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Tubers and Tumors Are CLIPped Together in Tuberous Sclerosis Complex

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Amplification of Human Interneuron Progenitors Promotes Brain Tumors and Neurological Defects

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Evolutionary development of the human brain is characterized by the expansion of various brain regions. Here, we show that developmental processes specific to humans are responsible for malformations of cortical development (MCDs), which result in developmental delay and epilepsy in children. We generated a human cerebral organoid model for tuberous sclerosis complex (TSC) and identified a specific neural stem cell type, caudal late interneuron progenitor (CLIP) cells. In TSC, CLIP cells over-proliferate, generating excessive interneurons, brain tumors, and cortical malformations. Epidermal growth factor receptor inhibition reduces tumor burden, identifying potential treatment options for TSC and related disorders. The identification of CLIP cells reveals the extended interneuron generation in the human brain as a vulnerability for disease. In addition, this work demonstrates that analyzing MCDs can reveal fundamental insights into human-specific aspects of brain development.

Commentary

Tuberous sclerosis complex (TSC) arises from heterozygous pathogenic variants in the *TSC1* or *TSC2* gene, resulting in hyperactivation of the mechanistic target of rapamycin (mTOR) pathway. Affected individuals manifest with various symptoms including refractory epilepsy, developmental delay, autism, cortical malformations, and tumor formation in brain, kidneys, heart, eyes, lung, and skin. In the brain, 3 main types of lesions are seen: cortical tubers, subependymal nodules (SENs), and subependymal giant-cell astrocytomas (SEGAs). Cortical tubers are foci of dysplastic cortex that can generate seizures and contain dysmorphic neurons and astrocytes and giant cells, large cells that express neural progenitor, neuronal, and glial markers. SENs and SEGAs are intraventricular brain tumors that can cause complications such as hydrocephalus, with SENs often progressing into SEGAs.

Prior TSC studies to determine how mTOR pathway hyperactivation alters cortical development have mostly utilized rodent models, but pathological brain lesions seen in humans with TSC are largely absent in $Tsc1^{+/-}$ and $Tsc2^{+/-}$ mice, and are a rare feature of the Eker rat model that contains a heterozygous *Tsc2* mutation.[1] In mice, homozygous mutations in *Tsc1/Tsc2* are necessary to recapitulate brain pathology. A predominant theory has been that a somatic "second hit" causing biallelic pathogenic variants in *TSC1* or *TSC2* initiates tumor and tuber formation in TSC. Sequencing of human tissue has revealed biallelic pathogenic *TSC1/TSC2* variants in most SENs and SEGAs, and less commonly in cortical tubers.[2] However, whether a second hit is necessary to initiate the formation of the tubers and tumors is unknown. Furthermore, tubers, SENs, and SEGAs have similar transcriptional signatures[2] suggesting a common cell type of origin, but identifying this cell remains elusive. In a recent study, Eichmüller et al. hypothesized that a second hit is not required and that a vulnerable cell type gives rise to TSC brain lesions because of *TSC1/TSC2* haploinsufficiency.[3] Here, the authors used human induced pluripotent stem cells (iPSCs) that were heterozygous for a pathogenic *TSC2* variant, along with isogenic controls, and differentiated the cells into human cerebral organoids (hCOs), three-dimensional cultures that resemble structural aspects of the developing fetal brain.

The authors found tuber-like and tumor-like lesions in $TSC2^{+/-}$ but not control hCOs starting around 105-130 days of differentiation. The tuber-like lesions contained dysmorphic neurons and astrocytes, as well as giant cells. The tumor-like lesions were hyperproliferative, displayed mTOR pathway

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hyperactivity, and expressed markers of neural stem cells, consistent with SENs. By single-cell RNA sequencing (scRNA-seq) on day 220 of *TSC2*$^{+/-}$ hCOs that were almost entirely comprised of tumor cells, almost all the cells were interneurons and interneuron progenitors. ScRNA-seq was also performed at 110 days, when the tubers and tumors were starting to emerge, and *TSC2*$^{+/-}$ hCOs had overrepresentation of interneurons and interneuron progenitors of an apparent caudal ganglionic eminence (CGE) origin. Tuber-like and tumor-like lesions consisted of different subtypes of CGE interneurons, but both subtypes originated from caudal late interneuron progenitors, termed CLIP cells. Immunoreactivity for CLIP cell markers was present in tumor-like and tuber-like lesions in the hCOs, and in SEGAs and giant cells from human TSC specimens.

Genotyping of the *TSC2* locus in tumor organoids revealed that most (50-70%) organoids were heterozygous, with a minority (30-50%) of organoids having copy-neutral loss-of-heterozygosity (cnLOH). This suggests that a second hit in *TSC2* is not necessary for tumor formation and that cnLOH can occur during tumor progression, as previously observed in most human specimens.[2] By immunoreactivity, *TSC2* was expressed in 98% of giant cells in tuber-like lesions, suggesting that a second hit is also unnecessary for the formation of tubers. The authors suggest that CLIP cells are particularly vulnerable to *TSC1/TSC2* haploinsufficiency because they have decreased basal *TSC1/TSC2* expression as determined by immunofluorescence and mass spectrometry. As the CLIP cells were found to have increased epidermal growth factor receptor (EGFR) expression, the authors treated *TSC2*$^{+/-}$ hCOs with an EGFR inhibitor and demonstrated a decreased tumor burden (albeit not as dramatically as treatment with the mTOR inhibitor everolimus).

These findings are remarkable because unlike the rodent TSC models, this human cell-based model is the first to recapitulate multiple CNS features (tubers and SENs) from a heterozygous mutation in *TSC2*. Tumors that resembled SEGAs were not found, but future analysis of later time points may reveal progression of SEN-like to SEGA-like tumors. One possibility is that a second hit in *TSC1/TSC2* causes the transition from SEN to SEGA. Interestingly, tumor-like lesion formation was favored by growth of the hCOs in a "high nutrient" hyperglycemic medium, while the growth of tuber-like lesions was favored by growth in a "low nutrient" lower glucose medium (but still hyperglycemic). Notably, high- and low-nutrient media differed in other ways, as the low nutrient medium contained neurotrophins that can activate the mTOR pathway. Further study of which components in the media favor formation of tubers or tumors would elucidate how the mTOR pathway is modulated by the growth environment, and how different metabolic states alter pathological processes in TSC.

While SENs and SEGAs from human specimens often express neuronal and glial markers, the lack of astrocytes in the tumor-like lesions supports the idea that SENs and SEGAs have a neural stem cell or neuronal origin[4]. A strength of this study is the usage of immunohistochemistry to show markers of CLIP cells in the hCOs as well as in fetal human TSC specimens. Finding that tubers originate from abnormal interneuron differentiation may reframe TSC as an interneuronopathy and has the potential to explain the epileptogenicity of these lesions.

Prior work using *TSC1*- and *TSC2*-mutant hCOs demonstrated a phenotype in homozygous but not heterozygous mutants.[5] This may be explained by a difference in how the hCOs were generated–the differentiation protocol used in the prior study used patterning factors to promote a dorsal/cortical fate, and therefore interneuron progenitors and interneurons are scarce or absent. For the present study, Eichmüller et al. used an "intrinsic" differentiation protocol that lacks patterning factors and generates a more heterogenous mixture of cells with dorsal and ventral forebrain specification. To confirm that tumor-like lesions arise from interneuron progenitors in the ventral forebrain, the authors performed additional hCO differentiations with patterning factors to promote either a dorsal or ventral fate and found that only the ventrally-fated hCOs contained tumors.

The presence of *TSC2* immunoreactivity in giant cells of tuber-like lesions is consistent with prior studies that did not find a second hit mutation in most human cortical tuber specimens.[2] However, immunoreactivity can be an unreliable readout of expression, and these data do not rule out the possibility that a subgroup of cells (perhaps separate from the giant cells) in the tubers contain a second hit as an initiating event and affect neighboring cells in a non-cell autonomous manner. The presence of a non-cell autonomous effect is proposed to explain why sequencing studies of brain lesions from other mTORopathies reveal somatic mosaicism with a low mutational burden.[6,7]

Several important questions remain unanswered. What causes focal disease in TSC, if not a genetic second hit? Are CLIP cells also the cell of origin in other mTORopathies like focal cortical dysplasia? Why do some CLIP cells form into tubers and others into SENs/SEGAs? Is it the timing of when the CLIP cell is affected, e.g., before or after migration from the ventral forebrain, or the microenvironment in the caudothalamic groove vs. the cortex? Recent data demonstrate that interneurons (including those expressing CGE markers) can be produced from dorsal progenitors in primary human cell cultures,[8] raising the possibility that 2 spatially distinct CLIP-like cell populations could give rise to TSC lesions.

This study affirms the power of human organoid models of neurodevelopmental diseases. Future studies utilizing these models may identify pathophysiological mechanisms causing neurological symptoms of TSC, such as seizures, and more targeted treatments for tumors including EGFR inhibitors. Further study of the cellular origin of TSC lesions will be enriched by scRNA-seq analysis of fetal and post-natal tubers, SENs, and SEGAs from human specimens.

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