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New frontiers in modeling Tuberous Sclerosis with human stem cell-derived neurons and brain organoids

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Abstract

Recent advances in human stem cell and genome engineering have enabled the generation of genetically-defined human cellular models for brain disorders. These models can be established from a patient's own cells and can be genetically engineered to generate isogenic, controlled systems for mechanistic studies. Given the challenges of obtaining and working with primary human brain tissue, these models fill a critical gap in our understanding of normal and abnormal human brain development and provide an important complement to animal models. Recently, there has been major progress in modeling the neuropathophysiology of the canonical "mTORopathy" Tuberous Sclerosis Complex (TSC) with such approaches. Studies using two- and three-dimensional cultures of human neurons and glia have provided new insights into how mutations in the *TSC1* and *TSC2* genes impact human neural development and function. Here we discuss recent progress in human stem cell-based modeling of TSC and highlight challenges and opportunities for further efforts in this area.

Keywords

Tuberous Sclerosis Complex; TSC1; TSC2; mTOR; cortical tuber; human pluripotent stem cells; brain organoids; neurons; astrocytes; disease modeling; CRISPR/Cas9

Introduction

Clinical presentation of TSC

Tuberous Sclerosis Complex (TSC) is a multisystem developmental disorder with a prevalence of about 1 in 6000 births worldwide. TSC causes benign tumors, called hamartomas, in multiple organs including the skin, lungs, kidney, and brain (Crino et al., 2006). Hallmark pathologies of TSC are cortical tubers, which are focal developmental malformations consisting of enlarged and dysplastic neurons, glia and giant cells in the cortex (Mizuguchi and Takashima, 2001). TSC is associated with significant neurological and psychiatric impairments which include epilepsy in about 80% of individuals and

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variable degrees of intellectual disability (Chu-Shore et al., 2010). TSC patients also have high rates of autism spectrum disorder, attention deficit hyperactivity disorder, and other behavioral and affective disorders (Curatolo et al., 2015). Epilepsy is a major concern in TSC as it can begin in infancy and becomes intractable in about two-thirds of patients (Thiele, 2010). In some cases, surgical resection of the affected brain tissue is required to mitigate seizures.

TSC can be treated with rapamycin (also called sirolimus) and its derivative everolimus, collectively known as rapalogues. Recent clinical trials with these drugs have shown benefit for treating epilepsy in TSC, with approximately 40% seizure reduction in 40% of individuals (French et al., 2016). Rapalogues are also effective at treating the glioneuronal brain tumors that occur in about 5–20% of TSC patients, called subependymal giant cell astrocytomas or SEGAs (Krueger et al., 2010). However, tumors may regrow if treatment is stopped (Miller et al., 2015). Clinical trials with rapalogues focusing on neuropsychological deficits and autistic symptoms in TSC are underway (www.clinicaltrials.gov), although a recent trial did not report significant improvements in neurocognitive functioning with six months of daily everolimus (Krueger et al., 2017). Systemic side-effects associated with chronic rapalogue use are prevalent but generally tolerated and include infections due to immunosuppression and stomatitis (French et al., 2016). Despite some success with rapalogues, there is still an unmet clinical need for TSC treatment and additional therapeutic approaches are required.

Biochemistry and genetics of TSC

TSC is caused by loss-of-function mutations in the genes *TSC1* (van Slegtenhorst et al., 1997) or *TSC2* (European Chromosome 16 Tuberous Sclerosis, 1993). These two genes encode for the proteins TSC1 and TSC2 that form a multimeric complex together with the protein TBC1D7 (Dibble et al., 2012), which represses mTOR complex 1 (mTORC1) signaling. Biochemically, TSC2 is a GTPase-activating protein (GAP) for the small GTPase Rheb, which prevents its activation of mTOR complex 1 (mTORC1) (Tee et al., 2003). TSC1 is a stabilizer of TSC2, preventing its degradation and enhancing its GAP activity (Tee et al., 2003). As an active signaling node, mTORC1 promotes anabolic cellular processes through a multitude of functions including stimulation of protein and lipid synthesis, cellular metabolic control, and suppression of autophagy, among others (Ben-Sahra and Manning, 2017; Saxton and Sabatini, 2017). Constitutive or deregulated mTORC1 activity, as in the case of TSC1/2 complex loss, causes increased cell growth, altered proteostasis, and transcriptional and translational alterations (Sarbassov et al., 2005). Loss of either TSC1 or TSC2 is sufficient to cause mTORC1 hyperactivity. However, loss-of-function mutations in *TSC2* tend to cause greater mTORC1 activation and are associated with more severe epilepsy and cognitive impairment (Jones et al., 1997; Dabora et al., 2001; Zeng et al., 2011; van Eeghen et al., 2012; Blair et al., 2018).

Patients with TSC have germline heterozygous mutations in *TSC1* or *TSC2*; however, pathological lesions including cortical tubers are variable and appear stochastically. A leading hypothesis to explain cortical tuber formation is the “two-hit” model (Crino, 2016). This model proposes that loss of heterozygosity due to a somatic second-hit mutation in

TSC1 or *TSC2* in a small population of neural progenitor cells (NPCs) causes altered differentiation, development and neuronal migration resulting in a focal malformation. In support of this model, second-hit mutations in *TSC1* or *TSC2* are frequently identified in TSC-related tumors and have been detected in some cortical tubers (Henske et al., 1996; Sepp et al., 1996; Smolarek et al., 1998; Au et al., 1999; Chan et al., 2004; Crino et al., 2010; Qin et al., 2010; Martin et al., 2017). In addition, studies in mouse and human cellular models have shown that complete loss of TSC1/2 function is required for the formation of enlarged, dysplastic neurons and glia, which do not develop from a heterozygous state (Meikle et al., 2007; Goto et al., 2011; Blair et al., 2018). That said, this idea has been controversial in the field as second-hit mutations have been identified in only a minority of tubers tested (Henske et al., 1996; Crino et al., 2010; Qin et al., 2010; Martin et al., 2017). This may reflect low allelic frequency of the somatic mutation (D'Gama et al., 2017), dilution of the second-hit cells by infiltrating glia, mutations in regulatory regions which are not assessed in exome sequencing studies (Tyburczy et al., 2015), or epigenetic changes.

Other mTORopathies

TSC is representative of a larger class of disorders called "mTORopathies", caused by mutations in mTOR pathway regulators that result in constitutive activation of mTORC1 signaling. In addition to TSC1 and TSC2, these regulators include PTEN, PI3K, AKT3, DEPDC5, STRADA, Rheb and others (Crino, 2015; Reijnders et al., 2017). Recently, it was shown that in addition to germline mutations, mTORopathies can arise through somatic brain mutations, including gain-of-function mutations in mTOR itself (Lee et al., 2012; D'Gama et al., 2015; Lim et al., 2015; Nakashima et al., 2015; Mirzaa et al., 2016; Moller et al., 2016; D'Gama et al., 2017). These somatic mTORC1-activating mutations have been identified in brain tissue from patients with focal cortical dysplasia and hemimegalencephaly. Notably, an mTOR-activating mutation detected in fewer than 10% of brain cells is sufficient to cause disease (D'Gama et al., 2017). These types of somatic mutations are more challenging to model than inherited mutations as they require a mechanism to induce them in a sparse population of cells and early in brain development. Given potentially similar underlying neuropathophysiology, disease mechanisms discovered in models of TSC are likely to provide insights into this greater class of mTOR-related disorders.

Rodent models of TSC

Rodent models have provided key insights into the consequences of Tsc1/2 loss on brain development and function. Germline knock-out mouse models demonstrated that complete loss of *Tsc1* or *Tsc2* is embryonic lethal (Onda et al., 1999) (Kwiatkowski et al., 2002), and subsequent conditional knockout models of *Tsc1* (Kwiatkowski et al., 2002) and *Tsc2* (Hernandez et al., 2007) have been developed. Different applications of Cre-recombinase using viral injections, in utero electroporation, or Cre-expressing mouse lines, have illuminated the effects of Tsc1 or Tsc2 loss on multiple neuronal types. Somatic mTOR-activating mutations have also been modeled in mice by sparse expression of mutant *MTOR*, constitutively active Rheb, or CRISPR/Cas9 constructs targeting *Tsc1* or *Tsc2* (Lin et al., 2016; Lim et al., 2017; Park et al., 2018; Nguyen et al., 2019). The Eker rat model of TSC, carrying a spontaneous loss-of-function mutation in *Tsc2* was shown to develop sporadic

cytomegalic neurons, glia, and SEGAs in aged or irradiated young animals to induce a "second-hit" (Eker et al., 1981; Takahashi et al., 2004; Wenzel et al., 2004).

Collectively, these rodent studies have shown that loss of Tsc1/2 function impacts multiple processes happening at different developmental time points. These include altered neuronal differentiation, survival, migration, morphology, excitability, synaptic plasticity, glial function, and behavior [for relevant reviews see (Tsai and Sahin, 2011; Costa-Mattioli and Monteggia, 2013; Crino, 2013; Magri and Galli, 2013; Lipton and Sahin, 2014)]. In general, complete loss of *Tsc1* or *Tsc2* is required to observe strong disease-related phenotypes. That said, heterozygous animals do exhibit some changes in synaptic function, neuronal excitability, and behavior (Ehninger et al., 2008; Auerbach et al., 2011; Sato et al., 2012; Tsai et al., 2012). While these rodent models have been and will continue to be powerful research tools, it is important to note that bona fide cortical tuber regions are not readily observed in animal models, suggesting that this pathology may result from unique aspects of human brain development.

Opportunities for human stem cell-based models of TSC

While the rodent and human brain exhibit overall similar developmental patterns and trajectories, there are unique aspects of human brain development that cannot be captured in animal models. In particular, the human cortex develops over a significantly protracted time period compared to mice and exhibits unique cell types and proliferative zones (Miller et al., 2019). For example, the dramatically increased complexity and size of the human cortex is thought to be due to a specific progenitor cell type called outer radial glia that forms the outer subventricular zone, which is not present in rodents (Hansen et al., 2010; Pollen et al., 2015). Notably, outer radial glia have been shown to exhibit high levels of mTORC1 activity and unique expression of mTOR-pathway components (Pollen et al., 2019), indicating an important role for mTOR signaling in human cortical development. The human brain comprises about 85 billion neurons and at the peak of neurogenesis 100,000 new neurons are generated each minute (D'Gama and Walsh, 2018). This massive cell proliferation also results in increased liability for somatic mutations, whose contribution to both normal and abnormal human brain development is becoming increasingly appreciated (D'Gama and Walsh, 2018).

To capture these unique aspects of human brain development and understand how alterations in TSC-mTOR signaling affect these features, human cellular models are needed. Recent advances in somatic cell reprogramming have allowed the derivation of human induced pluripotent stem cells (hiPSCs) from skin or blood cells from patients, which have the advantage of preserving patient-specific genetic information (Okano and Yamanaka, 2014). With the advent of site-specific nucleases as gene-editing tools, most notably CRISPR/Cas9, human pluripotent stem cells (either hiPSCs or human embryonic stem cells, hESCs) can be genetically engineered with good efficiency to generate disease models with targeted mutations (Jinek et al., 2013; Blair et al., 2016; Hockemeyer and Jaenisch, 2016). Combining these approaches, it is also feasible to genetically engineer patient-derived cells to either correct the mutation or introduce an additional mutation, thereby establishing a genetically controlled, isogenic disease model using a patient's own cells (Soldner et al.,

2011). These stem cells can subsequently be differentiated into numerous somatic cell types including neurons and glia for modeling brain disorders (Soldner and Jaenisch, 2018).

Depending on the differentiation protocol employed, two- or three-dimensional cultures comprising different lineages of neurons and glia can be established. Cortical excitatory neurons and astrocytes of the telencephalic lineage are key cell types of relevance for TSC as the dysplastic cells in tubers are positive for glutamatergic and astrocytic markers (Crino, 2013). These cell types can be generated through manipulation of endogenous neuroectodermal differentiation pathways either via inhibition of the dual-SMAD pathway (Chambers et al., 2009) or overexpression of transcription factors (Zhang et al., 2013). Studies with cell type-specific conditional knock-out mice have also highlighted cerebellar Purkinje cells as relevant to TSC pathophysiology, particularly the behavioral symptoms of autism (Tsai et al., 2012; Stoodley et al., 2017; Tsai et al., 2018). A human cellular differentiation protocol based on the addition of specific growth factors has recently been established for cerebellar Purkinje cells and specifically applied to disease modeling in TSC (Sundberg et al., 2018), as discussed below.

While differentiation has traditionally been done in two-dimensional (2D) monolayer cultures, protocols have recently been adapted for three-dimensional (3D) differentiation to generate brain spheroids or organoids (collectively called brain organoids here) (Kadoshima et al., 2013; Lancaster et al., 2013; Pasca et al., 2015; Qian et al., 2016). As discussed by Chen, Song & Ming in this special issue, 3D brain organoid models have advantages over 2D models including more complex cytoarchitecture and cellular niches that preserve cell-cell and cell-matrix interactions (Chen et al., 2019).

The approach of differentiating neurons and glia from human pluripotent stem cells generally operates on a human developmental timescale. For example, by transcriptional profiling, a 2–3 month old human brain organoid is roughly equivalent to a 16–19 post-conception week human brain (Pasca et al., 2015). This enables the observation and manipulation of human neural development in approximately real-time. For this reason, neurodevelopmental disorders such as TSC are particularly well-suited to this disease modeling approach. In the next section we will describe published work to date using human stem cell derived-neurons and brain organoids to investigate disease mechanisms in TSC.

Human neuron and brain organoid models of TSC

Forebrain excitatory neurons and glia in 2D cultures

Alterations in differentiation, signaling, and gene expression—Initial work in developing human neuronal models of TSC has focused on the differentiation of genetically engineered hESCs (Costa et al., 2016; Grabole et al., 2016; Blair et al., 2018), TSC patient-derived iPSCs (Li et al., 2017; Zucco et al., 2018; Nadadhur et al., 2019) or gene edited TSC iPSCs (Ebrahimi-Fakhari et al., 2016) into two-dimensional forebrain cultures. These studies were undertaken using a variety of neuronal differentiation methods, investigating the effects of *TSC1* or *2* reduction on neural precursors, neurons, astrocytes and, in one case, oligodendrocytes (Nadadhur et al., 2019).

Differentiation into neural precursors proceeded normally in each study with only minor differences observed such as increased neural rosette size in *TSC2*$^{-/-}$ cultures (Costa et al., 2016) and increased proliferation rate in *TSC2*$^{+/-}$ cultures (Li et al., 2017), although this was not observed in other studies (Zucco et al., 2018). In contrast to this normal early neural differentiation, terminal differentiation into neurons proved highly problematic for cells with complete loss of TSC1/2 complex function. Specifically, *TSC2*$^{-/-}$ cultures produced significantly lower numbers of cells expressing the neuronal markers HuC/D (Costa et al., 2016). Notably, loss of one copy of *TSC1* or *TSC2* was much less deleterious with cultures exhibiting either a minor decrease in HuC/D-positive cells (Costa et al., 2016; Zucco et al., 2018), or no decrease at all (Nadadhur et al., 2019). The differentiation defects in cells with loss of TSC1/2 may be due to a combination of increased neuronal death (Costa et al., 2016), delayed neuronal differentiation (Zucco et al., 2018), or a shift towards astro-glial fate (Costa et al., 2016; Grabole et al., 2016). Dissecting the potential mechanisms of altered differentiation will be an interesting avenue for future investigation with these models.

The expected hyperactivation of mTORC1, as indicated by increased phosphorylation of downstream targets including ribosomal protein S6, was observed in all studies. However the strong effects seen at every developmental stage in *TSC2*$^{-/-}$ cultures (Costa et al., 2016), were not consistently seen at the NPC stage in *TSC2*$^{+/-}$ cultures (Blair et al., 2018; Zucco et al., 2018). Transcriptome analysis through RNA sequencing of patient iPSC-derived heterozygous NPCs found 513 differentially expressed transcripts compared to a sibling control line. Gene ontology analysis indicated that these transcripts were primarily involved in neuron migration and development (Zucco et al., 2018). Independent RNA sequencing of isogenic, gene-edited *TSC2* heterozygous and homozygous cultures found very few differences between *TSC2*$^{+/-}$ and *TSC2*$^{+/+}$ cells (10 transcripts) but large differences between *TSC2*$^{-/-}$ and *TSC2*$^{+/+}$ with over 2000 transcripts differentially expressed (Grabole et al., 2016). It is possible that some of the differences between the patient iPSC and control line in the study by Zucco et al could be driven by genetic differences independent of the *TSC2* mutation. Analysis of additional TSC patient and control cell lines would be helpful to resolve this. In the *TSC2*$^{-/-}$ cultures in the Grabole et al study, groups of transcripts involved in astrogliosis, inflammation, and glycolysis were all upregulated, which corresponds to observations of poor mitochondrial function in gene edited iPSC-derived *TSC2*$^{-/-}$ neurons (Ebrahimi-Fakhari et al., 2016). The transcriptome of *TSC2*$^{-/-}$ cultures also closely corresponded with previous microarray studies of cortical tubers and SEGAs (Boer et al., 2008; Tyburczy et al., 2010).

Given the key involvement of mTORC1 signaling in mRNA translation, translational profiling may reveal further differences in TSC neural cultures that may occur independently of transcriptional changes. This will be an interesting avenue for future exploration. Related to this, a recent study showed that mTORC1 signaling and translation of the translational machinery is high in human pluripotent stem cells but is suppressed during neural differentiation (Blair et al., 2017). In addition, numerous changes in mRNA translation without a corresponding change in mRNA levels were observed across human neuronal development, highlighting the importance of translational control for developing neurons (Blair et al., 2017).

Impact on neuronal morphology and physiology—The most dramatic morphological differences were observed in homozygous *TSC2* knockout cells. $TSC2^{-/-}$ NPCs, neurons, and glia exhibited somatic hypertrophy and neurons displayed increased dendritic arborization (Costa et al., 2016; Blair et al., 2018). The effects of heterozygous *TSC1* or *2* loss were less clear for these cultures, with either no change in neuronal morphology (Costa et al., 2016; Zucco et al., 2018), minor increases in dendritic branching and no change in soma size (Nadadhur et al., 2019) or increases in both (Li et al., 2017). One note is that the study by Li and colleagues was based on a single cell line from one TSC patient compared to an iPSC line from an unrelated individual. It therefore remains to be determined whether phenotypic differences between these cell lines are due to the *TSC2* mutation or a result of cell line variability or genetic background.

Electrophysiological phenotypes were probed in a subset of studies with either whole cell electrophysiology, multi-electrode arrays (MEA), or calcium imaging (Costa et al., 2016; Nadadhur et al., 2019). Whole cell recordings showed a strong decrease in the frequency of spontaneous (sEPSCs) and miniature (mEPSCs) excitatory post-synaptic currents in both $TSC2^{+/-}$ and $TSC2^{-/-}$ neurons in a gene dose-dependent manner (Costa et al., 2016). However, mEPSC amplitude was increased in $TSC2^{-/-}$ neurons, suggestive of increased synaptic strength. $TSC2^{-/-}$, but not $TSC2^{+/-}$ neurons also had significantly reduced intrinsic excitability, consistent with their morphological alterations and changes in passive membrane properties (Costa et al., 2016). This decreased intrinsic and synaptic excitability in developing $TSC2^{-/-}$ neurons suggests that other circuit components, e.g. inhibitory neurons, may be required to generate hyperexcitability at the network level following loss of TSC1/2 function (Talos et al., 2012; Bateup et al., 2013). By contrast, MEA recordings of heterozygous cultures from patient TSC iPSCs did show increased spontaneous network activity, which was also reflected by the increased frequency, but not amplitude, of calcium transients in these cultures (Nadadhur et al., 2019). Discrepancies between these findings may reflect gene dose-dependent effects, cell line and culture variability (which could have significant effects on network activity levels and development), or inhibitory and excitatory neuron composition of the cultures, which was not explored in these studies.

Treatment with rapalogues and other mTOR inhibitors such as AZD-8055 reversed many of the phenotypes of TSC1 or 2 loss in forebrain neural cultures including altered electrophysiology (Costa et al., 2016; Nadadhur et al., 2019), aberrant morphology (Costa et al., 2016; Nadadhur et al., 2019), hyperactive mTORC1 signaling (Costa et al., 2016; Li et al., 2017; Zucco et al., 2018) and altered mRNA translation (Grabole et al., 2016).

Cerebellar Purkinje cells

While forebrain excitatory cultures deficient in *TSC1* or *2* have been the primary focus of most studies thus far because of their potential to develop into cortical tuber-like cells, cerebellar tubers can also form in some TSC patients (Jay et al., 1998; Vaughn et al., 2013). In addition, mouse studies have demonstrated the importance of Tsc1/2 function in cerebellar Purkinje cells for autism-related behaviors (Tsai et al., 2012; Stoodley et al., 2017; Tsai et al., 2018). To generate a cerebellar model for TSC, a new human Purkinje cell differentiation protocol was developed and hiPSC lines from three individuals with TSC

were generated, using cells from the parents or unaffected individuals as controls (Sundberg et al., 2018). In addition, this study made use of an established *TSC2* heterozygous patient iPSC line, which had been further genetically engineered to create a $TSC2^{-/-}$ cell line together with a repaired $TSC2^{+/+}$ control cell line (Ebrahimi-Fakhari et al., 2016). This strategy has significant advantages over the use of control iPSC lines from unrelated individuals as it provides an isogenic system in which cells have the same genetic background and differ only in the disease gene.

In this model, many of the same phenotypes as in forebrain cultures were observed including increased rates of NPC proliferation, upregulated expression of astroglial markers, increased cell death, increased cell size, hyperactivation of mTORC1 activity and decreased excitability of differentiated neurons (Sundberg et al., 2018). These properties were observed in both heterozygous and homozygous cultures with more severe deficiencies in $TSC2^{-/-}$ cells. Transcriptomic analysis again revealed more differential gene expression between homozygotes and controls than heterozygotes and controls, with similar differentially expressed transcripts as in forebrain cultures including altered mitochondria and autophagy genes (Grabole et al., 2016; Sundberg et al., 2018). Interestingly, in cerebellar cultures there was also decreased expression of mRNA processing genes, including many genes which are targets of FMRP, the protein disrupted in the neurodevelopmental disorder Fragile X Syndrome. Finally, treatment with mTOR inhibitors reversed all the observed phenotypic effects of complete *TSC2* loss (Sundberg et al., 2018).

3D brain organoid models of TSC

Recent developments in 3D differentiation techniques to generate human stem cell-derived brain organoids provide a new platform to investigate neurodevelopmental disorders in a physiologically relevant setting that can be maintained for long periods of time (Kadoshima et al., 2013; Lancaster et al., 2013; Pasca et al., 2015; Qian et al., 2016). Specifically, these models demonstrate cortical patterning, including the presence of human specific cellular niches (Pollen et al., 2019) and neuronal migration (Bershteyn et al., 2017). These features may be particularly relevant for TSC as cortical tubers are developmental malformations that reflect not only altered differentiation but also defective migration and patterning.

A recent study combined 3D neuronal differentiation with CRISPR/Cas9 genome-editing to investigate the “two-hit” hypothesis of cortical tuber development in human brain organoids (Blair et al., 2018). In addition to establishing a panel of hESC lines with constitutive loss-of-function mutations in *TSC1* or *TSC2*, the authors used CRISPR/Cas9 to create an hESC line with a constitutive mutation in one allele of *TSC2* and a conditional mutation in the other ($TSC2^{c/-}$). To generate a second-hit, Cre recombinase was added to $TSC2^{c/-}$ brain organoids to cause biallelic inactivation of *TSC2* and expression of a red fluorescent protein at a defined point during development. Applying sub-saturating amounts of Cre recombinase at a stage when NPCs were proliferating resulted in populations of $TSC2^{-/-}$ cells that developed alongside $TSC2^{c/-}$ cells (which are effectively heterozygous), analogous to what is hypothesized to happen in the developing cortex of TSC patients.

Many of the developmental differences seen in 2D neuronal culture were also observed in this model including a strong bias towards an astro-glial cell fate, altered cell morphology,

cytomegaly, and activation of mTORC1 signaling (Blair et al., 2018). These phenotypes became more apparent over developmental time, with relatively minor alterations at the neural precursor stage and greater abnormalities following terminal neuronal or astrocyte differentiation. This may be because mTOR signaling is normally high in stem cells (hPSCs and NPCs) and becomes strongly suppressed during neurogenesis. Failure to suppress mTOR signaling during neuronal differentiation due to loss of the TSC1/2 complex may alter proteostasis and interfere with transcriptional and translational programs. Within the organoids, the second-hit mutation resulted in the cell autonomous generation of multiple cell types found in cortical tubers including dysmorphic neurons, dysplastic astrocytes, and giant cells. Comparisons between wild-type controls and cells heterozygous or homozygous knock-out for *TSC1* or *TSC2* revealed differentiation defects and mTORC1 hyperactivation only in organoids with homozygous deletion of *TSC1* or *TSC2*, supporting the two-hit model. In addition, phenotypes tended to be more severe in organoids with complete *TSC2* loss-of-function compared to *TSC1*.

Chronic treatment of brain organoids with rapamycin prevented mTORC1 hyperactivation and cytomegaly of $TSC2^{-/-}$ cells (Blair et al., 2018). In addition, rapamycin treatment biased differentiation towards neuronal fates, indicating that mTOR signaling can bi-directionally control cell fate and thus have a major impact on nervous system development. In addition, while early treatment with rapamycin shifted cell fate, later treatment did not, demonstrating a critical window for mTOR to regulate cell fate decisions in developing brain organoids. Removal of rapamycin after early treatment caused the return of mTORC1 hyperactivity, indicating the potential necessity of chronic rapalogue use to fully treat TSC (Blair et al., 2018).

Summary of human neural models of TSC

Taken together, the studies described above demonstrate the power of human stem cell-based disease modeling for neurodevelopmental disorders such as TSC. In particular, human cell studies have led to the robust generation of tuber-like cells, including giant cells, *in vitro*, enabling future studies into the molecular mechanisms for this developmental abnormality in the context of human-specific neural development. Collectively, these studies have revealed profound impairments in neuronal differentiation and development due to *TSC1/2* mutations together with increased production of astro-glial cells. These findings are consistent with patient histology literature describing the altered cell morphology and molecular expression patterns of cortical tuber cells (Crino, 2013). In addition, findings in human neural models of TSC have supported the idea that alterations in astrocyte differentiation and function are important for TSC neuropathophysiology (Wong and Crino, 2012). Thus, these initial studies have established and validated new models for TSC that can be employed to perform mechanistic investigations into how TSC-mTOR signaling contributes to early human brain development in both normal and pathological states.

In addition to allowing the study of human-specific cell biology, a key advantage of human cellular systems over rodent models is the ability to observe and manipulate the very earliest stages of neural development. This is technically challenging in mice as germline loss of *Tsc1* or *Tsc2* causes embryonic lethality prior to brain development. To circumvent this,

brain-specific deletion of *Tsc1* or *2* can be utilized; however, this requires expression of Cre recombinase, which may not be expressed early enough in development to induce the full panoply of abnormalities that occur *in utero* in TSC. This could be a factor limiting the development of bona fide cortical tubers in rodent models. The ability to generate long-lived human neural cultures that develop from a stem cell state therefore provides a unique platform to perform early manipulations and to analyze their long-term consequences on neuron and astrocyte development.

Perspectives, challenges, and future directions

While human stem cell-based models for the neurological aspects of TSC have many compelling features, there are important caveats and considerations to this approach. In particular, while the ability to differentiate neurons from a stem cell state provides an opportunity to model the earliest stages of human brain development, it is important to note that these cells generally develop on a human time scale. Specifically, 3D brain organoids that are cultured for 2–3 months are transcriptionally similar to first or second trimester human fetal brain (Pasca et al., 2015). Therefore, the neurons and astrocytes in these models are reflective of an immature, fetal state. This is less of a problem, and indeed may be a useful feature, for studying abnormalities of brain development such as cortical tubers and related focal dysplasias. However, it can present a challenge when seeking to study aspects of TSC that may emerge later in development. Related to this, immaturity also applies to the functional properties of the neurons and astrocytes generated. For example, in 2D cortical cultures, neurons do not robustly fire action potentials until 7–10 weeks post-differentiation (Johnson et al., 2007). The maturation process can be accelerated by direct conversion of stem cells into functional neurons via forced expression of neurogenic transcription factors (Zhang et al., 2013). However, this type of protocol bypasses normal developmental stages that may be important to preserve in certain disease models. In brain organoids, mature electrophysiological activity is detected around 14 weeks and later (Yoon et al., 2019) and it may take >6 months post-differentiation for coordinated network activity to develop (Quadrato et al., 2017). In terms of astrocyte function, a recent single cell transcriptomics analysis of human cortical spheroids across development noted a shift in astrocyte gene signatures from fetal to post-natal around nine months in culture, with further maturation and development extending to spheroids grown for almost two years (Sloan et al., 2017). Therefore, if these more mature functional properties are required before pathophysiology emerges, human neural cultures may need to be maintained for very long periods of time.

Another consideration is that while undirected brain organoid models that generate multiple cell types exist (Lancaster et al., 2013), the variability and stochasticity with which different cell types are generated are a potential impediment to reproducibility required for disease modeling (Quadrato et al., 2017). As a result, the majority of differentiation protocols are directed, in that they are designed to generate particular neural cell type(s) arising from a specific developmental lineage. One therefore needs to consider what cell types are most pertinent to the disease and it may not be possible to have all relevant cell types present in a given model. This may be important in TSC, in which changes in several different cell types and non-cell autonomous effects may converge to produce disease phenotypes. For example, in mice, a growth factor secreted by Tsc1 KO neurons impairs oligodendrocyte

development, which in turn negatively affects the myelination of cortical neurons (Ercan et al., 2017). In addition, in a *Tsc1* conditional knock-out mouse model, changes in inhibitory synapses onto excitatory neurons are what drive hyperactivity of the hippocampal network (Bateup et al., 2013). Neuronal differentiation approaches tend to produce mainly glutamatergic or GABAergic neurons but generally not both as these arise from different lineages. Excitatory/inhibitory circuits have been modeled in 2D cultures by mixing separately derived excitatory and inhibitory neurons together (Yang et al., 2017), or in 3D cultures by fusing pallium (excitatory glutamatergic lineage) and subpallium (inhibitory GABA-ergic lineage) organoids together into an “assembloid” (Birey et al., 2017).

A particularly exciting aspect of human stem cell-based disease modeling is the option to develop patient-specific models. These have the major advantage of preserving patient-specific genetic information. The disadvantage is the challenge in finding an appropriate control as differences between cell lines generated from different individuals could reflect cell line variability or differences in genetic background unrelated to disease state. Gene editing approaches can be employed to generate isogenic cell lines with defined mutations. This approach can be done in the context of patient cells to capitalize on the advantages of both technologies, namely generating an isogenic disease model with patient-specific cells (Ebrahimi-Fakhari et al., 2016; Blair et al., 2018). Advances in gene editing have also facilitated more sophisticated genetic models in which conditional mutations can be generated that were previously only feasible in animal models (Pak et al., 2015; Blair et al., 2018). These conditional strategies may be particularly relevant to mTORopathies as somatic mutations in the mTOR pathway can lead to disease (D’Gama et al., 2017). In this case, using patient-derived cells to establish a model is not possible, as the somatic mutation would not necessarily be present in the fibroblasts or blood cells used for somatic cell reprogramming. Advanced gene editing can also be used for the expression of additional tools such as genetically encoded calcium indicators to monitor neuronal activity, optogenetics proteins for neuronal activation or silencing, or fluorescent cellular organelle reporters (Roberts et al., 2017).

In summary, the concurrent technical developments of neuronal differentiation from hPSCs, iPSC creation from human somatic cells, and genome engineering have facilitated the modeling of neurodevelopmental disorders in a laboratory setting. In particular, by applying these techniques to TSC, new human cellular models have been developed to not only answer questions about the developmental origins of the disorder but also give biological insights into treatment. We anticipate that the continued use and refinement of these models in conjunction with animal models will eventually lead to more effective and less invasive treatment for TSC and related mTORopathies.

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