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James H. Millonig *Editors*

Neurodevelopmental Disorders

Employing iPSC Technologies to Define and Treat Childhood Brain Diseases

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About the Editors

Emanuel DiCicco-Bloom, MD, is a Professor of Neuroscience and Cell Biology and Pediatrics (Child Neurology and Neurodevelopmental Disabilities) at Rutgers Robert Wood Johnson Medical School (RWJMS) in New Jersey and a member of graduate programs in Cell and Developmental Biology, Neuroscience, and Toxicology at Rutgers University. He graduated *summa cum laude* from Princeton University, received his M.D. from then Cornell University Medical College, and trained in Pediatrics and Neurology at New York Hospital-Cornell Medical Center, joining RWJMS in 1990.

Dr. DiCicco-Bloom has broad experience performing basic and translational research on neurodevelopmental disorders including animal models and human induced pluripotent stem cells (iPSCs). As an active child neurologist, his research focuses on defining molecular and cellular pathways that regulate the production of neuronal cells (neurogenesis) during brain development, and how related abnormalities contribute to developmental disorders including autism. He investigates how growth signals, genetic factors, and environmental toxins impact cell proliferation, survival, and fate determination during brain development using rat and mouse neural stem cells in culture and in vivo, with a focus on the cerebral cortex, cerebellum, and hippocampus. To begin defining mechanisms that are more directly relevant to human disorders, recent collaborative studies with Co-Editor James H. Millonig, Ph.D. have focused on creating human induced neural stem cells (NPCs) from people with autism, to determine their neurobiological signatures. Significantly, by comparing NPCs from two forms of autism, including idiopathic and genetically defined (CNV 16p11.2 Deletion Syndrome), these studies reveal a common neurobiological phenotype consisting of reduced neural process outgrowth and cell migration in autism compared to control NPCs. Moreover, dysregulation of the mTOR signaling pathway appears to be central to these phenotypes, as pathway manipulation both corrects autism deficits and recreates abnormalities in relevant controls. This new era of exploring neuropsychiatric conditions in human neurons may provide more relevant cellular and molecular pathways on which to target therapeutic interventions that may be "personalized" to the needs of the specific individual.

Dr. DiCicco-Bloom has long served the autism scientific and advocacy communities, providing scientific expertise to federal agencies including NIH, DOD, NSF, and IACC, and disease advocacy organizations including National Alliance for Autism Research, Autism Science Foundation, Autism Speaks, Autism Tissue Program, Brain Canada, Rett Syndrome Foundation, and Simons Foundation. He Chaired the Scientific Program Committee of the 2008 International Meeting for Autism Research (IMFAR) meeting (London) and Co-Chaired 2010 (Philadelphia), and has long served the IMFAR Program Committee. For the Society for Neuroscience, he has served on many committees (PECC, GPA, Audit, Rigor and Reproducibility Working Group) and as a Councilor. Currently, he is the Chair of the NIH Developmental Brain Disorders Study Section, is a member of the DOD Autism Research Program and the American Brain Coalition (ABC) Board of Directors, and serves as Scientific Advisor to the Eagles Autism Foundation. He serves on the editorial boards of Autism Research, Molecular Autism, and other developmental neuroscience journals, and has authored numerous research articles and book chapters in neuroscience, child neurology, and psychiatry.

James H. Millonig, PhD, is an associate professor in the Department of Neuroscience and Cell Biology and resident member in the Center for Advanced Biotechnology and Medicine at Robert Wood Johnson Medical School—Rutgers University.

Dr. Millonig graduated from The University of Rochester *magna cum laude* with a B.S. in Biochemistry. After college, he received an M.Sc. degree from Oxford University, University College studying *B. subtilis* sporulation. Dr. Millonig matriculated in the Princeton University's Molecular Biology PhD program and performed his thesis research in the lab of Shirley Tilghman Ph.D., *President Emerita* of Princeton University. During his Ph.D., he was trained in genetics, mouse development, and molecular biology. Subsequent post-doctoral research, using mouse genetic approaches to study cerebellar development, was performed in Mary E. Hatten's laboratory at The Rockefeller University. He joined the faculty at Rutgers in 1999.

Dr. Millonig has studied CNS development throughout his career applying molecular genetic approaches to understand basic mechanisms and disease. His work in collaboration with Emanuel DiCicco-Bloom M.D. and Linda Brzustowicz M.D. demonstrated the genetic association of *ENGRAILED2* (*EN2*) with autism spectrum disorder and defined autism-like behaviors in mouse models that could be reversed by pharmacological treatment. His lab also identified the orphan GPCR, Gpr161, as an unknown, important regulator of neural tube closure and lens development. Forward mouse genetic approaches demonstrated that Gpr161 functions through the retinoic acid (RA) pathway. His lab is currently studying rare human mutations in the RA metabolic pathway and Gpr161 and their impact on development. More recently, Dr. Millonig and Dr. DiCicco-Bloom have applied iPSC-based approaches to study idiopathic and 16p11.2 Deletion Syndrome. They have defined neurodevelopmental phenotypes in culture and are applying numerous omic strategies to define the downstream signaling and cell biological pathways affected in

autism. He is the recipient of many research awards including Basil O'Connor Starter Research Award, NARSAD Young Investigator Award, and the Thomas A Edison Patent Award for medical diagnostics. He has authored over 100 abstracts, papers, and grants related to autism and neurodevelopmental disorders. He has been an active reviewer for many scientific journals including Molecular Psychiatry, Translational Psychiatry, and eLife. For the NIH, he has been an *ad hoc* member of Clinical Neuroplasticity and Neurotransmitters and Genetics of Health and Disease, was a standing member of the NIH Developmental Brain Disorders Study Section (2010–16), and has also contributed to NIH Loan Repayment Program, BRAIN, and NIH Director's Early Independence Awards initiatives.

He is currently the Director of the Rutgers—RWJMS—Princeton University MD/PhD program and is the senior associate dean in the Rutgers School of Graduate Studies. He was co-PI on an NIH Director's Fund Broadening Experiences Scientific Training (BEST) training grant. In these roles, Dr. Millonig has developed numerous educational initiatives including the BEST program called iJOBS, which includes all Rutgers campuses across NJ that have reached 836 trainees or 16,258 person-hours in 4 years.

Modeling Neurodevelopmental Deficits in Tuberous Sclerosis Complex with Stem Cell Derived Neural Precursors and Neurons

Maria Sundberg and Mustafa Sahin

1 Background of Tuberous Sclerosis Complex

1.1 Neurology of TSC

TSC is an autosomal dominant, genetic disorder that profoundly affects development of different organs, including the central nervous system (CNS). TSC is classified as a rare disorder with a prevalence of 1:6000 live births worldwide [1–3]. The genes affecting the occurrence of TSC are *TSC1* and *TSC2*, encoding for proteins that form a complex regulating the mechanistic target of the rapamycin (mTOR)-pathway [4, 5].

During the development of the CNS, TSC is characterized by formation of cortical and cerebellar tubers, low grade astrocytic tumors known as subependymal giant cell astrocytomas (SEGAs) and small non-cancerous lesions known as subependymal nodules (SENs). Epilepsy is very common within TSC patients affecting >80–90% of the population [6]. Thus, TSC is considered to be one of the most common genetic causes of epilepsy. Clinically, epilepsy affects the lives of the vast majority of TSC patients, and without effective treatment it can negatively affect the patient's brain development. In general, the majority of treatments used for epilepsy suppress seizures in the short-term but do not provide long lasting cure for the deficits within the neural circuitry, and the mechanisms that cause epileptogenesis in TSC patients are still under investigation. Autism spectrum disorder (ASD) is diagnosed in approximately 40–60% of patients with TSC [7, 8]. Previous studies have indicated that children with idiopathic ASD and children with TSC and ASD have similar behavioral and cognitive deficits, indicating common circuit-level dysfunction [9, 10]. In addition, although several cognitive- and behavioral-therapies exist

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for TSC patients with cognitive impairment and autism, there are currently no treatments that permanently reverse these deficits. Thus, novel approaches are crucially needed to provide more effective and safe treatments for TSC patients.

In recent years several research studies have shown that human pluripotent stem cells, such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are an ideal tool for characterization of human neuronal development in vitro in neurodevelopmental and neurodegenerative disorders [11–19]. In addition, TSC-patient derived hiPSC-derived neurons can be used for disease phenotyping at the cellular and genetic level and for screening of compounds for the development of new drug interventions. Although TSC is a multi-organ disorder that affects development of the eye, heart, lung, kidneys, skin, and brain, here we are going to concentrate on the neurological manifestations of TSC and describe recent advances in the human stem cell derived neuronal models of TSC. We are also going to introduce Tsc1/2-deficient animal models whose characterization has supported the establishment and validation of the disease phenotyping assays for human stem cell derived neuronal cells in vitro.

1.2 mTORC1 and mTORC2

mTOR is a conserved serine/threonine kinase that is formed by two functionally distinct complexes: mTORC1 and mTORC2 [20]. Hyperactivation of mTORC1 greatly affects the development of neuronal cells and is a key factor associated with neurodevelopmental deficits in TSC. mTORC1 complex consists of several subunits, including nonobligate protein proline-rich AKT1 substrate 40 kDa (PRAS40), DEP domain containing mTOR-interacting protein (DEPTOR), regulatory associated protein of mTOR complex 1 (RAPTOR), mammalian lethal with sec13 protein 8 (mLST8), telomere maintenance 2 (TEL2), and TELO interacting protein 1 (TTI1). mTORC1 phosphorylates eukaryotic translation initiation factor 4E binding protein (EIF4EBP1) and protein kinase S6-kinase1 (S6K) which affects mRNA translation, cell growth, cell proliferation, and differentiation [20]. In the regulation of mTORC1, TSC1 and TSC2 are accompanied by TBC1D7 [21], which is a member of the TBC-domain containing proteins and an important subunit of the tuberous sclerosis TSC1-TSC2 complex. TSC2 functions as a GTPase activating protein (GAP), which regulates hydrolysis of GTP into GDP on the GTPase Ras homolog enriched in the brain (RHEB). RHEB-GTP activates mTORC1 and stimulates phosphorylation of 4EBP1 and S6K [22]. Mutations in either *TSC1* or *TSC2* can cause loss of function of the TSC1-TSC2-protein complex causing increased RHEB-GTP activation leading to mTORC1 overactivation in cells [22].

mTORC2 complex also consists of several components, including telomere maintenance 2 (TEL2), TELO interacting protein 1 (TTI1), protein observed with rictor (PROTOR), DEPTOR, mammalian stress-activated protein kinase 1 (mSin1), mLST8, and rapamycin-insensitive companion to mTOR (RICTOR) [20]. The mTORC2 regulates cellular cytoskeleton organization and affects phosphorylation of AKT1 by phosphorylation of Ser-473. mTORC2 is insensitive for short-term

rapamycin treatment, but it is affected by long-term exposure to high doses of rapamycin. mTORC2 is activated by the TSC1-TSC2 complex along with several growth factors and nutrients such as insulin. mTORC2 is involved in cell growth, cytoskeleton organization, and activation of protein kinases. mTORC2 has also been involved in morphological and functional development of neurons [23, 24]. A summary of the mTORC1 and mTORC2 pathways and interacting molecules are presented in Fig. 1.

To develop effective treatment options for TSC, much research has been done with human pluripotent stem cell derived neuronal cells in vitro. In the next section, we will discuss the current research advances that have been made with disease phenotyping of TSC-deficient human pluripotent stem cell derived neuronal cells and how the mTOR-pathway inhibitors affect development of human neurons in vitro.

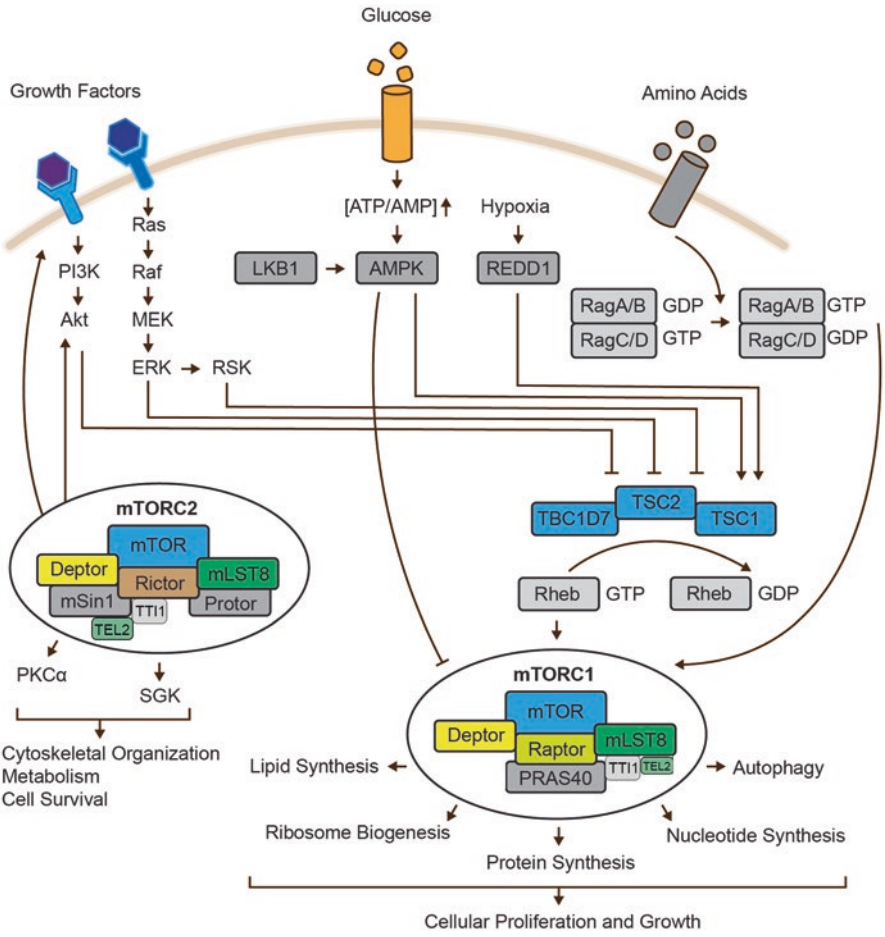

Fig. 1 Schematic presentation of the regulation of the mTORC1 and mTORC2 activation in the cell

2 Neural Stem Cell Development in TSC

2.1 TSC2 Deficiency in hESCs and hiPSCs

To study the neural stem cell development in humans, hESCs and hiPSCs are an ideal source of pluripotent stem cells that can be differentiated in vitro into all three germ layer cell types of the human body [25, 26]. Several neuronal differentiation protocols have previously been developed to drive these cells towards neuronal fate [27, 28]. To study specifically the neurodevelopmental deficits of TSC in vitro, a genetically engineered hESC-derived neuronal cell model has been created with zinc-finger nucleases to induce mutations in one *TSC2* allele ($TSC2^{+/-}$) or both *TSC2* alleles ($TSC2^{-/-}$) [29]. In addition, our group has created an allelic series of *TSC2* mutation in isogenic hiPSCs lines [17] by correction of the $TSC2^{+/-}$ mutation in TSC-patient derived hiPSCs, using the CRISPR-Cas9 gene editing method [30–32], or by inducing a mutation into second allele of the TSC2-patient hiPSCs ($TSC2^{+/-}$) to generate a biallelic deletion in *TSC2* ($TSC2^{-/-}$) with the TALEN method [33].

Several differentiation protocols have been employed to drive the neuronal differentiation of these pluripotent stem cell lines in vitro. For example, TSC2-deficient pluripotent stem cells have been differentiated into neural precursor cells in suspension cultures to form neural aggregates [17] or by using the rosette formation method [29] along with the dual-SMAD-inhibition protocol using noggin and SB-431542 [27]. Noggin specifically blocks BMP-pathway and enhances neuroectodermal differentiation of pluripotent stem cells, and SB-431542 blocks proliferation by inhibiting transforming growth factor beta-superfamily (TGFβ3) including activin receptor like kinases. For cerebellar neural precursor differentiation, cells have been treated with basic fibroblast growth factors (bFGF, FGF8b) and with specific GSK3β inhibitor CHIR-99021 [17]. Further neuronal maturation of PCs was induced in neurobasal medium with B27 and N2 supplements and with BDNF and T3. For differentiation of GABAergic and glutamatergic neurons, BDNF, GDNF, cAMP, and ascorbic acid were used [29]. In addition, to generate induced neurons (iN-neurons) hiPSCs have been transfected with doxycycline inducible NGN2-vector, and cells were selected with puromycin for further differentiation into cortical neurons in neurobasal medium with B27, BDNF, NT3, and laminin [34]. These neuronal cells have been characterized with various analyses, including the gene expression profiling, protein expression profiling, proliferation and differentiation capacity, morphological development, mitochondrial function, synaptic formation, and electrophysiological activity of the TSC2-deficient neural cells. Schematic presentation of the human pluripotent stem cell line derivation and neuronal differentiation for disease phenotyping of TSC in vitro is shown in Fig. 2.

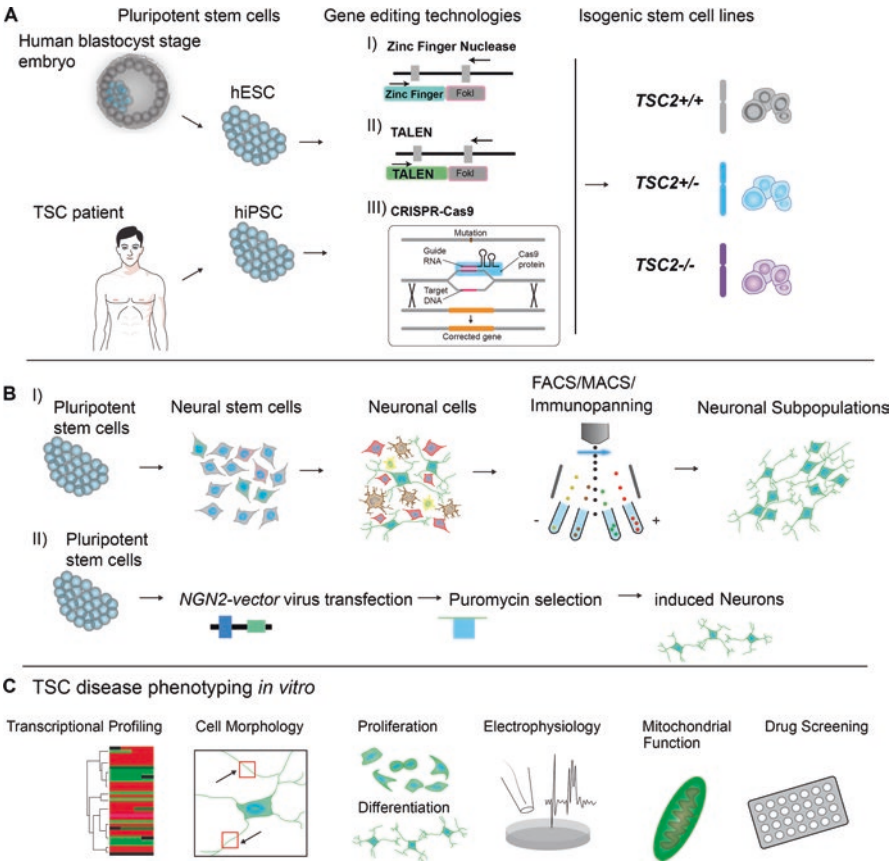

Fig. 2 (**a**) Schematic presentation of human pluripotent stem cell line derivation and gene editing technologies. (**b**) Neuronal differentiation of human pluripotent stem cell lines. (**c**) TSC disease phenotyping assays in vitro

2.2 *Effects of mTORC1 Pathway Activation in Human Neural Stem Cell Proliferation*

During CNS development SENs and SEGAs are typically formed in the TSC-patient brain. These non-malignant tumors consist of giant cells and dysplastic cells that originate from abnormally differentiated neural stem cells (NSCs) during development. Several mouse models have been previously created with *Tsc1* or *Tsc2* mutations in Nestin-Cre-positive NSCs or Emx1-Cre-positive NSCs [35–37]. Although these mice have abnormal cortical lamination and cellular abnormalities that resembled SENs in the cortex, these mouse models have not reliably recapitulated formation of SEGAs [35–37]. Thus, human stem cell models are useful for answering questions surrounding abnormal differentiation of TSC-patient derived

NSCs and the molecular mechanisms and gene-expression deficits causing formation of SEGAs or abnormal function of neuronal networks in TSC.

Several studies have evaluated mTORC1 activity in TSC2-mutant stem cell derived neural precursor cells (NSC/NPCs with *TSC2*$^{+/-}$ and *TSC2*$^{-/-}$) using protein expression analyses of phosphorylation of ribosomal protein S6, 4EBP1, and AKT [17, 29, 38]. According to these studies, the NPCs derived from hESCs with heterozygous loss of TSC2 (*TSC2*$^{+/-}$) did not show significant increase in the pS6 levels compared to control cells (*TSC2*$^{+/+}$) [29]. However during further neuronal differentiation both *TSC2*$^{+/-}$ and *TSC2*$^{-/-}$ neuronal cells had increased pS6 levels and p4EBP1 levels compared to control cells [29]. Similar results were observed in TSC-patient derived hiPSC-cerebellar cell studies, which showed that early neuronal precursor cells with *TSC2*$^{+/-}$ did not display a significant increase in pS6 levels compared to control (*TSC2*$^{+/+}$). However, after later stages of neuronal differentiation (>32 days in vitro) the *TSC2*$^{+/-}$ cells had increased pS6 levels compared to control cells [17]. In both of these human stem cell derived-precursor and -neuronal cell studies, the homozygous *TSC2*$^{-/-}$ cells displayed more robust and significant disease phenotypes, with increased pS6 levels already at the early NSC stage [17, 29]. Similar results were reported by Li et al. with *TSC2*$^{+/-}$ hiPSC-derived NPCs [38]. These data suggests that heterozygous mutation of TSC2 affects cellular function less severely compared to complete loss of TSC2 via biallelic mutation.

To study cell proliferation in vitro in TSC2-deficient NSCs, Costa et al. [29] characterized the size and morphology of the hESC-derived neural rosettes. Their results showed that *TSC2*$^{-/-}$ rosettes were larger due to the increased number of neural precursor cells in the rosettes compared to control *TSC2*$^{+/+}$ neural rosettes. This study also showed that in the developing neuronal cell population the *TSC2*$^{+/-}$ and *TSC2*$^{-/-}$ neural cell populations had upregulated expression of genes related to neural stem cell and precursor proliferation (*NESTIN, SOX2, PCNA*) [29]. Consistent with these findings, increased proliferation of TSC-patient hiPSC-derived NPCs was also described with increased BrdU-incorporation in TSC2-deficient cells or with increased expression of proliferation marker Ki67 in TSC2-deficient cerebellar precursor cell populations [17, 38]. These proliferation deficits were further characterized by detection of increased expression of CYCLIN D1 [17], which is a key regulator of cellular proliferation in NSCs [39, 40]. Abnormal regulation of CYCLIND1/CYCLIND2 has been described as an important factor in the development of megalencephaly–polymicrogyria–polydactyly–hydrocephalus syndrome [41, 42]. Similarly to the pathophysiology of TSC, megalencephaly–polymicrogyria–polydactyly–hydrocephalus syndrome results in increased growth of neuronal tissue and formation of enlarged balloon cells in the patient brain [41, 42]. In addition, abnormally increased neuronal proliferation and brain size have been described in children with ASD [43, 44]. Thus, the abnormal neural precursor cell proliferation via CYCLIND1/CYCLIND2 and mTORC1 pathway dysregulation creates an environment which predisposes the brain towards tuber formation and abnormal brain growth. The human stem cell derived neural precursor models described here, effectively recapitulated these cellular abnormalities and molecular deficits in vitro. Interestingly, we and other researchers have shown that mTORC1-pathway inhibition with rapamycin treatment rescued both the hESC-derived NSCs and the hiPSC-derived NPC proliferation deficits in vitro [17, 29, 38].

These results suggest that the early developmental abnormalities seen in TSC-patient neural cells may be reversible with correctly timed inhibition of mTORC1.

2.3 Differentiation Capacity and Morphological Development of TSC-Deficient Stem Cell Derived NPCs

Abnormal cellular proliferation of TSC-deficient neural precursor cell populations also suggests imbalance in neuronal cell differentiation capacity of the cells. Previously created *Tsc1 or Tsc2* deficient rodent models have displayed enhanced development of neural progenitors and premature differentiation and degeneration of neurons, as well as increased astroglial differentiation of the neural progenitors [36, 45, 46]. We and others have compared these findings to human pluripotent stem cell derived neuronal data [17, 29, 38]. Decreased expression of post-mitotic neurons that express neuronal proteins HuC/D were discovered in $TSC2^{-/-}$ and $TSC2^{+/-}$ hESC-derived neural cell populations at day 14 of differentiation compared to control neural cell populations [29]. Also, decreased expression of neural cell adhesion molecule (NCAM) was detected in $TSC2^{-/-}$ hiPSC-derived neural precursor cell populations at day 16 and day 30 of differentiation [17]. In line with this finding, increased astroglial differentiation was detected in these cellular populations in both hESC-derived TSC2-deficient ($TSC2^{+/-}$, $TSC2^{-/-}$) neural cell populations [29] and TSC-patient derived hiPSC-neural cell populations ($TSC2^{+/-}$, $TSC2^{-/-}$) [17, 38] during the differentiation process shown with increased GFAP expression.

In addition to an altered neural cell fate, the mTORC1 pathway overactivation in TSC leads to increased protein synthesis and cellular growth, which dysregulates normal morphological development of the neurons, as shown in Tsc1/2 deficient rodent models with increased branching and number of axons [36, 45, 47]. Consistent with these studies, the human neuronal cells derived from TSC patients or TSC2-mutant hESCs displayed enlarged soma size and abnormal dendritic branching with an increased number of neurites compared to control neurons [17, 29, 38]. These in vitro findings with human stem cell derived neural cells are also consistent with in vivo findings from TSC-patient brains where abnormal mTORC1-pathway activation causes neural cell overgrowth, abnormal neuronal lamination, tuber formation, and increased glial cell differentiation and SEGA formation [48–50].

2.4 Abnormal Purkinje Cell Differentiation of TSC2-Deficient hiPSCs

Cerebellar damage and deficits, including loss of Purkinje cells (PCs), have been associated with occurrence of autism [51–54]. Also, mouse models with loss of *Tsc1* or *Tsc2* specifically in the PCs display autistic-like behavioral deficits, including abnormal social behavior, ultrasonic vocalizations, cellular spine development,

and PC functionality [46, 55]. Thus, to study the molecular deficits predisposing autism, it is important to take a closer look at how stem cells give rise to the differentiation of PCs and which pathways are important for their normal development and function in the cerebellum.

To create PCs in vitro, mouse embryonic stem cells have been differentiated towards cerebellar lineage with a high concentration of bFGF and insulin [56]. This study described a method of differentiation of Neph3-positive (KIRREL2) precursor cells that were sorted with FACS for enrichment of L7 (PCP2) positive PCs, and these cells were co-cultured with mouse embryonic granule cell progenitors for final PC maturation in vitro [56]. Transcription factors that were upregulated in the cerebellar progenitors were *Wnt1*, *Fgf8*, *En2*, *Lhx5*, *Corl2* (*SKOR2*), which are important for PC precursor development [56]. Similarly, hESCs have been differentiated into PCs using high bFGF and insulin in vitro, and co-culturing the cells with mouse cerebellar cells or cerebellar tissue sections [57, 58]. This protocol also included a cell sorting step that facilitated enrichment of KIRREL2+ PC precursors in vitro [57]. These methods have been used to differentiate spinocerebellar ataxia patient hiPSCs into PCs in vitro [59]. However, reliable repetition of these methods with different hESCs or hiPSC-lines has been challenging. Thus, our group has recently developed an improved differentiation protocol for derivation of human PCs from hiPSCs in vitro [17]. In this new protocol, hiPSCs are first cultured on suspension spheres to form neural precursor aggregates, in the presence of noggin and SB-43752 that induces dual SMAD-inhibition and drives the cells towards neural lineage by inhibiting the BMP4 and TGFβ3-pathways [27]. To further induce the midbrain/hindbrain boundary development, the cells were differentiated with a specific GSK3β-pathway inhibitor, CHIR-99021 [17]. In addition, transcription factors FGF8b and bFGF were added to the cells to guide them to express cerebellar lineage markers [17]. With this method, the hiPSCs were differentiated efficiently towards cerebellar lineage, and they expressed the transcription factors *EN1*, *EN2*, *HOX1*, *GBX2* and proteins such as KIRREL2, SKOR2, LHX1A [17]. At day 30 of differentiation > 20% of the cells expressed PCP2. The developing PC populations were sorted with THY1+ selection, and co-cultured in the presence of mouse cerebellar granule neurons towards PCs. After 120 days of differentiation 60–90% of cells were PCP2 positive, which indicated efficient human stem cell derived PC production in vitro [17].

To further analyze the differentiation capacity of TSC2-mutant patient derived hiPSCs into PCs, the cerebellar lineage marker expression profiles were characterized during the differentiation process. The TSC2-deficient hiPSCs displayed decreased *GBX2* expression at early stages of cerebellar differentiation, and the expression levels of proteins SKOR2 and LHX1A were also downregulated. Increased astroglial derivation of the precursor cells was also detected, as described above [17, 29, 60]. This study also showed that post-mitotic neuronal markers THY1 and PCP2 were reduced during *TSC2*$^{-/-}$ hiPSC-derived cerebellar precursor cell differentiation into PCs in vitro [17]. Morphological abnormalities of THY1+ TSC2-deficient PCs were detected with increased soma size and increased number of neurites compared to control PCs. These deficits were mTORC1-dependent, and

they were rescued with long-term rapamycin treatment in vitro. These results suggest that developmental deficits of TSC2-mutant PCs could contribute to the development of ASD in patients with TSC.

3 Gliosis and Myelination in TSC

3.1 Astrocyte Differentiation from TSC-Deficient Neural Precursor Cells

During spontaneous differentiation of TSC2-deficient pluripotent stem cell derived NPCs towards neuronal lineage, the TSC2-mutant cells have significantly lower capacity to differentiate into neurons and increased capacity to differentiate towards astroglial cells [60]. Gene expression analyses of TSC2-deficient hESC-derived NPCs revealed that TSC2-mutation increased activation of inflammatory pathways and increased cellular metabolism in these cells. At the gene- and protein expression level the TSC2-deficient neural cells had increased synthesis of angiogenic factors that are important inducers of vasculogenesis in the cells [60]. Interestingly, this study also described that inhibition of mTOR-pathway hyperactivation with rapamycin rescued the translational deficits, but did not affect the mRNA expression levels in the TSC2-mutant cells [60]. Grabrole et al. detected upregulation of genes regulating glial fate, *NF1B* and *NF1X,* and downregulation of the pro-neuronal marker *MASH1* in TSC2-mutant NPCs compared to control cells. During further differentiation of the TSC2-deficient NPCs, the expression of astrocyte markers *GFAP and* phospho-STAT3, and glial precursor markers CD44 and CD184, were significantly increased in TSC2-mutant neural cell populations compared to control populations [60]. Similarly, a previous study of TSC-patient hiPSC-derived neural cell populations has discovered that heterozygous mutation of TSC2 was sufficient to alter the neural differentiation capacity of the NPCs towards an astroglial cell fate [38]. This study also detected increased expression of proliferative BrdU+ astroglial cells and increased mTORC1-pathway activation in astrocytes in the TSC-patient derived cell populations compared to control cell populations [38]. Increased astrocyte differentiation capacity has also been described in TSC-patient hiPSC-derived cerebellar precursor cell populations with increased CD44 and GFAP expression during differentiation [17]. Taken together, these studies indicate that loss of function of TSC2 during differentiation of the human NPCs leads to increased astroglial precursor cell differentiation and decreased neuronal differentiation and maturation in vitro [17, 29, 38, 60]. Increased astroglial differentiation of the TSC-deficient NPC populations is also consistent with clinical data showing increased formation of astrocytomas and tubers with increased number of astroglial cells in the TSC-patient brain [61].

To gain a greater understanding of the gene expression changes of the human TSC2-deficient astrocyte enriched cell populations, researchers compared their

dataset with two microarray datasets from previous studies of mouse models of reactive astrogliosis; ischemic-stroke-model and LPS-mediated neuroinflammation model [60, 62]. This comparison revealed significant overlap of the differently expressed genes among *TSC2*$^{-/-}$ human neural cells versus the mouse reactive astrocyte-models. Further analyses of the enriched gene sets revealed that pathways related to metabolic and inflammatory responses were upregulated in the TSC2-deficient hESC-derived astrocyte enriched cell cultures [60].

Strong correlation has also been detected between gene ontology analyses of the hESC-derived TSC2-deficient neural cell populations compared to microarray gene expression analyses of SEGAs and cortical tubers of TSC patients [60, 63]. Pathways related to neo-angiogenesis and inflammation (interferon-induced guanylate binding proteins *GBP1* and *GBP2*) are significantly enriched in the TSC2-deficient neural cell populations and in cortical tubers and SEGAs. Interestingly, increased expression of angiogenic markers, vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), at the gene- and protein-level in TSC2-deficient neural cultures suggests that TSC2-deficient tuber forming cells have capacity to induce vascularization during development of the SEGAs [60]. These data suggest that development of anti-angiogenic treatment options for TSC with VEGF-receptor blocking drugs could be used to prevent vascularization of tumors and to inhibit growth of astrocytomas during differentiation of the TSC-deficient patient cells. Moreover, increased inflammatory signaling in astrocytes has previously been linked to mTOR-pathway hyperactivation and occurrence of epilepsy in a *Tsc1-GFAP*-conditional knock-out mouse [64]. Treatment of these mice with anti-inflammatory agent epicatechin-3-gallate partially improved their viability and reduced the number of seizures [64]. It is possible that combining existing mTORC1-pathway inhibitors together with anti-inflammatory drugs and anti-angiogenic drugs could provide more efficacious treatments for TSC patients. Such combined treatments could work simultaneously to inhibit reactive astrocyte activation, hyperexcitability, and vascularization of the SEGAs in the TSC-patient brain.

3.2 Development of Oligodendrocytes and Myelination Deficits in TSC

Oligodendrocytes are myelin forming cells of the central nervous system. Previous mouse studies have shown that the mTOR-pathway has an important role in oligodendrocyte development [65], and mTOR affects myelination through regulation of myelination production related protein synthesis, lipid metabolism, and cholesterol consumption in oligodendrocytes [66, 67]. In TSC patients, hyperactivated mTOR signaling dysregulates myelination of the axons in the cortex and large white matter tracts, which has been detected in neuroimaging studies [68–70]. In line with these clinical findings, mTORC1-pathway hyperactivation in Tsc1/2-deficient mice affects differentiation of glial precursor cells leading to increased astroglial differentiation and altered differentiation of oligodendrocytes, causing myelination deficits in the

CNS [71, 72]. A mouse model of specific loss of *Tsc2* in oligodendrocyte precursor cells displayed reduced numbers of Olig2+ oligodendrocytes in the cortex and corpus callosum compared to a control mouse brain. In addition, the Tsc2-deficient mouse oligodendrocyte precursors are drawn towards an astrocyte cell fate instead of maturation into oligodendrocytes [71]. This finding is in line with previous mouse studies, where hyperactivation of that mTORC1-pathway in Tsc1-deficient oligodendrocytes leads to hypomyelination of axons [72]. At the molecular level, the deficient mTORC1-pathway hyperactivation and loss of Tsc1/2 in mouse oligodendrocytes have been shown to reduce the expression of transcription factors that regulate cholesterol consumption and lipid production, which leads to deficient myelin production [71, 72]. Interestingly, mTORC1 plays a more critical role in the myelination regulation than mTORC2 alone [72, 73]. This has been shown in raptor (mTORC1) mutant mouse oligodendrocytes where myelin protein expression and lipogenesis were downregulated significantly compared to control cells, whereas in rictor (mTORC2) mutant oligodendrocytes myelination or lipogenesis were not altered as significantly compared to control cells [72].

As described above, altered myelination in TSC appears to be a multifactorial process. To summarize, firstly Tsc1 or Tsc2 deficiency regulates glial precursor fate decisions leading to decreased number of oligodendrocytes, and by negatively affecting expression of myelin producing transcription factors in the oligodendrocytes [71, 72]. The second cause of myelination deficits in TSC is due to non-cell autonomous effects, where abnormalities in the TSC-deficient neuronal cells lead to abnormal development of oligodendrocytes and deficits in myelin production [74, 75]. The molecular mechanism behind this effect has previously been described in a mouse model of specific loss of Tsc1 in cortical neurons [74, 75] and with TSC-patient hiPSC-derived TSC2-deficient neurons [74]. Mouse model showed decreased myelination of the CNS due to upregulation of an inhibitory signaling mediator known as connective tissue growth factor (CTGF). Increased expression of CTGF was also detected in TSC2-deficient patient hiPSC-derived neurons [74]. These myelination deficits were partially rescued by deletion of CTGF in the neurons [74].

To study oligodendrocyte development in human stem cell models, several differentiation protocols have been developed to derive oligodendrocytes from pluripotent stem cells [76–80]. These methods include several different growth factors that have been detected originally in mouse embryonic oligodendrocyte development and specification, such as SHH, bFGF, PDGF-AA, IGF-1, RA, T3, and NT3 [81]. The differentiation of human pluripotent stem cells to oligodendrocytes usually takes around 75–120 days in vitro, and includes both suspension culturing of glial-spheres and adherent culturing of the maturing oligodendrocyte precursor cells [76–80]. TSC-patient hiPSC derived oligodendrocyte precursors cells have shown increased proliferation capacity and decreased maturation in co-cultures with patient neurons, and rapamycin treatment rescued these deficits. These TSC-patient hiPSC derived oligodendrocyte models are valuable for characterization of the molecular pathways driving the myelination deficits in the TSC-patient brain [82].

4 Cellular Homeostasis and Viability in TSC

4.1 Mitochondrial Dysfunction in TSC

Mitochondria are cell organelles that regulate cell respiration and energy consumption via production of ATP. Normal mitochondrial function is important for cells and especially for differentiating neuronal cells that require a lot of energy for synapse formation and for development of electrophysiologically active neuronal networks [83]. In TSC, the hyperactivated mTOR-pathway dysregulates the normal neuronal development and function, and it has been detected that mitochondrial function is impaired in Tsc1 and Tsc2-deficient hippocampal and cortical neurons [83, 84]. Specifically, oxidative stress was increased in the Tsc-deficient neurons [83, 84]. In addition, in the Tsc2-deficient neuronal axons the number of mitochondria was decreased compared to control axons. Mitochondrial respiration was also altered in the Tsc2-deficient rodent neurons [83]. These disease phenotypes were also studied in TSC-patient derived hiPSCs that were differentiated into cortical neurons with the NGN2-overexpression iN-protocol [34], both from TSC-patient derived hiPSCs ($TSC2^{+/-}$) and from the TALEN induced biallelic-mutant hiPSCS ($TSC2^{-/-}$), and compared to parental control cells ($TSC2^{+/+}$) [83]. In contrast to rodent data, no differences in mitochondrial respiration were detected in TSC2-deficient patient derived cortical neurons ($TSC2^{+/-}$) compared to control neurons ($TSC2^{+/+}$). However, the $TSC2^{-/-}$ hiPSC-derived cortical neurons had significant reduction in the mitochondrial function compared to control neurons ($TSC2^{+/+}$) [83]. Further staining and intensity analyses of the mitochondrial membrane potential with tetramethyl-rhodamine-ethyl-ester dye showed significant reduction in the membrane potential of the $TSC2^{-/-}$ hiPSC-neurons compared to control neurons ($TSC2^{+/+}$), although significant changes were not detected in the patient derived cortical neurons ($TSC2^{+/-}$) compared to control neurons. In a more detailed analysis, this study also discovered accumulation of mitochondria in the soma of the TSC2-deficient human neurons ($TSC2^{-/-}$). In addition, there was abnormal axonal transport of the mitochondria back to the soma in the Tsc2-deficient neurons, leading to decreased presynaptic mitochondria in these neurons [83]. These results suggest that dysfunctional mitochondria and decreased energy production at synapses may contribute to the impaired synaptic development in TSC-deficient neurons.

4.2 Autophagy in TSC

Autophagy is a cellular process that maintains catabolic processes in the body, by breaking down dysfunctional and damaged cells and their organelles and by releasing degraded molecular components in order for new cells to reform. Growth factor depletion, nutrient depletion, oxidative stress of mitochondria, endoplasmic reticulum-stress, toxic accumulation, and infection can induce autophagy in cells

[85]. Autophagy regulates cell death via interaction with necrotic and apoptotic pathways. Autophagy also functions as a main regulator of recycling damaged or aged mitochondria in the cells, in a process called mitophagy [85]. Autophagy activation may lead to apoptosis and cell death in neurodegenerative [86, 87] and neurodevelopmental diseases [88], where abnormal protein accumulation and organelle dysfunction are present. Typically, mTOR-pathway activation suppresses autophagy in the cells [89]. In brains of ASD patients, it was reported that a hyperactivated mTOR-pathway reduced autophagy activation[90]. This study also showed that deficient autophagy activation affected spine pruning and caused increased spine density in ASD patient neurons compared to control neurons [90]. Related to these findings, impaired autophagy activation has been detected in both TSC-patient brain tissues and in mouse models with Tsc1 mutations, where dysfunctional autophagocytosis is linked to increased epileptogenesis in TSC [91]. In Tsc2-deficient mouse embryonic fibroblasts, autophagy activation was decreased compared to control cells, leading to autophagy substrate p62 accumulation. In addition, combinatorial treatment of $TSC2^{-/-}$ tumors with mTORC1-inhibitor and autophagy inhibitor blocked tumorigenesis more effectively than treatment with either one alone in vivo [92]. Our group has shown that autophagy activation was regulated in opposite directions in the Tsc2 deficient fibroblasts and in the Tsc2-deficient neurons [93]. Tsc2-deficient rat cortical neurons displayed accumulation of the autophagic marker LC3-II and of the autophagy substrate p62 [93]. There was also impaired recruitment of autophagosomes to damaged axonal mitochondria in *Tsc2*-deficient neurons [83]. Similar findings were found in the TSC-patient hiPSC-derived cerebellar cultures, where mitochondrial reactive oxygen species (mROS) and LC3II expression were significantly increased in the $TSC2^{-/-}$ hiPSC-derived PC precursors compared to control ($TSC2^{+/+}$) PC precursors [17]. This may be due to accumulation of damaged mitochondria and increased oxidative stress in the cells, which may lead to increased apoptosis of the TSC2-deficient developing human cerebellar neurons. This molecular deficit is in line with previous data showing reduced volume of the cerebellum of TSC patients [94] and reduced PC number in ASD affected brains [53, 54].

5 Autism and Epilepsy in TSC

5.1 MTOR-Pathway in Autism and Epilepsy

Common features of autism include repetitive behaviors, restricted interests, and a prominent deficit presenting as abnormal and impaired social interactions [95–97]. Previous studies have shown that there exists a prevalent correlation between epilepsy and ASD [98–100]. In general, over 30% of autistic patients also have epilepsy [98–100]. Strikingly, from the TSC patients with deficient mTOR-pathway regulation 80–90% have epilepsy [6], and autism has been described to be present

in approximately 40–60% of TSC patients [7, 8]. Additionally, deficient expression of another mTOR-pathway suppressor called phosphatase and tensin homolog (PTEN) has been associated with development of epilepsy and autism in both patient and mice models in vivo [101–104]. Also, TSC2 deficient hiPSC-derived NGN2-induced cortical neurons with biallelic loss of TSC2 have shown hyperactive network formation, synchronization, and increased mTOR-pathway activation, which were rescued with rapamycin treatment [105]. Taken together, this data suggests that there might exist shared pathological mechanisms that lead to development of both epilepsy and autism, and this mechanism may be linked to hyperactivation of the mTOR-pathway.

5.2 *Tsc-Deficient Rodent Models of Epilepsy and Autism*

Previously, researchers have created several rodent models to study the molecular link between epilepsy and autism. Here we are briefly introducing a few of them as an example of in vivo models. One of these models is a *Tsc2*$^{+/-}$ haploinsufficient rat model that displayed a reduced tendency for novel objects and social exploration in open field studies [106]. When both naïve and *Tsc2*$^{+/-}$ rats were exposed to kainic acid to induce epilepsy, both groups showed reduced social interaction due to increased anxiety [106]. These results suggest that epilepsy in both *Tsc2*$^{+/-}$ mutation and healthy background caused increased tendency for development of autistic-like social deficits in behavior [106]. These deficits were rescued with treatment of the animals with mTORC1-inhibitor rapamycin [107]. Moreover, our research group has previously generated a mouse model of Tsc2 with conditional expression of Tsc2 hypomorphic and *Tsc2*-null alleles together with synapsin-I-cre (SynIcre), which resulted in restricted expression of Tsc2 mutation only in the neurons of the mouse brain [108]. In the Tsc2 mutant mouse brain, the cortical neurons were larger than control neurons, and this difference was related to increased mTORC1-pathway activation and pS6 expression with increased protein synthesis [108, 109]. Importantly, these mice demonstrated behavioral deficits including altered social interaction and ASD-like behavior, anxiety, and epilepsy [108, 109].

Similar to Tsc2-deficient mouse models, researchers have also created a mouse model with specific loss of Tsc1 in neurons and showed that these mice displayed seizures, deficits in neuronal myelination, and enlarged cortical and hippocampal soma size in the brain [75]. Specific mTOR-inhibitors rapamycin and everolimus were sufficient to rescue these disease phenotypes in the mouse model of neuronal loss of Tsc1 [110]. In addition, another mouse model of *Tsc1* gene mutation in serotonergic neurons has displayed autistic-like behaviors and epilepsy related to insufficient neurotransmission of 5-HT. In this study, seizures increased hyperactivation of the mTOR-pathway by increasing pS6 levels in the cells [111]. Hyperactivation of the mTOR-pathway alone without epilepsy also caused autistic-like behaviors in the mouse model of *Tsc1*$^{flox/flox}$*;Slc6a4-cre*, where Tsc-deficiency is specific to 5-HT neurons [111]. Previously, our lab created a mouse model with specific loss of *Tsc1*

in Purkinje cells (PCs), which displayed autistic-like behavioral deficits, including decreased interests in social novelty and increased self-grooming habits, abnormal vocalizations, and deficits in motor learning tasks [46]. At the cellular level the Tsc1-mutant PCs had enlarged soma size and increased pS6 expression, increased spine density, and increased cellular stress compared to control PCs [46]. These mice did not have seizures, but this study showed that cerebellar deficits caused by loss of Tsc1 and mTORC1-pathway activation in PCs are connected to the development of ASD, and mTORC1-pathway inhibition with rapamycin could partially rescue these deficits [46]. Similar results were obtained using Tsc2-knockout specifically in PCs [55]. In summary, these Tsc1/Tsc2 mice models provide evidence that neuron specific loss of Tsc1 or Tsc2 causes mTORC1-hyperactivation leading to both epilepsy and ASD-like phenotypes suggesting a shared pathological origin of these neurological deficits in TSC.

Although these rodent models have been valuable in studying the link between epilepsy and autistic-like behaviors in vivo, these models do not provide insights into the molecular and genetic deficits that drive disease development and manifestation in TSC-patient derived neurons. Thus, in the following sections, we will review the molecular signaling studies in TSC related epilepsy and autism in diagnostically and therapeutically relevant human stem cell derived neuronal cell models.

5.3 *Genes Associated with Neural Differentiation and Synaptic Development in TSC2-Deficient Human Neurons*

To characterize the effects of TSC2 mutations on synaptic development of human neurons, researchers have previously established an allelic series of TSC2-mutations in isogenic hESC-lines with control $TSC2^{+/+}$ and zinc-finger-nuclease-induced $TSC2^{+/-}$ and $TSC2^{-/-}$ [29]. This cellular model described deficient neuronal differentiation and altered synaptogenesis in human stem cells with decreased expression of a group of genes related to synaptic transmission and autism [29], including: *CNTNAP2*, *NLGN3*, *KCC2,* and *RBFOX1*. More specifically, the *CNTNAP2* gene codes contactin-associated protein-like 2, which belongs to the neurexin family of proteins, and functions as a cell adhesion molecule and receptor in the nervous system. The neurexin family of proteins are expressed in myelinated axons, and they regulate neuron and glia interactions during development of the nervous system. Deficits in the expression of this gene have been associated with several neurodevelopmental disorders, which are highly associated with autism and intellectual disability, epilepsy, ADHD, schizophrenia, and Tourette syndrome [112]. NLGN3 is a neuroligin belonging to a family of post-synaptic adhesion molecules that regulate formation, maturation, and function of synapses. Previous studies have indicated that mutations in *NLGN3* and *NLGN4* are associated with autism [113] and induced point-mutations in *NLGN3* altered synaptic functionality in mice hippocampal and cortical neurons [114]. KCC2 is a potassium chloride cotransporter 2 that is coded

by the SLC12A5 gene and operates as a transporter of chloride in mature neurons, in addition to regulating neuronal inhibition [115, 116]. Decreased KCC2 expression reduces activation of GABA receptors by increasing intracellular Cl^- levels and depolarization of the cells, which leads to decreased inhibitory signaling of the GABAergic cells. In the studies of Rett-syndrome, researchers have shown that stem cell derived neurons from Rett-patients had deficient MeCP2 expression which caused loss of KCC2 expression and delayed developmental switch of GABAergic cells from excitatory to inhibitory [117]. Interestingly, previous studies have also described that loss of function of KCC2 causes infantile epilepsy. Downregulation of KCC2 expression is also associated with idiopathic and acquired epilepsy and neurodevelopmental disorders with seizures [115]. Thus, the downregulation of *KCC2* in TSC2-mutant human stem cell derived neurons [29] could cause reduced inhibitory signaling of the GABAergic neurons and lead to hyperactivation of excitatory neurons resulting in seizures in TSC patients. *RBFOX1* gene expression has also been shown to be downregulated in TSC2 deficient human neurons compared to control neurons [29]. RBFOX1 also known as Ataxin-2-binding protein 1 (A2BP1) or FOX1 is coded by the *RBFOX1* gene which is located in chromosome region 16p13 in humans [118]. Several studies have shown that deficient RBFOX1 expression is highly associated with autism, and other neurodevelopmental disorders, including epilepsy [119–123].

In addition, detailed gene expression analyses of TSC2 deficient hESC-derived neural precursor cells have discovered downregulation of genes related to neuronal maturation and synapse formation, with increased expression of genes related to astroglial differentiation, and increased expression of genes related to inflammation and metabolism [60]. This is a relevant finding, since previous pathological studies have shown that in the cortical tubers of TSC patients, inflammatory genes were also enriched, whereas synaptic transmission related genes were decreased [63], and both of these datasets contained interferon-inducible genes *GBP1* and *GBP2* [60, 63]. When these in vitro data of TSC2-mutant hESC-derived neural cells was compared to microarray data of SEGAs from TSC patients, correlation in the gene expression profiles was even more significant. Angiogenic factors and inflammation were increased in both groups, whereas the neuronal expression was decreased significantly within both of these groups of genes. In general, the TSC2-deficiency in hESC-derived neural populations drives the cells towards an astrocyte cell fate during the differentiation process, and thus the gene expression profiles were also more strongly correlated with the astrocytomas than with the datasets from cortical tuber biopsies [60]. When one compares this hESC-derived neuronal cell population data to transcriptional gene expression profiling of hiPSC-derived TSC2-deficient PCs with TSC2-gene dosage dependent weighted co-expression analyses [17], there is significant overlap within the gene expression profiles [17, 60].

Gene ontology analyses of the TSC-patient hiPSC-derived PC precursors revealed that genes whose expression were increased in $TSC2^{-/-}$ cells compared to control cells were related to mitochondria function, protein transport, and autophagy. In contrast, the group of downregulated genes in the $TSC2^{-/-}$ cells were genes related to mRNA transportation and production [17]. This led to further comparison of

genes expressed in TSC-patient hiPSC-derived PCs [17] to the gene expression data of FMRP targets in two previous transcriptional gene expression studies [124, 125]. FMRP is coded by *Fragile-X-mental-retardation 1* gene *FMR1*. FMRP functions as an mRNA binding protein and regulator of mRNA trafficking within axons together with motor proteins, and it also mediates protein translation, synaptic protein expression, and synapse formation in axons [124–131]. In addition, several genes related to the development of ASD are enriched in the FMRP target gene group. When the TSC2-deficient hiPSC-derived PC data was compared to these previous gene expression analyses [124, 125], it was discovered that FMRP target genes and target genes of FXR1 were significantly enriched in the co-expression group of downregulated genes in the $TSC2^{-/-}$ hiPSC-derived PCs [17]. These results suggest that both the mTOR-pathway and FMRP regulates the same genetic pathways that are involved in the neuronal dysfunction predisposing to ASD and FXS.

5.4 Synaptic Function and Excitability of TSC2-Deficient Human Neurons

Whole cell patch-clamping is a conventional method that has been utilized for several decades for characterization of functional properties of individual neurons in vitro. The first study to describe the effects of loss of function of TSC2 in the functionality of hESC-derived neurons, discovered that TSC2-deficient neurons ($TSC2^{+/-}$ and $TSC2^{-/-}$) displayed decreased excitability compared to control neurons ($TSC2^{+/-}$) [29]. This study also described reduced glutamatergic synapse formation and function in $TSC2^{-/-}$ neurons compared to control $TSC2^{+/+}$ cells, with decreased frequency of spontaneous excitatory post-synaptic currents (sEPSCs) and miniature excitatory post-synaptic currents (mEPSCs) [29]. These functional deficits were rescued with long-term rapamycin treatment in vitro, which confirmed involvement of mTOR-pathway regulation [29]. This functional data is in line with previously described altered functional and behavioral deficits in the SynCre-Tsc2-mouse model [108, 109]. Importantly, the $TSC2^{-/-}$ hESC-derived neuronal cells displayed similar functional deficits to dysplastic and enlarged neurons in cortical tubers, by being significantly hypoexcitable compared to healthy control neuronal cells [29]. These in vitro findings support previous clinical studies, which have described that formation of cortical tubers predispose the brain towards development of epilepsy in TSC patients by disturbing the formation of neuronal network connectivity and functionality during brain development [132, 133].

In line with these studies, our group has developed a TSC-patient hiPSC-derived PC model, which displayed significant synaptic deficits and electrophysiological abnormalities in the TSC2-deficient PCs compared to control PCs in vitro [17]. According to this study, the TSC-patient hiPSC-derived PCs with specific TSC2-mutations showed significantly increased capacitance and input resistance compared to control PCs [17]. This finding was consistent with the increased cellular size of TSC2-deficient cells

compared to control cells. Interestingly, the TSC2-deficient patient PCs and *TSC2*$^{-/-}$ PCs had lower firing rates compared to control cells. In addition, TSC2-deficient patient PCs and *TSC2*$^{-/-}$ PCs were significantly hypoexcitable compared to control PCs. Although it is important to note that these cells are only modeling human cerebellum development in vitro, the detected disease phenotypes in patient neurons with specific loss of TSC2 are consistent with the functional phenotypes detected previously in Tsc1/2-deficient mice cerebellar PCs [46, 55]. Functional analyses of Tsc1-mutant PCs in the mouse cerebellum slices have shown reduced spontaneous firing rates in the *Tsc1*$^{+/-}$ and *Tsc1*$^{-/-}$ PCs compared to control PCs *Tsc1*$^{+/+}$ [46].

At the molecular level, TSC2-deficient hiPSC-derived PCs have reduced expression of pre- and post-synaptic proteins: synaptophysin and PSD95, and PC specific glutamate receptor δ2 [17]. Importantly, we also detected decreased expression of FMRP protein, in TSC2-deficient hiPSC-derived PCs, which affects synaptic development and functionality of the cells [17]. TSC2-deficient hiPSC-derived GABAergic PCs also had decreased synaptic transmission, which was detected by decreased frequency of mEPSCs in *TSC2*$^{-/-}$ PCs compared to control *TSC2*$^{+/+}$ hiPSC-derived PCs [17]. These functional phenotypes of human PCs are regulated by mTORC1 pathway hyperactivation, since long-term rapamycin treatment rescued these deficits in TSC-patient hiPSC-derived PCs [17]. TSC and FXS are two similar neurodevelopmental disorders that lead to neuronal deficits and ASD. Thus, these studies suggest that there might exist a synergy in mTORC1 pathway regulation and FMRP expression in TSC and FXS. In general, decreased inhibitory neuron activity in the development of the neural network can cause imbalance between inhibitory and excitatory signaling and predispose to dysfunctional neural circuitry formation in TSC and ASD. Although more research needs to be done to characterize the detailed molecular mechanisms affecting the neuronal development and network functionality during inhibitory and excitatory neuron differentiation in TSC-patients brain, here we have summarized the disease phenotypes detected in the TSC2-deficient pluripotent stem cell derived neuronal cells in vitro: Fig. 3, Table 1.

6 Future Prospects

6.1 Pharmacological Treatment of Neurological Deficits in TSC

To date, several preclinical animal studies have been conducted with mTOR-inhibitors rapamycin and everolimus to rescue the behavioral, functional, and cellular deficits in TSC [110, 134]. These studies show that rapamycin treatment increased the lifespan of the animals and decreased occurrence of seizures in the mice [110, 134]. In addition, in clinical studies with TSC patients, everolimus reduced the frequency of the seizures and improved the quality of life of the patients, and the treatment was well tolerated [135]. Other clinical studies have shown that

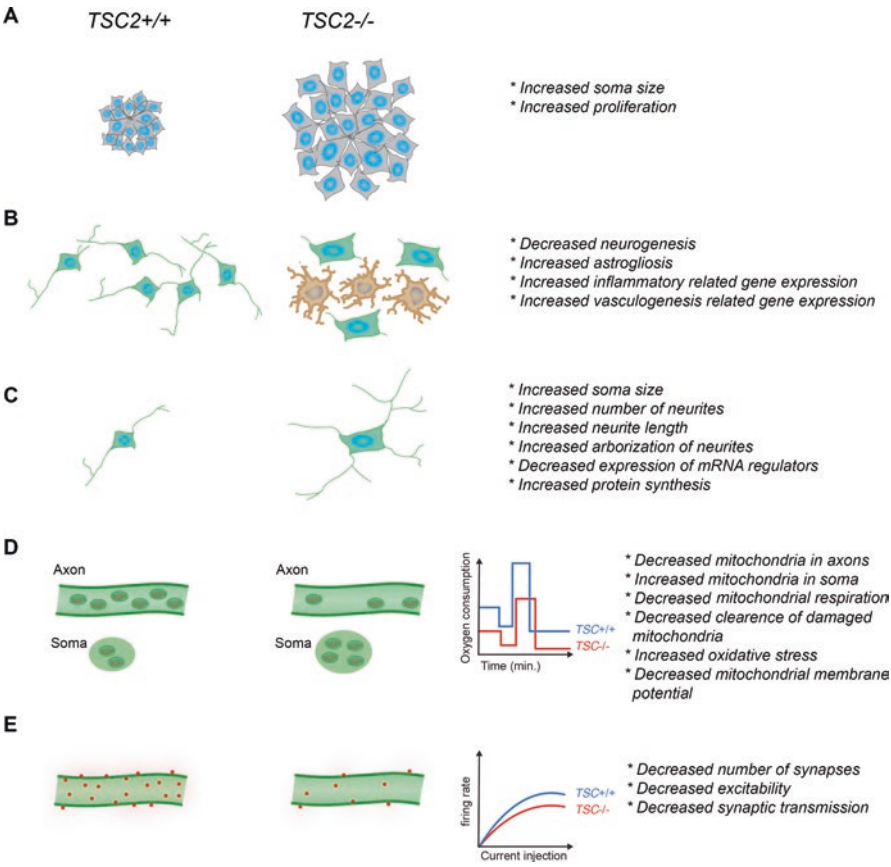

Fig. 3 Schematic presentation of TSC2-deficient cellular phenotypes in vitro. (**a**) Control and TSC2-deficient neural precursor cells. (**b**) Control and TSC2-deficient developing neuronal cultures. (**c**) Control and TSC2-deficient maturing neurons, (**d**) Mitochondrial development and function in control and TSC2-deficient neurons, (**e**) Synapse development and electrophysiological activity of the control and TSC2-deficient neurons

long-term everolimus treatment reduced seizure frequency in ≥50% TSC patient populations [136, 137] and > 50% of the TSC patients became seizure free [137]. Related to this, it has been reported that treatment of TSC patients with sirolimus (rapamycin) decreased frequency of seizures by 41% [138], although significant improvements were not discovered in this study, and cognitive development of the patients did not improve [138]. Taken together, these studies indicate that mTOR-inhibitors may have beneficial effects for the treatment of epilepsy in TSC patients, although more controlled, long-term clinical studies are needed to confirm these findings.

Clinical studies have also shown that mTOR-inhibitors are effective in slowing the growth of SEGAs and renal angiomyolipoma in TSC patients [139, 140]. Although mTOR-inhibitors possess clear advantageous effects for TSC patients,

Table 1 Human pluripotent stem cell derived neural cell cultures as a platform to study TSC

TSC-mutant human stem cells	Stem cell derived neural cell types	Gene deficits in TSC2-mutant human neural cells	Cellular deficits in the TSC2-mutant human neural cells	References
– Isogenic hESCs (*TSC2*+/+) with zinc-finger nuclease-induced allelic series of: *TSC2*+/– *TSC2*–/–	– NPCs and neural rosettes – Mixed population of GABAergic neurons and glutamatergic neurons	– Increased expression of genes regulating NSC proliferation – Downregulation of genes regulating neuronal maturation and synapse formation – Downregulation of genes related to synaptic transmission – Dysregulation of ASD-related genes	– Hyperactivation of mTORC1 pathway in NPCs and neurons – Increased rosette area and abnormal cellular structure – Increased soma size – Increased dendritic arborization – Reduced firing rate – Reduced frequency of sEPSCs and mEPSCs – Rapamycin rescued the early proliferation deficits and soma size in NPCs – Rapamycin rescued the synaptic deficits and functionality of neurons	Costa et al. [29], *Cell Rep*.
– Isogenic hESCs (*TSC2*+/+) with zinc-finger nuclease-induced allelic series of: *TSC2*+/– *TSC2*–/–	– NPCs – Mixed population of neurons and astrocytes	– Increased astroglial genes – Decreased glutamatergic and GABAergic neuron markers – Increased inflammatory induced genes – Increased expression of angiogenic genes	– Reduced neuronal maturation – Increased astrogliosis – Increased active inflammatory response – Increased metabolic activity – Increased ribosome occupancy and protein synthesis – Increased angiogenic protein expression – Rapamycin rescued the translational deficits	Grabole et al. [60], *Genome Med.*

(continued)