) cell cultures to a virus during the expansion phase of neural progenitor cells (NPCs). The results of these elegant experiments show that a second hit to the TSC2 gene resulted in abnormal neural progenitors, neurons, and glia. Treatment with rapamycin was able to reverse most of the phenotypes. This article uses a unique strategy to model a second hit that can be applied to other models of disease.

The first series of experiments demonstrated that homozygous, but not heterozygous, mutations in TSC1 and TSC2 increased the phosphorylation of proteins that correspond to increased mTORC1 activity (p-S6 and p-4E-BP1). Next, hESCs were differentiated into 2D cultures to examine the impact of homozygous mutations on NPCs. Homozygous mutations led to increased mTORC1 activity and the development of hypertrophic, nestin-positive NPCs. Most phenotypes were more robust in TSC2 mutations, which is the gene most commonly mutated in human TSC.

When the authors created 3D, $TSC2^{-/-}$ cortical spheroids, they found a delayed or reduced expression of neuronal markers (MAPK and NeuN) and increased expression of glial markers (GFAP and S100B). In WT cortical spheroids, mTORC1 activity decreased during the developmental switch from

cellular proliferation to differentiation; however, a similar reduction was not found in the $TSC2^{-/-}$ spheroids.

With respect to cellular morphology, the neurons and glia examined from $TSC2^{-/-}$ spheroids were larger, hypertrophic, and dysmorphic, likely resulting from increased mTORC1 activity. $TSC2^{-/-}$ spheroids exhibited increased p-STAT3, a molecule implicated in astrocyte differentiation. Interestingly, rapamycin treatment reduced the expression of glial markers and increased the expression of proteins found in neurons. Thus, they conclude that $TSC2^{-/-}$ causes increases in mTORC1 activity, which was found to be gliogenic.

The approach to model a second hit is perhaps the most impressive aspect of the study. By creating a constitutive heterozygous TSC2 knock out in one allele and a Cre-inducible knockout version of the gene on the other allele, the investigators could deliver a lentivirus containing either Cre or GFP at a concentration resulting in sparse infection. This strategy allows control over 2 important variables including the timing of the second hit and examination of the cell-autonomous contribution toward altered cellular morphology. They also applied this strategy to cells from a patient with TSC with a heterozygous mutation in the TSC2 gene. Collectively, these experiments showed that biallelic inactivation of the TSC2 gene results in dysplastic neurons and glia that are enlarged and hypertrophic. Although these morphological alterations were reversed upon treatment with rapamycin, the decision regarding cell fate (ie, glial bias) was not reversible with delayed treatment (>day 80). Furthermore, hyperactivity of mTORC1 reemerges upon withdrawal of rapamycin, suggesting the requirement of continuous treatment.

The 3D cortical spheroid preparation is a nascent technology, and a large degree of uncertainty remains regarding how well it models a healthy human embryonic cortex, let alone a diseased one. Unlike 2D cultures, cortical spheroids preserve the organization of proliferative zones containing progenitor cell regions and radial glia that give rise to mature neurons. Furthermore, cortical spheroids possess regions similar to superficial and deep layers of the human neocortex.[1-3] This preservation of gross anatomical structure of a young cortex permits investigators to probe cellular and network activity using whole-cell patch-clamp recordings, multielectrode arrays, and voltage-sensitive dye and/or calcium imaging. However, cortical spheroids are devoid of vasculature, which limits their growth likely due to reduced oxygenation and other factors normally supplied by blood vessels.

In this study, the authors demonstrate that $TSC2^{-/-}$ mutant cortical spheroids contain enlarged NPCs, and hypertrophic neurons and glia. Indeed, tubers found in patients are more than just dysmorphic cells, but also consist of gross deviations in cortical structure such as a loss of cortical lamination and disorganized cytoarchitecture. Several of these features were previously reported in TSC2 knockout animal models.[4,5] While it is reassuring that the current study corroborates these previous findings, whether the study sheds light on cortical tuber formation is an open question, since this hallmark feature of TSC is likely multidimensional.

One caveat with the second-hit theory of TSC is the lack of evidence thereof. Resected tissue from patients has not been able to provide evidence of somatic, second-hit mutations in TSC1 or TSC2. As the authors mention, this may be due to the inability to detect such cell-autonomous effects in samples that contain heterogeneous cell types. With the advances in single-cell genetic screening, detecting these rare cells will likely become possible in the future and will either corroborate the second-hit model or provide other clues regarding the expression of TSC phenotypes in patients.

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