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TITLE: Understanding the Role of TSC1/2 in Cerebellar Purkinje Neurons

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Boston, MA 02115-5724

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14. ABSTRACT

Tuberous sclerosis complex is a multisystem autosomal dominant disorder caused by mutations in either TSC1 or TSC2 genes. Previously our group has shown that Tsc1 knock-out mice Purkinje cells are involved in the development of autistic-like features for these mice. In addition, at BCH we have collected fibroblasts and derived pluripotent stem cell lines from TSC-patients and unaffected familial controls. We have developed differentiation protocol for generation of human Purkinje cells from iPSCs. We have studied mTOR-pathway hyperactivation in TSC2-deficient patient iPSC-derived PCs compared to control cells. We are also analyzing the proliferation and differentiation rate of TSC2-deficient neural precursor cells compared to control cells and studying electrophysiological properties of TSC2-deficient iPSC derived Purkinje cells. According to our preliminary functional data, the TSC-patient iPSC-derived PCs and healthy control iPSC-derived PCs exhibit GABAergic inhibitory synaptic currents, which can be blocked with bicuculline. These cells also exhibit glutamatergic excitatory synaptic currents, which can be blocked with CNQX. The disease phenotyping with TSC deficient iPSC-derived PCs is important for the future development of new pharmacotherapy for TSC-patients with autism.

15. SUBJECT TERMS
autism, tuberous sclerosis, cerebellum

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1. INTRODUCTION:

Tuberous sclerosis complex (TSC) is a multisystem autosomal dominant disorder caused by mutations in either *TSC1* or *TSC2* genes. TSC patients are often affected with developmental delay and epilepsy and approximately half of patients with TSC display symptoms of autism spectrum disorder (ASD). Although much research has been conducted, the neural circuitry and molecular mechanism underlying autism remain unclear. Specific cerebellar defects have been seen in TSC patients, suggesting a crucial role for the cerebellum. Cerebellar pathology can be found in 1 in 3 patients with TSC, and studies correlate cerebellar pathology with ASD symptomatology in patients with TSC. Purkinje cells are the sole output neuron of the cerebellum, and previously we have shown that *Tsc1* mutant Purkinje cells cause autistic-like behaviors in mice. The objective of this study is to establish a novel platform to characterize *TSC1/2*-mutation related neurodevelopmental disorders and ASD related cellular dysfunctions in *in vitro* and *in vivo* models of human patient specific iPSC-derived Purkinje cells. The results of this study will provide important insights about the molecular mechanism underlying neurodevelopmental disorders. In the future, the results of this study can be used to establish novel therapeutic targets for treatment of TSC patients diagnosed with or at risk of developing ASD.

2. KEYWORDS: Human iPSC, TSC1/2, Purkinje cells, disease phenotyping, autism, cerebellum.

3. ACCOMPLISHMENTS:

Specific Aims:

Aim 1: To compare the transcriptional profiles of mouse and human PCs with and without TSC1/2 expression. As a first step, we will carry out RNAseq in PC's isolated from Tsc1-knockout mouse and control mice and compare the gene expression profiles to identify differences in expression of transcription factors during PC development. Using the protocol developed by the Hatten lab for differentiating hES cells into PCs, we will select iPSC clones that express the Pcp2 bacTRAP tag (*Pcp2-Egfp-L10a*), differentiate them into PCs and assay the transcriptomes of iPSC-derived PCs from TSC and unaffected patients. We will then use bioinformatics to identify gene pathways that differ in Tsc1 mutant mouse and human PCs compared with wild type/unaffected cells.

Aim 2: To genetically correct TSC mutations in patient-specific iPSC lines and rescue the disease phenotypes in patient specific neurons *in vitro*. We will employ CRISPR-Caspase 9 (Cas9) genome editing techniques to correct TSC1/2 mutations in patient-specific iPSC lines and evaluate the reversibility of any observed phenotypes. The TSC1/TSC2-deficient iPSCs will be differentiated into neuronal cells to establish comprehensive cellular phenotypic analysis compared to isogenic controls, including cell vulnerability to a range of insults, oxidative stress, and electrophysiological properties reflecting cellular excitability.

Aim 3: To evaluate functionality and neural circuit formation capacity of human patient derived cerebellar precursor and PCs *in vivo* in Tsc1-PC knockout mice. The goal is to characterize the functionality and differentiation capacity of human iPSC-derived cerebellar precursors and PCs *in vivo*. We will study if human TSC patient neurons and control neurons survive, and develop normally, integrate into the correct layer of the mouse cerebellum and show functionality. We will analyze dendritic arborization, axon outgrowth, and use electrophysiology to measure neurons excitability. If successful, this research will provide a critical new model system for analyzing disorders that involve human cerebellar circuit development. This study will provide proof of concept that loss of PCs is responsible for the behavioral deficits in the Tsc-deficient animals.

One deviation we have made from the proposed studies as initially submitted is that we have focused our human cell work on the TSC2 gene. There are several reasons for this decision. First, TSC2 mutation is much more common than TSC1 mutation among TSC patients. Second, severity of ASD is higher among those with TSC2 mutation compared to those with TSC1 mutation. Finally and most importantly, as the iPSC field has matured it has become clear that one needs 3-4 patients with the same genetic mutation to show reproducibility of one's findings. So, instead of dividing our iPSC lines between TSC1 and TSC2 patients, we focused on collecting a large number of patients with TSC2 mutations and utilized iPSC lines from these patients for the project.

Studies and Results

For Aim 1. In order to analyze gene expression specifically from Purkinje cells within a mixed culture of cells, a clonal Pcp2-EGFP-L10a TRAP-hESC line was generated using lentivirus. The EGFP-L10a TRAP tag is expressed both during initial differentiation and following isolation of PCs and co-culture with mouse granule cells (Figure 1). RNA isolated from PCs at various stages of differentiation are being assayed by microarray. Currently, we are using metagene analysis and other bioinformatic analysis methods we used previously to examine stage-specific changes in actively transcribed genes during murine GC development (Zhu et al., 2016) using datasets from *Tg(Pcp2EGFoL10a)* TRAP mice. We are especially interested in whether reactomes for chromatin remodeling genes will change during PC differentiation. We will then be able to use metagene analysis to compare metagenes that change during murine PC development with datasets from hES-PC derived cells at different states of differentiation. To date, we have obtained microarray data from one set of hES-PCs cells that express the Pcp2 TRAP tag. Comparison of these datasets will allow an estimation of neuronal maturity as well as variations between mouse and human gene expression. These datasets will serve as a baseline from which TSC patient PCs can be compared. Techniques and vectors for the generation of TRAP TSC-iPSC lines have been transferred from The Rockefeller University to Boston Children's Hospital.

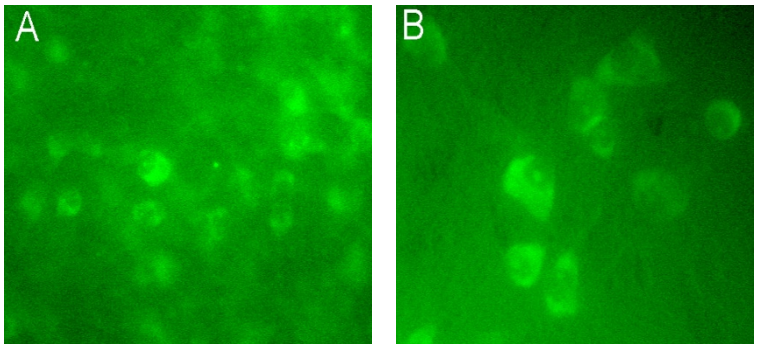


Figure 1. Live imaging of the Pcp2-EGFP-L10a TRAP-hESC line expressing GFP after 20 days of differentiation (A) and following isolation of hPSC-PCs on day 24 of differentiation followed by 24 days of co-culture with mouse granule cells (B). The EGFP-L10a fusion protein is localized to the nucleolus and the perinuclear space, as expected.

At BCH, we have collected mRNA samples from control and TSC2 deficient patient derived hiPSC-derived $THY1^{+}$ selected premature PCs *in vitro and* analyzed the transcriptional profiles of these cells. We performed co-expression analyses of the genes according to the TSC2-gene allele dosages (Figure 2). Then, we performed gene ontology analyses for the TSC2-gene dosage dependent gene expression modules, and we detected increased expression of genes in categories related to mitochondria function, protein transport, and autophagy, which were upregulated in the TSC2-mutant cerebellar cells compared to control cells (M67, Figure 2). In the other co-expression module (M99), we detected enriched expression of genes in categories related to mRNA processing and transport and FMRP target gene group, as well as targets of FXR1, which were significantly decreased in the TSC2-mutant cerebellar cells compared to control cells (M99, Figure 2). We also confirmed these findings with qRT-PCR analyses of the selected genes from these categories (Figure 2E), and we showed that rapamycin treatment rescued expression of some of these genes in the TSC2-mutant PCs ($TSC2^{-/-}$) vs control cells (Figure 2E) (Sundberg et al 2018).

Interestingly, we also detected significant overlap of the TSC-deficient cerebellar cell gene expression modules compared to previous datasets of TSC2-deficient hESC-derived neural lineages, which implicated that there are common shared genes regulated by TSC2-deficiency during neuronal development (Sundberg et al 2018).

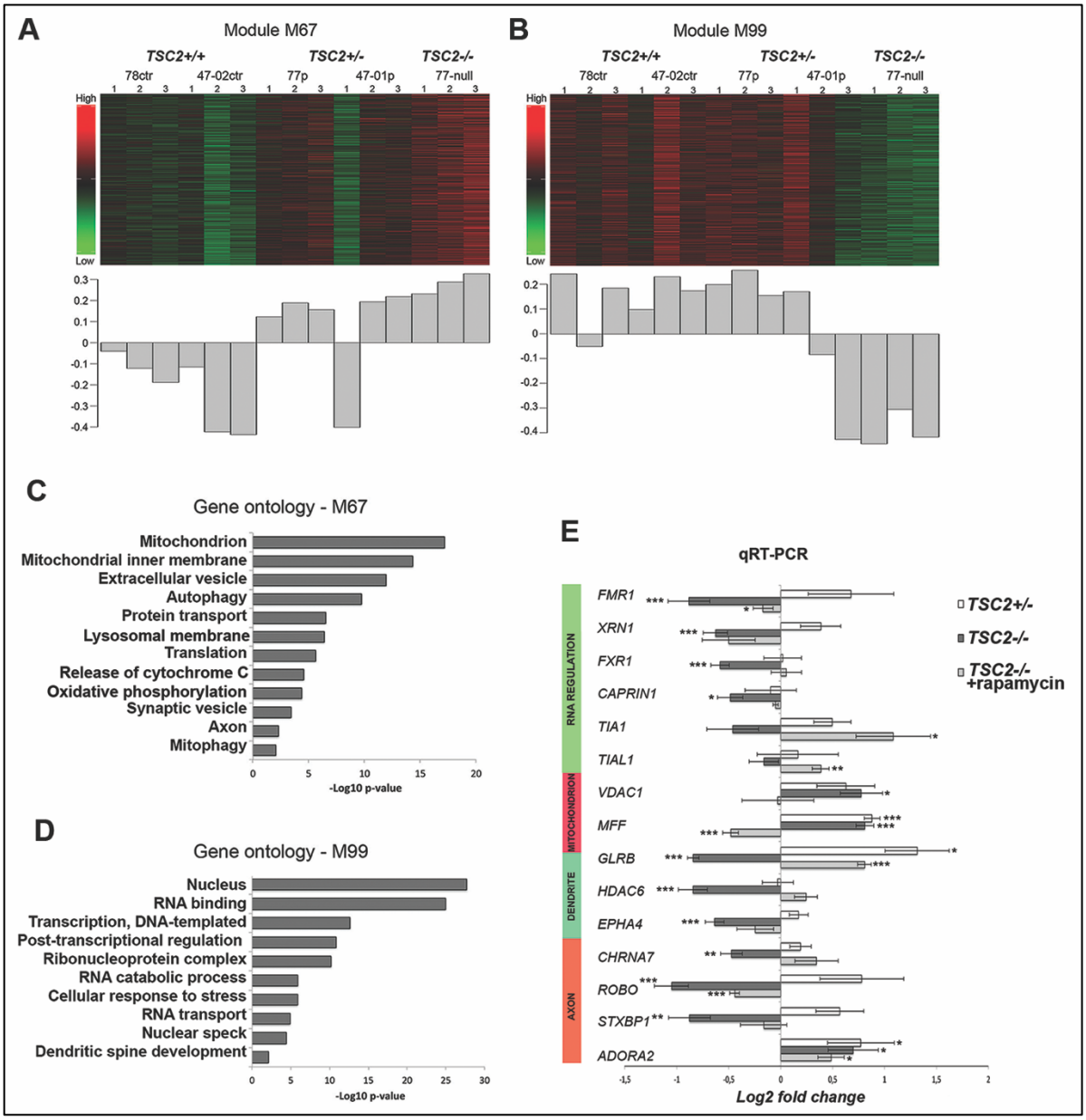


Figure 2. Gene expression profiling of TSC2-deficient hiPSC-derived PCs. A-B. Heatmaps and first principal components of the co-expressed groups of transcripts with the strongest positive (M67) and negative correlation with TSC2 genotype (M99). The heatmap depicts scaled expression of all transcripts within the co-expression group, where green is low relative expression and red is high relative expression. The bar graph shows the first principal component of the expression of the transcripts within the co-expression module. C-D. Gene ontology analysis for genes belonging to the co-expression modules with strongest positive and negative correlation with TSC2 genotype. Gene ontology terms were selected for specificity and non-redundancy. The x axis represents –log10 p-value for enrichment of the GO term calculated by DAVID. E. qRT-PCR analyses from pooled data of two patients (47-01 and 77), two controls (47-02 and 78), and three replicates of TSC2-null lines,

and rapamycin-treated TSC2-null cells, *p<0.05, **p<0.01, ***p<0.001 .(from Sundberg et al 2018).

For Aim 2. At BCH, we have successfully generated CRISPR-cas9-mediated correction of TSC2-microdeletion in TSC-patient (*TSC2*$^{+/-}$) derived hiPSC line (Figure 3) (Sundberg et al 2018). We used puromycin selection for isolation of the CRISPR-cas9 and ssODN transfected cells, and with this method we were able to isolate 5 individual hiPSC clones that contained the corrected TSC2-gene. The gene correction was confirmed with insertion of silent mutation of DNA-cutting restriction enzyme to the CRISPR-cas9 transfected cells, followed by Sanger-sequencing of the correction site (Figure 3B-C). The selected hiPSC-clones had normal karyotype (Figure 3D), and they expressed pluripotency markers SSEA-4, OCT4, NANOG, TRA1-60 (Figure 3E).

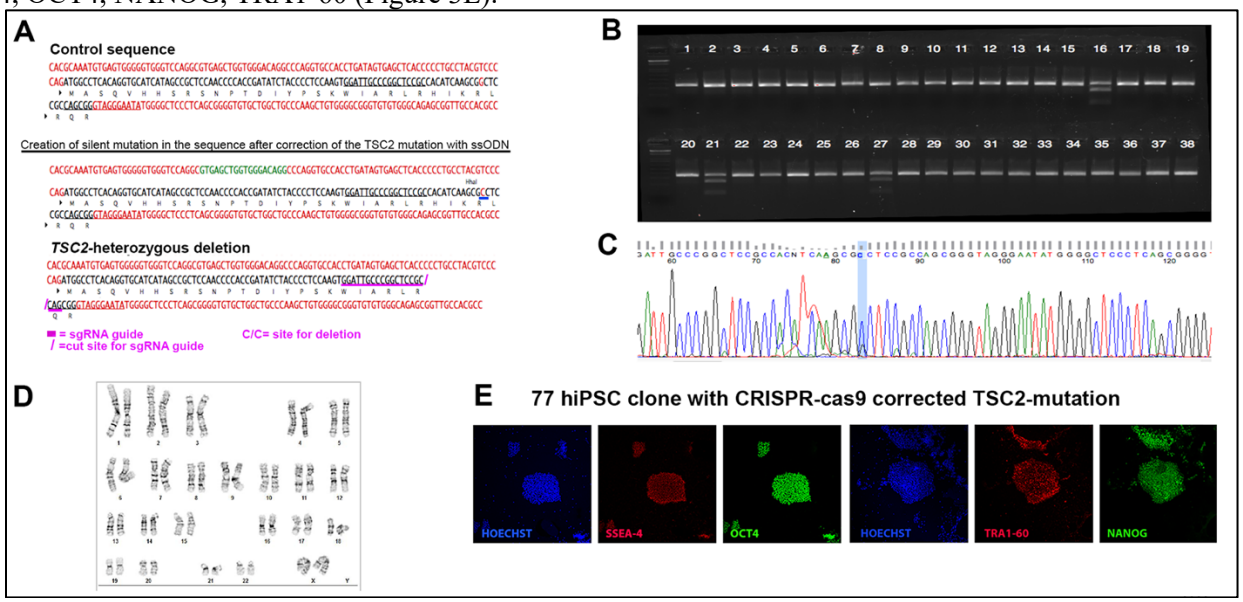


Figure 3. A. Sequence of the TSC2-gene and CRISPR-cas9-sgRNA design for correction of the TSC2-mutation. ssODN was used for correction of the microdeletion and a silent mutation was created into ssODN sequence to include restriction site for the DNA-restriction enzyme Hha1. B. DNA restriction enzyme Hha1 was used to screen the CRISPR-cas9-sgRNA and ssODN transfected and puromycin selected hiPSC-clones. Clones 16, 21, and 27 were cut with Hha1, which indicated successful incorporation of the ssODN. C. Sanger-sequencing was used to confirm corrected sequence. D. Representative data of normal karyotype of CRISPR-cas9 corrected *TSC2*+/+ hiPSC-clone. E. CRISPR-cas9 corrected *TSC2*+/+ hiPSCs were positive for pluripotency markers; SSEA-4, OCT4, TRA1-60, NANOG (Sundberg et al 2018).

We have also characterized the cerebellar cell differentiation and PC differentiation capacity of the CRISPR-corrected (*TSC2*$^{+/+}$) hiPSCs in comparison to isogenic TSC2-mutant cell lines with heterozygous and homozygous microdeletion of the TSC2 (*TSC2*$^{+/-}$, *TSC2*$^{-/-}$, Figure 4). We evaluated the gene-dosage effect of the TSC2-loss on the cerebellar precursor cell development and PC differentiation capacity, proliferation, and synaptic development *in vitro*. We detected decreased proliferation of the CRISPR-corrected cerebellar precursor cells (*TSC2*$^{+/+}$) compared to precursor cells with TSC2-mutations (Figure 4A-B). We also detected higher expression of SKOR2 in the CRISPR-corrected cerebellar precursors (*TSC2*$^{+/+}$) compared to precursor cells with TSC2-mutations (Figure 4C-D). Correction of the TSC2 mutation also decreased the mTORC1-pathway overactivation in the isogenic *TSC2*$^{+/+}$ cells, by decreasing the pS6 expression levels significantly compared to cells with heterozygous or homozygous mutation of the TSC2 (Figure 4E-F). We also detected decreased expression of FMRP and decreased synaptic marker expression of PSD95, SYP1, GRID2 in TSC2 mutant cerebellar cells compared to CRISPR-corrected isogenic cells (*TSC2*$^{+/+}$), which implicated that correction of the TSC2-mutation rescued the discovered disease phenotypes *in vitro* (Figure 4) (Sundberg et al 2018).

For the functional characterization of the hiPSC derived PCs *in vitro,* we used whole cell patch clamping method. We discovered that in the co-cultures with mouse granule neurons the hiPSC-derived PCs with TSC2-mutations (*TSC2*$^{+/-}$, *TSC2*$^{-/-}$) were hypoexcitable and had decreased synaptic activity (decreased frequency of mEPSCs) compared to control hiPSC-derived PCs (*TSC2*$^{+/+}$, non-isogenic familial control or gender matched control) (*Sundberg et al 2018*). We also detected that these functional deficits were partly rescued with rapamycin treatment of the TSC2-mutant PCs (Sundberg et al 2018). In the future studies, we are going to characterize the functional properties of the CRISPR-corrected isogenic hiPSC-clones in parallel with isogenic patient hiPSC-lines with heterozygous TSC2-mutations or with bi-allelic TSC2-deletions. For these studies we will optimize multi-electrode-array methods for characterization of the network activity of the hiPSC-derived PCs *in vitro*.

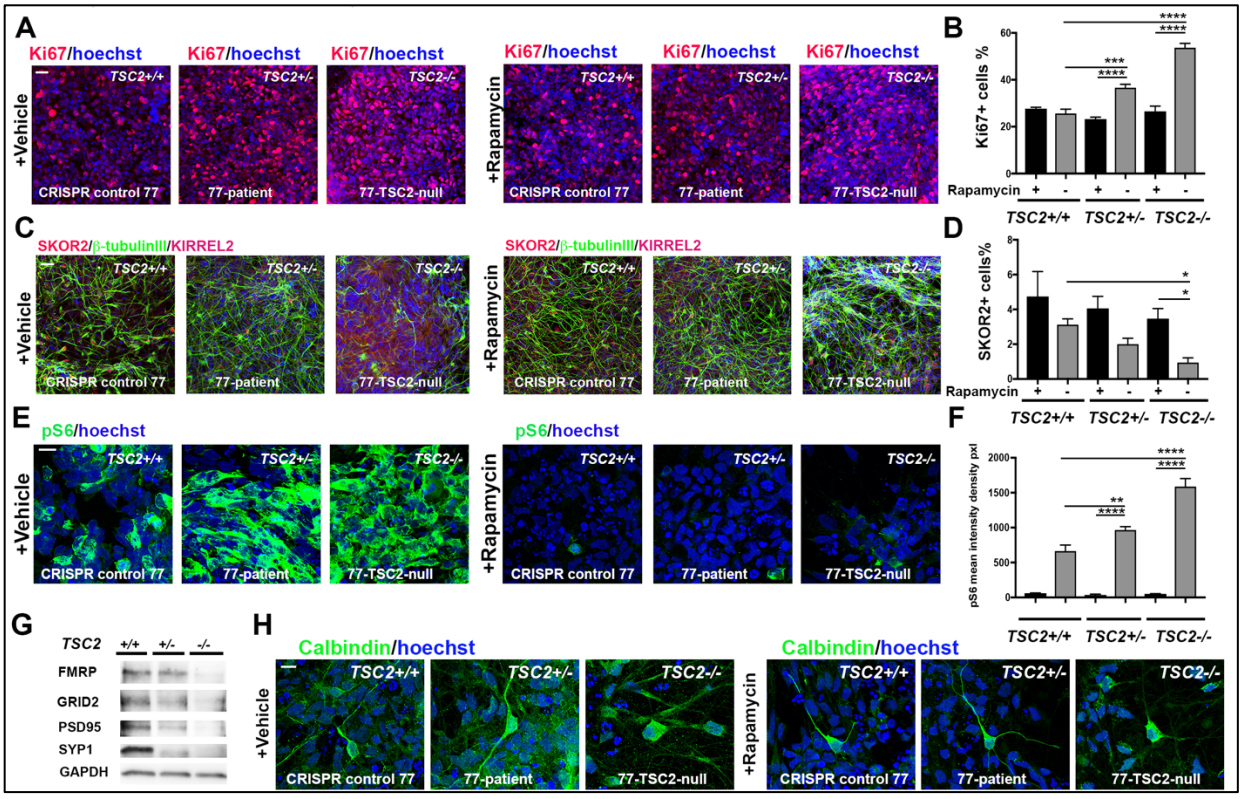
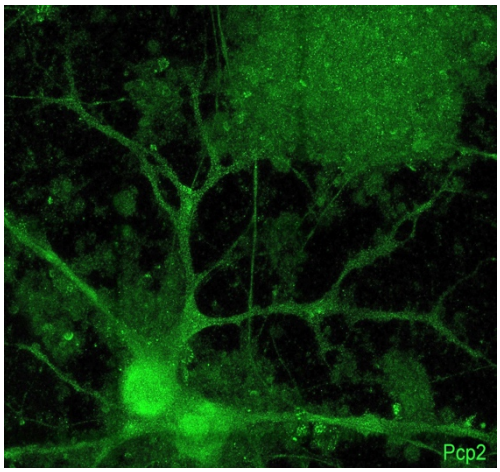


Figure 4. TSC2-gene-dosage effect on the cerebellar precursor cell proliferation and differentiation in isogenic hiPSCs in vitro. A. Ki67-positive cells were calculated at day 24 of cerebellar precursor differentiation, scale bar 25 µm. B. Quantification of Ki67+ cells from total cell population showed increased Ki67+ cell number in TSC2-deficient cell population (TSC2-/-) compared to isogenic-control cells (TSC2+/+). Rapamycin treatment (20nM, for 2 weeks) rescued the hyperproliferation of TSC2-deficient cells. C. SKOR2/b-tubulinIII and KIRREL2 expression at day 24 of cerebellar differentiation showed increased SKOR2 expression in isogenic-control cell population compared to TSC2-null cell population. TSC2- null cell population had increased expression of KIRREL2-positive cells compared to isogenic control cells, scale bar 25 µm. D. Quantification of SKOR2+ cells from total cell population showed decreased SKOR2+ cell number in TSC2-deficient cell populations (TSC2+/−, TSC2−/−) compared to isogenic- control cells (TSC2+/+). E. Immunocytochemical staining of pS6 expression in isogenic control (TSC2+/+) and TSC2-mutant THY1+ hiPSC-derived PC precursor cells (TSC2+/−, and TSC2−/−), scale bar 15 µm. F. Quantification of the pS6 mean intensity density revealed significantly increased pS6-expression in TSC2-deficient THY1+ hiPSC-PC precursors compared to isogenic control cells. Rapamycin treatment for two weeks during the differentiation process decreased pS6-expression in these cells. Statistical analyses were done using two-way ANOVA followed by post-hoc-test. Data are presented as mean ± SEM. G. THY1+ hiPSC-PCs at day 49 of differentiation were characterized for the synaptic marker expression with western blot method. Increased expression of FMRP, GRID2, PSD95, and SYP1 were detected in CRISPR-



cas9 corrected 77 isogenic control cells (TSC2+/+) compared to TSC2-deficient 77-patient (TSC2+/−) and 77-TSC2-null (TSC2−/−) hiPSC-PCs. H. Representative CALB1-positive hiPSC-derived PC-precursors at day 50 of differentiation, isogenic CRISPR-cas9 corrected cells (TSC2+/+), 77-patient hiPSC derived-cells (TSC2+/−), 77-TSC2-null hiPSC-derived cells (TSC2−/−), scale bar 8 µm. (Sundberg et al 2018).

Work at Rockefeller is continuing to explore ways to speed up maturation of hPSC-PCs, with an extended dendritic arbor seen 79 days after isolation and co-culture with mouse granule cells (Figure 5).

Figure 5. Confocal imaging of hPSC-PCs labeled with Pcp2 antibody following isolation of PCs on day 24 of differentiation followed by 79 days of co-culture with mouse granule cells shows an extended dendritic arbor typical of mature Purkinje cells.

For Aim 3. At Rockefeller University, we have begun developing methods for implanting hPSC-PCs into the neonatal mouse cerebellum. Preliminary results show good survival and proper targeting of hPSC-PCs into the cerebellum for up to 1 week post implantation however neurons failed to survive past this point. As implantation of human cells into a mouse may cause xenograft immune rejection, we are currently testing implantation into NOD/SCID immunodeficient mice.

Implantation into the neonatal mouse has proven unsuccessful. While hPSC-PCs could be implanted within the cerebellum of postnatal day 0-2 mice, they did not survive for more than one week. The use of NOD/SCID immunodeficient mice to block immune rejection did not promote survival of hPSC-PCs. Possible reasons for the lack of hPSC-PC survival and integration into the mouse could be differences in developmental age of hPSC-PCs compared to the mouse or differences between mouse and human neurons. Some studies suggest that implantation of PCs into mouse models deficient in host PCs aids graft incorporation and could be a route for further investigation.

What opportunities for training and professional development has the project provided?

During this period Dr. Sundberg has participated in several neuroscience conferences and presented the results of this research project in poster formats. These conferences have provided for Dr. Sundberg valuable opportunities to learn different aspects of stem cell research and neuroscience research from the leaders of this research field. In addition, this project has provided opportunities for the Sahin lab to establish new collaborations with several researchers in the neuroscience community of the Harvard Medical School, Boston.

How were the results disseminated to communities of interest?
We published a manuscript in 2018: Sundberg et al., 2018 Mol Psychiatry.

What do you plan to do during the next reporting period to accomplish the goals?
This is the final report of our study.

4. IMPACT:

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

- Changes in approach and reasons for change.
- Actual or anticipated problems or delays and actions or plans to resolve them.
- Changes that have a significant impact on expenditures.
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to Report.

Changes in approach and reasons for change
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

6. PRODUCTS:
• Publications, conference papers, and presentations

Poster presentations:
1. Event: The Neurodevelopmental Disorders Inaugural Symposium, October 22nd, 2018.
Poster Title: Stem cell derived Purkinje cells from TSC-patients have altered synaptic development and hypoexcitability associated with reduced FMRP levels and rescued by mTORC1-inhibition.
Authors: Maria Sundberg [1], Ivan Tochitsky [1], David Buchholz [2], Kellen Winden [1], Ville Kujala [3], Deniz Cataltepe [1], Daria Turner [1], Min-Joon Han [1], Clifford J. Woolf [1], Mary E. Hatten [2], and Mustafa Sahin[1]

2. Event: Folkman Research Day at Boston Children's Hospital, May 23rd 2018.
Poster Title: Stem cell derived Purkinje cells from TSC-patients have altered synaptic development and hypoexcitability associated with reduced FMRP levels and rescued by mTORC1-inhibition.
Authors: Maria Sundberg [1], Ivan Tochitsky [1], David Buchholz [2], Kellen Winden [1], Ville Kujala [3], Deniz Cataltepe [1], Daria Turner [1], Min-Joon Han [1], Clifford J. Woolf [1], Mary E. Hatten [2], and Mustafa Sahin[1]

3. Event: ISSCR-meeting June 14th 2017, Boston.
Poster Title: Characterization of TSC2 deficient human iPSC derived Purkinje cells in an in vitro model of autism.
Author list: Maria Sundberg [1], Ivan Tochitsky [1], David Buchholz [2], Kellen Winden [1], Ville Kujala [3], Deniz Cataltepe [1], Daria Turner [1], Min-Joon Han [1], Clifford J. Woolf [1], Mary E. Hatten [2], and Mustafa Sahin[1]

4. Event: HSCI as KickSTARTER | 12th Annual HSCI Malkin Retreat, May 16th 2017.
Poster Title: Characterization of TSC2 deficient human iPSC derived Purkinje cells in an *in vitro* model of TSC and autism. **Authors**: Maria Sundberg [1], Ivan Tochitsky [1], David Buchholz [2], Kellen Winden [1], Ville Kujala [3], Deniz Cataltepe [1], Daria Turner [1], Min-Joon Han [1], Clifford J. Woolf [1], Mary E. Hatten [2], and Mustafa Sahin[1]

5. Event: Translational Neuroscience Center Symposium, April 6, 2017.
Poster Title: Characterization of TSC2 deficient human iPSC derived Purkinje cells in an *in vitro* model of TSC and autism. **Authors:** Maria Sundberg [1], Ivan Tochitsky [1], David Buchholz [2], Kellen Winden [1], Ville Kujala [3], Deniz Cataltepe [1], Daria Turner [1], Min-Joon Han [1], Clifford J. Woolf [1, 2], Mary E. Hatten [2], and Mustafa Sahin[1]

Affiliations of authors in posters: 1. Department of Neurology, F.M. Kirby Center for Neurobiology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
2. Laboratory of Developmental Neurobiology, The Rockefeller University, New York, NY, USA
3. Harvard John A. Paulson School of Engineering and Applied Sciences, Boston, MA, USA.

Journal publications.

1. **Title:** Purkinje cells derived from TSC patients display hypoexcitability and synaptic deficits associated with reduced FMRP levels and reversed by rapamycin. **Journal:** *Mol.Psychiatry. 2018 Feb 15. doi: 10.1038/s41380-018-0018-4.* **Authors:** Maria Sundberg [1], Ivan Tochitsky [1], David Buchholz [2], Kellen Winden [1], Ville Kujala [3], Deniz Cataltepe [1], Daria Turner [1], Min-Joon Han [1], Clifford J. Woolf [1], Mary E. Hatten [2], and Mustafa Sahin[1]

Affiliations: 1. Department of Neurology, F.M. Kirby Center for Neurobiology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
2. Laboratory of Developmental Neurobiology, The Rockefeller University, New York, NY, USA
3. Harvard John A. Paulson School of Engineering and Applied Sciences, Boston, MA, USA.

Books or other non-periodical, one-time publications.
Nothing to report.

Other publications, conference papers, and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.).
Use an asterisk (*) if presentation produced a manuscript.

- **Website(s) or other Internet site(s)**
  Nothing to report.
- **Technologies or techniques**
  Nothing to report.
- **Inventions, patent applications, and/or licenses**
  Nothing to report.
- **Other Products**
- Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
Provide the following information on participants:

- what individuals have worked on the project?
- has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?
- what other organizations have been involved as partners?

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).
• Provide the name and identify the role the person played in the project. Indicate the nearest whole person month (Calendar, Academic, Summer) that the individual worked on the project. Show the most senior role in which the person worked on the project for any significant length of time. For example, if an undergraduate student graduated, entered graduate school, and continued to work on the project, show that
person as a graduate student, preferably explaining the change in involvement.
Describe how this person contributed to the project and with what funding support. If information is unchanged from a previous submission, provide the name only and indicate "no change".

-This is a collaborative project between Boston Children's Hospital and Rockefeller University.

Name: Mustafa Sahin
Project Role: PI
Nearest person month worked: 1.2 cal
Contribution: As PI, Dr. Sahin has supervised all aspects of the research plan and coordinated the communication with Dr. Hatten's lab at Rockefeller University.

Name: Maria Sundberg
Project Role: postdoctoral fellow (BCH)
Nearest person month worked: 6 cal
Contribution: Dr Sundberg carried out progress reported in the Aim 2 on design of the CRISPR-cas9-mediated gene correction to TSC2-mutant hiPSC-line and neuronal differentiation and characterization of the corrected hiPSC-clone. She has also optimized functional characterization of these lines with multi-electrode platform and collected samples for transcriptional profiling of the maturing hiPSC-PCs *in vitro*.

Name: Mary Elizabeth Hatten
Project Role: Co-PI
Nearest person month worked: 1.2
Contribution: Dr. Hatten supervised the work done on Aims 1,2 and 3 and coordinated the research plan with Dr. Sahin at Boston. She also traveled to Boston to meet with personnel there are review progress as well as to plan a publication that is currently in preparation.

Name: David Buchholz
Project Role: Subcontract Postdoctoral Fellow
Nearest person months worked: 6
Contribution: Dr. Buchholz carried out the progress reported in Aims 1, 2 and 3 on generating hES cell lines expressing a Pcp2 TRAP line, on further developing a protocol for differentiating Purkinje neurons and on developing a methodology to implant immature hES-derived PCs into mouse cerebellar cortex.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state "Nothing to Report."
If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission.
Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Name: Mustafa Sahin
Project Role: PI

New Active Support:

Developmental Synaptopathies Associated with TSC, PTEN, and SHANK3 Mutations
Supplement to 5U54NS092090
NIH/NINDS
Grants Management Specialist: Melissa R Copeland
08/01/2018 – 07/31/2019 – 0.12 calendar months
Annual Direct
This study is investigating how head circumference and organ system involvement in TSC, as proxy measures for brain overgrowth and extent of cellular involvement, correlate with epilepsy severity and neurodevelopmental measures in older children with TSC.

Translational Postdoctoral Training in Neurodevelopment (PIs Nelson and Sahin)
T32 MH112510-01
NIHM / National Institute of Health
6001 Executive Boulevard, Room 7115, MSC 9645
Contract/Grant Officer: Michele Pearson (Program Specialist)

07/01/17-06/30/22 0.48 calendar (no salary)
DC/year **Specific**
Aims:

Neurodevelopmental disorders such as Autism Spectrum Disorders, Attention Deficit Hyperactivity Disorder, and Intellectual Disability are estimated to impact ~ 1 in 6 children in the United States. Unfortunately, currently available treatments have only limited impact on short and long-term outcomes for most NDDs - highlighting the immense unmet medical need in this area. The Translational Postdoctoral Training in Neurodevelopment (TPND) Program at Boston Children's Hospital will train post-doctoral neuroscience investigators (MD, PhD or MD/PhD) to develop and implement new medical and behavioral treatments for a range of neurodevelopmental disorders impacting children and their families.

Mapping the phenotype in adults with PMS (PI: Sahin)
Phelan-McDermid Syndrome Foundation
200 Capri Isles Blvd, Suite 7F
Venice, FL 34292
Contract/Grant Officer: Priscilla Hackstadt
03/01/17-02/28/19 (NCE) 0.12 cal
DC/year
Specific Aims:
Goal: Enrolling site and regulatory site for study on PMS on adult patients.
Specific Aims:
Aim 1 To comprehensively characterize adults with PMS using standardized medical, cognitive, and behavioral measures. Aim 2 To identify genetic factors which contribute to diverse phenotypes in patients with PMS.

Characterization of the disease phenotypes of Ataxia Telangiectasia patient Purkinje cells in vitro (PI: Sahin and Lerou)
The National Ataxia foundation
Address: 600 Hwy 169 South, Suite 1725, Minneapolis, MN 55426
Contract/Grant Officer: Sue Hagen; Patient and Research Services Director
01/01/18 – 12/31/18 0.12 Calendar
DC/year
Specific Aims:
The goal of this collaborative project between the Lerou- and Sahin- labs is to characterize the synapse development and functionality of human Purkinje cells derived from A-T-patient hiPSC and characterize NRXN1 splicing variations in A-T patient neural cells and tissues.

Completed Support:

Salivary miRNA Biomarkers in Neurodevelopmental Disorders
(PI: Sahin)
Motion Intelligence, Inc.
505 Irving Ave, Suite 2102
Syracuse, NY 13210
Contracting/Grant Officer: Richard Uhlig
11/11/2016 – 7/1/2018 – 0.12 calendar months
Annual Direct
This study is aimed at identifying a diagnostic salivary miRNA profile for four single gene neurodevelopmental disorders (MECP2, FMRP, TSC, and CDKL5).
No overlap.

RAD001 and mTOR Novartis Tool Compound 1 (NTC1) testing in Tsc2KC+ mice, (PI: Sahin)
Novartis Institutes of Biomedical Research
250 Massachusetts Avenue
Cambridge, MA 02139
Contracting/Grant Officer: Marjorie Eiref
Email: Marjorie.eiref@novartis.com
11/02/2016 – 09/30/2018 – 0.12 calendar months

This project will pilot neuroPk study to select appropriate dose of compounds in Tsc2KC+animals, evaluate effect of RAD001 and WB-26-VB80 on epileptiform EEG activity in male Tsc2KC+ hypomorphic mice, and harvest tissues for bio chemistry and final PK analysis.

Neuronal excitability in TSC
(PI: Sahin)
Lam Therapeutics Inc.
530 Old Whitfield Street, Guilford, CT 06437
Contracting/Grant Officer: Henri Lichenstein (President & CSO)
05/19/2016 – 12/19/2017 – 0.01 calendar months
Annual Direct
The goal is to test the efficacy of genetic anti-seizures medications on human TSC neurons.

Molecular Profiling of the Tuberous Sclerosis Brain and Patient Blood Cells (Sahin, M. PI/Mentor)
F. Hoffmann-LaRoche, LTD
Grenzacherstrasse 124
4070 Basel, Switzerland
Contract/Grant Officer: Jason Hannon
03/01/16 - 10/31/18 0.012 calendar
DC/year
Specific Aims:
The overall goal of this study is to generate a comprehensive molecular profile of the cortical-inhibitory and excitatory neurons and cerebellar Purkinje cells from rodent wild type and TSC modulated brain and to perform molecular profiling of blood cells from TSC patients.

Name: Mary E. Hatten
Project Role: Co-PI
New Active Support:

Starr Tri-Institutional Stem Cell Initiative Hatten (PI) 07/01/2016- 6/30/2019
Funding Agency: Starr Foundation
Title: Role of Tet and Chromatin Remodeling Genes in Human Cerebellar Neuron Synapse Formation and Function.
The major goal of this project is to study the effects of genetic perturbation of chromatin-modifying factors on the differentiation and synaptic physiology of hESC-derived cerebellar neurons. We will use CRISPR/Cas9 constructs to remove the Tet and other chromatin remodeling genes in hESC-derived GCs and PCs. Subsequently we will express these constructs in hESC-GCs or PCs and assay whether targeting Tet or chromatin remodeling genes affects cerebellar synapse formation when the human cells are co-cultured with mouse target. For targeted genes that decrease synapse formation by 50%, Hatten will use TRAP methodology to assay changes in gene expression, focusing on changes in axon guidance (dendrite formation) and ion channel genes, genes that we previously showed are altered by activation of Tet1/3 (Xhu et al, 2016). We will also transplant relevant differentiated hESC clones into mouse cerebellum to test their ability to integrate and form dendrites. Finally, Ryan will use a suite of biophysical approaches to examine the functional properties of synapses formed between hESC-derived cerebellar neurons and defined postsynaptic targets in vitro.
Role: PI (Co-PIs, Joseph G. Gleeson, M.D.; Timothy A. Ryan, Ph.D.)

Completed Support:
5R01NS051778-09 Hatten (PI) 05/01/2011-02/29/2016
Funding Agency: NIH/NINDS

Title: Role of Cdc42 and Par6 Polarity Complex in CNS Neuronal Migration
The goal of this research is to examine the role of the mPar6α polarity complex in the migration of CNS neurons along glial fibers during cerebellar development. The specific aims of this research are to investigate the other components of the mPar6α complex, the atypical PKCζ and Par3, to use genetic methods for chromophore-assisted inactivation of mPar6α in granule neurons and to examine upstream and downstream signaling pathways for the mPar6α complex in CNS glial-guided migration.

Role: PI

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Rockefeller University (as above, no change).

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each
- other's site); and
- Other.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

1. **Original copy of the article**: Purkinje cells derived from TSC patients display hypoexcitability and synaptic deficits associated with reduced FMRP levels and reversed by rapamycin. **Journal:** *Mol.Psychiatry. 2018 Feb 15. doi: 10.1038/s41380-018-0018-4.* **Authors:** Maria Sundberg [1], Ivan Tochitsky [1], David Buchholz [2], Kellen Winden [1], Ville Kujala [3], Deniz Cataltepe [1], Daria Turner [1], Min-Joon Han [1], Clifford J. Woolf [1], Mary E. Hatten [2], and Mustafa Sahin[1]. **Affiliations:** 1. Department of Neurology, F.M. Kirby Center for Neurobiology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA. 2. Laboratory of Developmental Neurobiology, The Rockefeller University, New York, NY, USA. 3. Harvard John A. Paulson School of Engineering and Applied Sciences, Boston, MA, USA.

2. **Abstract of the poster presentation in the ISSCR-conference, 06/14/17, Boston:**

Title: Characterization of TSC2 deficient human iPSC derived Purkinje cells in an in vitro model of autism.
Author list: Maria Sundberg, Ivan Tochitsky, David Buchholz, Kellen Winden, Deniz Cataltepe, Min-Joon Han, Clifford Woolf, Mary E. Hatten, Mustafa Sahin.

Abstract:
Tuberous sclerosis complex (TSC) is a neurodevelopmental disorder causing cortical tuber formation, epilepsy and autism. TSC1/2 mutations dysregulate the mechanistic target of rapamycin (mTOR) pathway and disturb neuronal protein synthesis, which leads to the development of ASD approximately in 50 % of all TSC-patients. Previous PET imaging studies have indicated that cerebellar deficits, such as cerebellar lesions and glucose hypermetabolism in deep cerebellar nuclei, are associated with a higher prevalence of autism in TSC-patients. However, the exact cellular deficits that occur during development of autism hasn't been investigated in detail with patient derived neural cells. We have previously created a mouse model lacking the Tsc1 gene specifically in cerebellar Purkinje cells (L7-Cre+;Tsc1f/f). This mouse model displayed autistic-like behavioral deficits including increased repetitive behaviors, decreased social interaction and cellular abnormalities (Tsai et al, 2012, Nature). Together, the clinical findings from TSC-

patients and the phenotype of the Tsc1 conditional knock-out mice indicate that cerebellar Purkinje cells have a crucial role in the development of ASD. To characterize the disease phenotypes of TSC at the cellular and molecular level we have developed a novel differentiation protocol for generation of Purkinje cells from human iPSCs with patient specific TSC2-mutations. In this study, we derived pluripotent stem cell lines from three TSC-patients and four unaffected familial controls or age-matched controls for neuronal differentiation in vitro. We find that TSC2-deficient patient iPSC-derived PCs have mTOR-pathway hyperactivation that was detected with increased levels of phospho-S6 and phospho-S6 kinase. Our comprehensive RNA sequencing analyses revealed several interesting genes related to neuronal differentiation deficits of TSC2-mutant iPSC-derived Purkinje cells. We also detected deficits in the electrophysiological properties of hiPSC-derived PCs. According to our data, inhibition of mTOR-pathway may improve the electrophysiological properties of TSC2-deficient human Purkinje cells, and could be a target for pharmacotherapy for treatment of ASD in TSC.