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TITLE: A Subependymal Giant Cell Astrocytoma (SEGA) Mouse Model

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14. ABSTRACT
The overall goal of the research proposed is to test the hypothesis that Tsc2 deletion in SVZ NSCs hyperactivates mTOR and elevates translation of mRNAs that generates a stochastic unstable stem-like state which can be stabilized with mTOR inhibitors or retinoic acid. This grant seeks to generate, characterize, and test therapeutic compounds a novel mouse model of Tuberous Sclerosis Complex (TSC). The TSC model produces mice having abnormal neuron migration, neuron morphology, hyperactivation of the mTORC1 pathway and most notably, the previously elusive subependymal giant cell astrocytoma (SEGA). The grant will help generate a better understanding of how SEGAs form and when and to test the extent that SEGAs can be permanently pharmacologically ablated. The grant will help address two TSCRP focus areas. First, this grant will help us to gain a deeper knowledge of TSC signaling pathways and the cellular consequences of TSC deficiency by examining molecular pathways and translational abnormalities in TSC SEGA cells. Second, this grant will test and facilitate therapeutics, biomarker, and clinical trials research. We will study a new class of third generation mTORC1 inhibitors tethered to rapamycin which are exemplified by the compound RapaLink1. We will determine the extent that RapaLink1 permanently reduces SEGA size and frequency. We will simultaneously study an FDA approved molecule called all-trans retinoic acid (ATRA). This annual report highlights research progress over the first period.

15. SUBJECT TERMS
Tuberous Sclerosis Complex (TSC), TSC2, Tuberin, Subependymal giant cell astrocytoma, SEGA, RapaLink1, all-trans retinoic acid, neural stem cell, mTORC1

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1. Introduction

Tuberous Sclerosis Complex (TSC) is a multi-system neurodevelopmental disorder[1–3]. Patients with TSC are born with brain malformations[1,4]. TSC is not common but serves as a model for understanding brain disorders because the genes that cause TSC are known, key altered molecular pathways have been identified, and a rational therapeutic approach has been developed[3]. Thus, lessons learned in TSC may be applied to similar diseases such as focal cortical dysplasia[5,6]. Mutations in *TSC1* or *TSC2* cause TSC[7,8]. Lesions called subependymal nodules (SENs) are found in the majority (~85%) of patients[1,9]. SENs are slow growing lesions found within the subependymal zone (subventricular zone, SVZ) that protrude into the ventricles and arise in a region where neural stem cells (NSCs) are found. SENs may grow during the neonatal period[4,7]. 20% of SENs transform into subependymal giant cell astrocytomas (SEGAs)[4,9,10]. SENs greater than 10 mm are considered SEGAs. SEGAs lead to hydrocephalus, increased intracranial pressure, can cause seizures, and can lead to death[1,9,10]. TSC1/2 encode for hamartin and tuberin which form a GTPase activating protein kinase that when inactivated leads to activation of the protein kinase mTOR[11]. mTOR inhibitors are a frontline treatment for TSC and reduce SEGA size[12]. Major limitations to mTOR inhibitors (rapamycin, rapalogs including sirolimous, and everolimous) are that their effects are reversible, may not effectively eliminate neurological manifestations, and have side effects including immunosuppression[12]. Most importantly, treatment with rapalogs is a life-long sentence because cessation of therapy is associated with SEGA regrowth[12].

Tsc1/2 gene mutations cause SENs and SEGAs. Histological profiles of TSC SEGAs and SENs overlap and there is currently no molecular marker that distinguishes between the two, although generally speaking, size (greater than 10 mm) and serial growth distinguishes between the two[3]. SVZ NSC *Tsc1* deletion in mice caused mTOR hyper-activation, neuron heterotopias, and small ventricular growths representing SENs[13,14]. Well-defined SEGAs were not generated following *Tsc1* deletion from SVZ NSCs. One possible reason that SEGAs are not generated following *Tsc1* deletion is that loss of *Tsc1* is insufficient for SEGA generation in mice. In contrast, dominant mutations in *Tsc1* (hamartin) that occur in patients might cause SEGAs by inhibiting *Tsc2* (tuberin). However, loss of *Tsc2* (tuberin) function may cause SEGAs in mice (and patients).

Indeed, TSC2 mutations are associated with severe brain lesions and a poor prognosis[1,9]. *TSC2* mutations are overwhelmingly more frequently associated with SEGA formation. For example, a study demonstrated out of 207 patients with SEGAs, only 22 patients had *TSC1* mutations whereas 185 had *TSC2* mutations[23]. It is important to remember that tuberin is the catalytic part of the GAP complex and tuberin and hamartin have biochemical, physiological, and clinically relevant differences. *Tsc2* deletion causes more severe phenotypes than *Tsc1* in mice, but *Tsc2* deletions have only recently been performed in embryonic NSCs and GFAP positive postnatal astrocytes[24–27]. A recent report suggested that SVZ NSC *Tsc2* deletion generates lesions resembling TSC SEGAs[28].

The overall goal of this project is to generate, characterize, and test therapeutic compounds in a novel mouse model of Tuberous Sclerosis Complex (TSC). The TSC model removes *Tsc2* from NSCs and will recapitulate key features of TSC including abnormal neuron migration, neuron morphology, hyperactivation of the mTORC1 pathway and most notably, subependymal giant cell astrocytomas (SEGAs). The goal of this funding cycle is to generate a better understanding of how and when SEGAs form. Two major tasks are being executed and are scheduled to be completed by month 24 as outlined in the SOW (listed under item #3 Accomplishments). Major task 1 is **Characterization of SEGAs** to determine the cellular composition and changes that occur in this TSC model along the subventricular zone-olfactory bulb neurogenic axis and Major task 2 is to **Determine the Molecular Effects of *Tsc2* Deletion on Neural Stem Cells and SEGAs.**

2. Keywords

Tuberous Sclerosis Complex (TSC), TSC2, Tuberin, Subependymal giant cell astrocytoma, SEGA, RapaLink1, all-trans retinoic acid, neural stem cell, mTORC1

3. Accomplishments

Major Tasks and % Completion

Major Task 1: Characterization of SEGAs	% Complete
Subtask 1: Cellular Composition Time Course of Focal SEGA Model- created via electroporation (genotypes: $Tsc2^{wt/wt}$, $Tsc2^{fl/wt}$, and $Tsc2^{f/f}$) • Mice are sacrificed at P7, P30, and P60 • Quantify changes in the number of NSCs, neuroblasts, and astrocytes between P7 to P30 • Determine whether SEGAs are dynamic and whether the cellular composition is changing using P60 time point. • Perform IHC of brains to determine whether there are defects in differentiation and migration, stain for NSCs and astrocytes • Measure the size, frequency, and location of SEGAs via confocal and IHC imaging. • The number of GFP and RFP double positive neurons or GFP negative/RFP positive neurons (Neu-N) will be determined for control and Tsc2 null cells.	40%
Subtask 2: Cellular Composition Time Course of Global SEGA Model- created by treating N-$Tsc2^{fl/wt}$ mice with tamoxifen (genotypes: $Tsc2^{wt/wt}$, $Tsc2^{fl/wt}$, and $Tsc2^{f/f}$). • Mice will be sacrificed at P10, P30, and P60 • Confocal images of SEGAs will be taken to identify changes in SVZ cell composition. Evaluate neurons (Neu-N) and neuroblasts (dcx) to determine whether there are defects in differentiation and migration as in Subtask 1. • Perform co-staining for GFAP/GS and Nestin and counter-stained for TO-PRO-3. • Perform IHC of brains. • Measure the size, frequency, and location of SEGAs via confocal and IHC imaging. • The number of Neu-N/RFP and EdU/RFP positive neurons will be quantified for control and Tsc2 null cells at P10, P30, and P60	40%
Milestone(s) Achieved: Quantification of SEGA number/size/and cellular composition (% cell type) at three ages.	
Local IACUC Approval	Complete
ACURO Approval	Complete
Mouse Breeding	Complete
Validation of Tools/Supplies	Complete
Specific Aim 2: Determine the Molecular Effects of *Tsc2* Deletion on Neural Stem Cells and SEGAs	
Major Task 2: Molecular pathways characterized by IHC	

Subtask 1: Three litters of newborn P0 N-Tsc2$^{f/f}$, N-Tsc2$^{f/wt}$, or N-Tsc2$^{wt/wt}$ mice will be injected with tamoxifen. Mice are sacrificed at P7, P30, and P60.	33%
Subtask 2: IHC- Sections will be stained for pS6, pUlk1, or p4EBP. These same sections will be stained for Nestin and GFAP.	33%
Major Task 3: Translational Profiling in TSC SEGAs	0%
Subtask 1: Prepare mice - P0 N-Tsc2$^{f/f}$, N-Tsc2$^{f/wt}$, or N-Tsc2$^{wt/wt}$ mice will be injected with tamoxifen. Mice will be sacrificed at P7.	
Subtask 2: Generate and sequence RNA libraries.	
Subtask 3: After mapping and unique exon hit count calculations, downstream differential expression analysis will be performed using DESeq2 to confirm differential mRNA expression.	
Milestone(s) Achieved: Quantification of mTOR activity in cell types at three ages.	
Specific Aim 3: To Determine efficacy of Rapalink1 and ATRA (retinoic acid) on SEGAs	0%
Major Task 3: Treatment and assessment of response of SEGAs to treatment.	0%
Subtask 1: Prepare animal model - use global N-Tsc2$^{f/f}$ mice. Mice will be injected at P0 with 20 µg/g Tamoxifen twice per day.	
Subtask 2: Treatment - at 60 days, animals will be randomized, assigned a unique identification number, and treated for 5 days a week with Rapalink-1 (6 mg/kg), retinoic acid (5 mg/kg), or vehicle (DMSO, 0.1% in sunflower seed oil) for 4 weeks or until death.	
Subtask 3: Survival curves will be established. Mice treated with either drug or vehicle will be assessed for signs of toxicity based on body weight, food/water intake, hair loss, and activity.	
Subtask 4: IHC for markers of mTOR activity and cell markers. Samples will be stained for GFAP and Nestin marker proteins to quantify NSCs. Nestin and GFAP staining will be analyzed as described in aim 1.	

1. Major activities. Major goals for Aim 1 span months 6-12. Completed goals are scheduled for month 24.

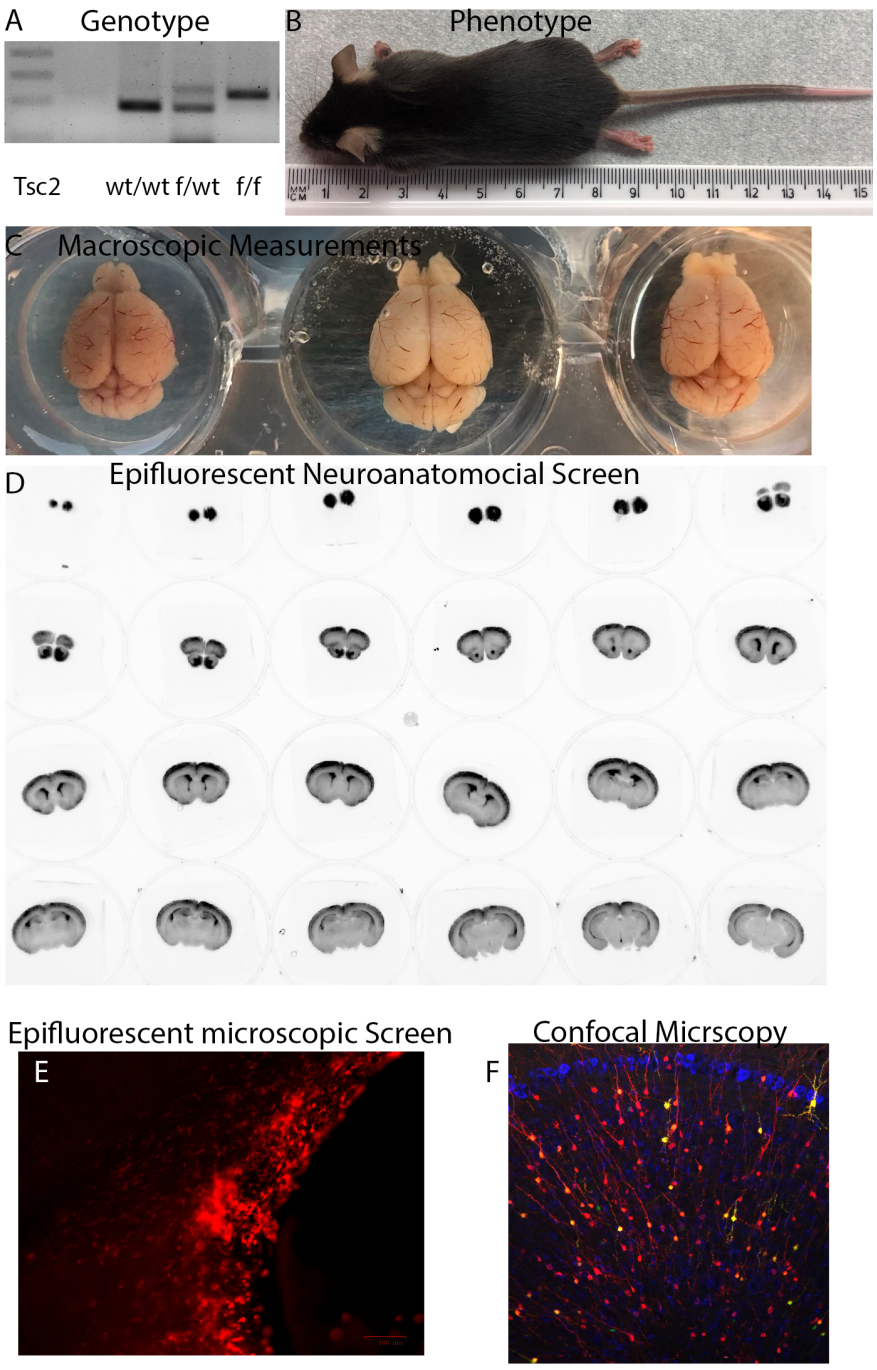

Figure 1. Data Acquisition Pipeline. A) Neonatal mice were electroporated or injected with tamoxifen. Mice were genotyped for Tsc2 wild-type (wt) or floxed (f) alleles, reporters, or nestin-CRE-ER^{T2}. Genotypes were roughly mendelian to date, see text. B) Mouse size, weight, and general health were documented, and photographs taken. C) Brains were removed and assessed for gross changes in anatomy including examination of brains and mice for hydrocephalus and ventriculomegaly. D) Chemidoc MP imaging of mouse coronal brain sections to localize potential growths was performed. Note the high intensity along the neurogenic axes including the SVZ and hippocampus as well as robust labeling in the cerebral cortex. E) Mouse sections were examined for changes to the SVZ, RMS, OB (and regions of novel defects including striatum). F) Immunohistochemical analysis and confocal microscopy was performed as indicated in SOW.

To date we have performed 40% of experiments for Major task 1 subtask 1 and subtask 2. Subtask 1 experiments are to electroporate mouse pups that are born to *Tsc2*$^{flox/wt}$ x tomato$^{+/+}$ x *Tsc2*$^{flox/wt}$ x tomato$^{+/+}$ crosses. Subtask 2 experiments are to inject tamoxifen into mouse pups from Nestin-CRE-ER^{T2} x *Tsc2*$^{flox/wt}$ x tomato$^{+/+}$ crosses. Mice are sacrificed at postnatal (P) day P7/10, P30, or P60. Major efforts are focused on P7 and P10 timepoints. The reason to focus on those time points is that Major task 2 seeks to determine the mechanism by which SEGAs form by performing translational profiling of cells at the time point of ~P10. Focused experiments at the earliest time points will guarantee that the *Tsc2* gene is removed and that the mTOR pathway is activated prior to translational profiling. 33% of experiments have been performed for Major task 2. We have performed screening and imaging of mice, brains, brain sections, and immunohistochemistry for major cellular markers and mTOR substrates. Mice at additional time points are scheduled for sacrifice.

2. Specific Objectives.
   a. To quantify SEGA number/size/and cellular composition (% cell type) at three ages.
   b. To quantify mTOR activity in cell types at three ages.

c. To assess the response of SEGAs to treatments.

3. Significant Results.

Triple and double transgenic mouse colonies were generated. Mouse identities were determined by genotyping PCR. Mice from $Tsc2^{f/wt}$ x $Tsc2^{f/wt}$ experimental crosses were born at approximately expected mendelian ratios (26.3% Tsc2 f/f, 52.6% Tsc2 f/wt, 21.1% wt/wt).

We developed a pipeline for which genotype, phenotype, neuroanatomical, and immunohistochemical analysis could be applied to CRE or tamoxifen injections. Recombination was confirmed by multiple modes of imaging including by Bio-Rad Chemidoc MP imaging, ZOE fluorescent section imaging, and immunohistochemistry followed by confocal microscopy (**Figure 1**).

Mice were electroporated at P0-1 with CRE recombinase and GFP encoding plasmids and sacrificed at P7 (**Figure 2**). Robust recombination was documented including the SVZ at P7 with virtually all electroporated GFP positive cells being RFP positive. Recombined cells along the SVZ included Sox2 positive neural stem cells (**Figure 2**). Staining for Nestin and glutamine synthetase was also performed. Electroporation of CRE was confirmed to selectively target cells in the SVZ as determined by confocal imaging and immunohistochemistry for Nestin and Sox2. The SVZ was expanded as indicated by an increase RFP and Sox2 positive cells in the SVZ. Changes in the structure/anatomy of the SVZ were documented. The prevalence and statistical significance of these findings is being quantified.

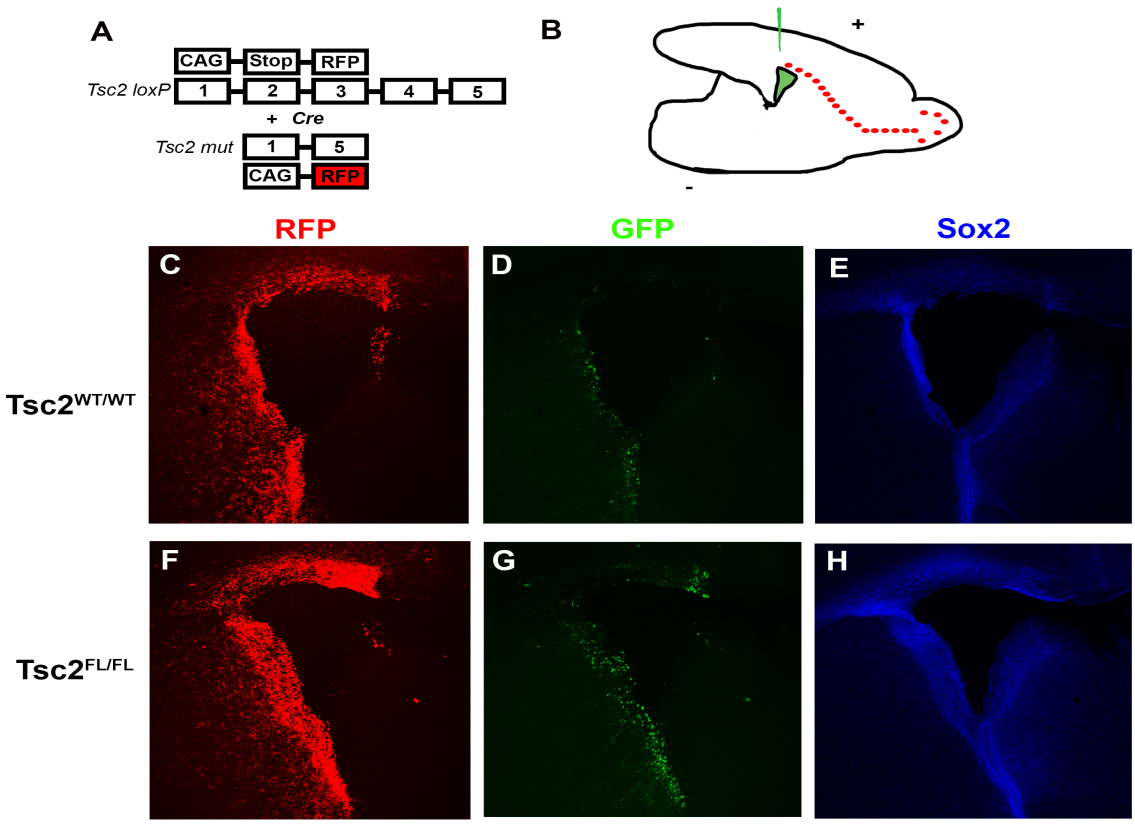

Figure 2. Electroporation and SVZ NSCs. (A) Schematic diagram of genotypes before and after recombination with *Cre*. Recombination resulted in the loss of exons 2-4 from the *Tsc2* gene and the activation of an RFP reporter gene. (B) Schematic diagram of neonatal electroporation (C-H) Neonatal electroporations of *Cre* and GFP (green) at P0 result in NSC labeling at postnatal day 7 in $Tsc2^{WT/WT}$ (C-E) and $Tsc2^{FL/FL}$ (F-H) mice. CRE resulted in recombination and RFP positive (red) NSCs that were also Sox2 positive (blue) c.

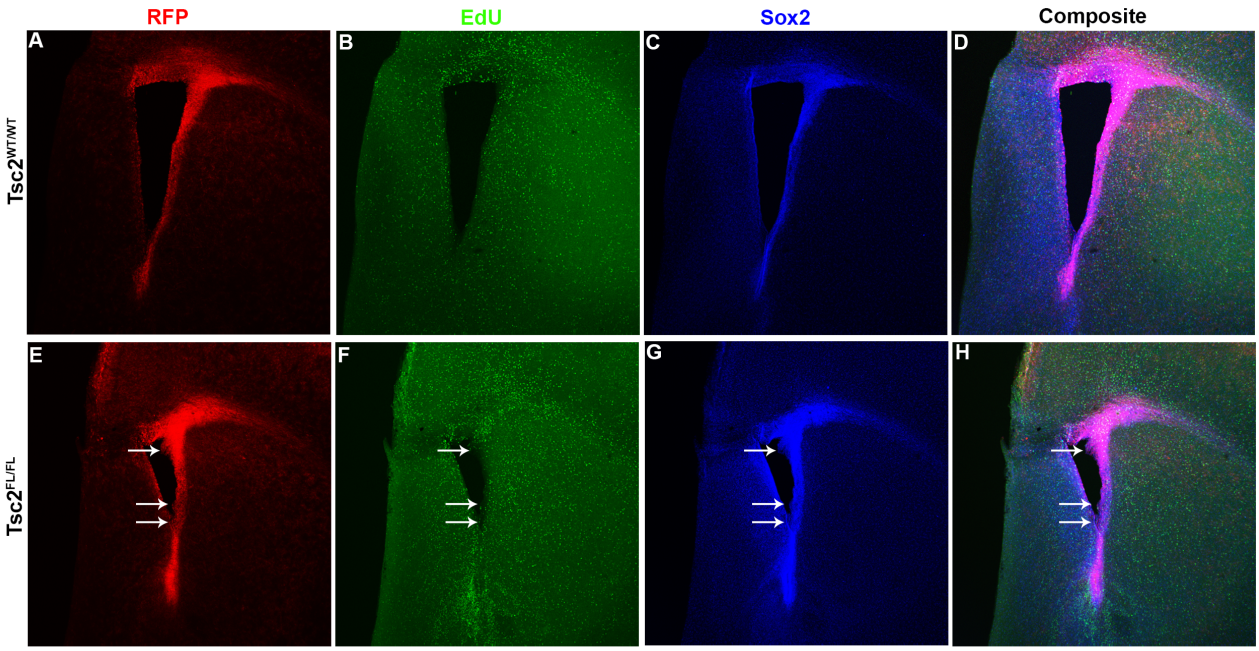

Figure 3. Tamoxifen injections and SVZ NSCs. Neonatal tamoxifen injection results in robust SVZ NSC labeling at postnatal day 10 in *Tsc2*$^{WT/WT}$ (A-D) and *Tsc2*$^{FL/FL}$ (E-H) mice. CRE resulted in recombination and RFP positive (red) NSCs that were also Sox2 positive (blue). Arrows point to anatomical anomalies in the SVZ.

Nestin-CRE-ER^{T2} was also confirmed to target neural stem cells including in the subventricular zone after tamoxifen injection (**Figure 3, 5**).

Neural stem cells (Sox2) were infrequently labeled by the thymidine marker EdU at P10 (**Figure 3**). However, by P10 doublecortin (DCX) positive neuroblasts had been generated and can be found in the rostral migratory stream and core of the olfactory bulb (**Figure 4**). Neurons (NeuN) in the OB were prevalent in Nestin-CRE-ER^{T2} mice but not as prevalent in electroporated mice. The most plausible explanation for these results is that dividing neural stem cells generated neuroblasts that matured into neurons and that the infrequent labeling is caused by the eventual dilution of EdU. We also found ectopic neurons in the RMS. The prevalence and statistical significance of these findings is being quantified.

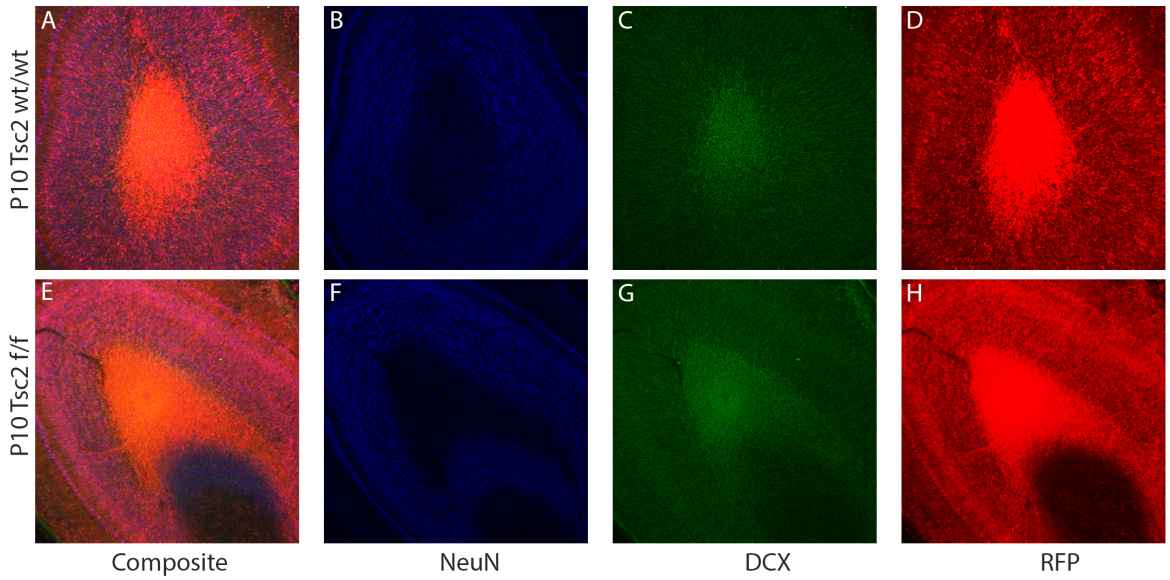

Figure 4. Tamoxifen injections and SVZ NSCs. Neonatal tamoxifen injection results in robust RFP labeling of neuroblasts and neurons at postnatal day 10 in *Tsc2*$^{WT/WT}$ (A-D) and *Tsc2*$^{FL/FL}$ (E-H) mice. CRE resulted in recombination and RFP positive (red) neuroblasts that were DCX positive (green) in the core of the olfactory bulb as well as granule cell neurons that were NeuN positive (blue).

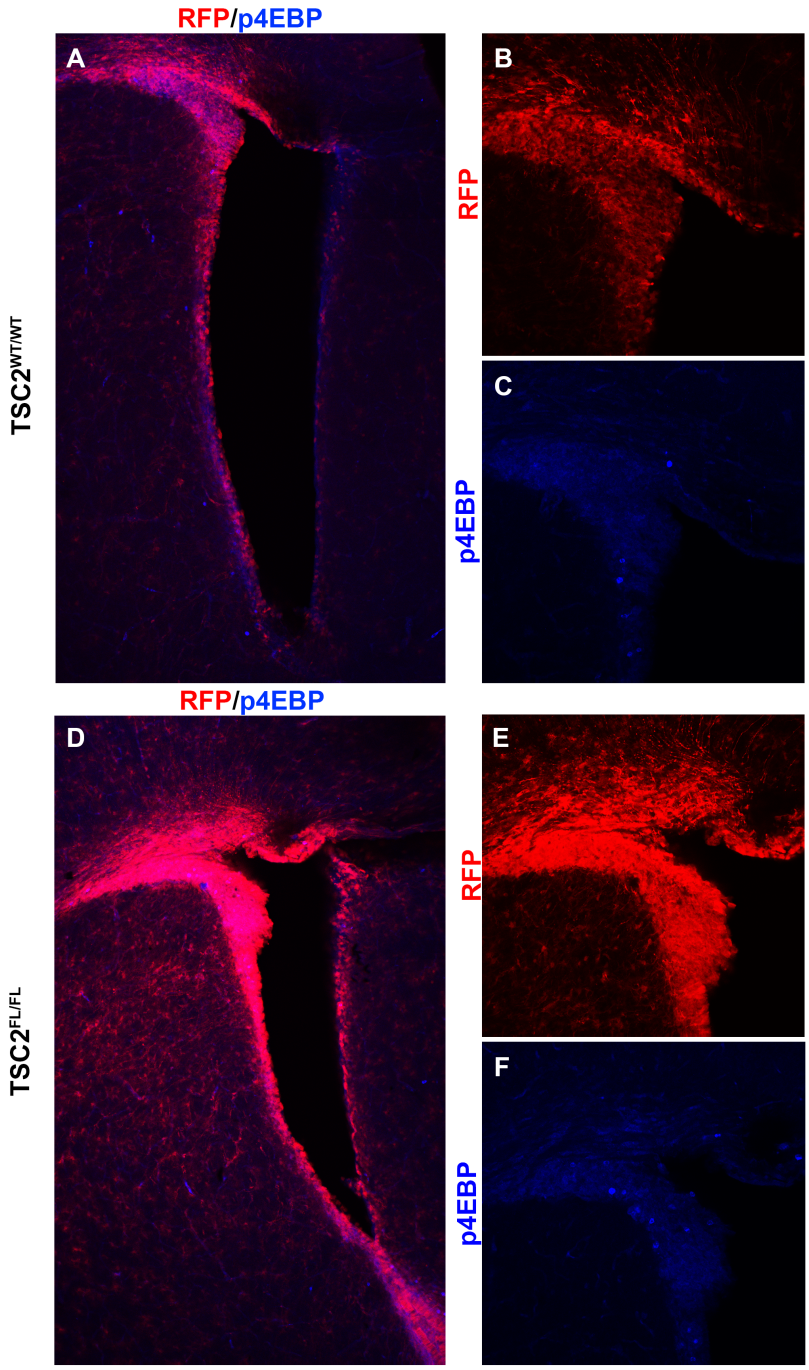

Figure 5. Subventricular zone mTORC1 activity. A. Composite image from 20x images of a coronal brain section of a nestin-CRE-ER^{T2} x Tsc2$^{wt/wt}$ x tomato mouse at P10 demonstrating phospho (p)4EBP (blue) and recombination and expression of tomato (RFP, red) **B.** 20x image of dorsolateral SVZ from A **C.** 20x image of dorsolateral SVZ from A depicting p4EBP (blue). Note diffuse and high round cell staining. **D.** Composite image from 20x images of a coronal brain section of a nestin-CRE-ER^{T2} x Tsc2$^{f/f}$ x tomato mouse at P10 demonstrating phospho (p)4EBP (blue) and recombination and expression of tomato (RFP, red) **E.** 20x image of dorsolateral SVZ from D. **F.** 20x image of dorsolateral SVZ from D depicting p4EBP (blue).

Immunohistochemistry for the phosphorylated forms of the mTORC1 substrates Ulk1, S6, and 4E-BP was performed (**Figure 5**). Apparent increases in mTORC1 activity were identified including along the SVZ. Cell type markers including Sox2 and Nestin or NeuN and DCX are also co-stained with mTORC1 substrates. Taken together, mTORC1 is likely active in these cells at this time point, although the extent that specific substrates are phosphorylated depends on the cell types. Quantification of the changes caused by loss of Tsc2 are ongoing.

4. Key outcomes.

A poster entitled, “A Mouse Model of Subependymal Giant Cell Astrocytomas” was presented at the International TSC Research Conference 2021. The conference includes families affected by TSC, medical professionals, industry and government stakeholders, students, and scientists. This meeting was a virtual meeting.

Over the next reporting period we have the following plans:

A. Animal colony and cage numbers (have been and) will continue to be increased to accommodate for the number of pups born in each litter.

B. Two additional doctoral students and the research assistant will assist in executing aims to increase productivity.

C. Major task 1. Over the next reporting period we will continue to determine how loss of *Tsc2* function affects development by electroporating CRE recombinase or using triple transgenic mice by examining P30/60 time points. We will analyze our data as indicated in the proposal.

D. Major task 2. Immunohistochemistry for cell type markers and phosphorylated forms of mTORC1 substrates will be completed. Polysome profiling will be performed followed by RNA sequencing to determine how mRNA translation is affected by mTORC1 hyperactivation.

4. Impact

Nothing to report.

5. Changes/Problems

Challenges predominantly relate to the COVID-19 pandemic and the effect on personnel, personnel expenditures, and animal facilities. Clemson University and animal facility operating procedure changes reduced the total number of animals in a cage. The projected numbers of mice/litter were lower than expected but by increasing cage numbers we can overcome this challenge. Mouse cages were also moved to a new room in the animal facility by facility personnel during a renovation which reduced mouse litters. Mouse cages have been moved back into place and productivity is increased. Additional mice were purchased to facilitate expansion of the colony. Two measures can be taken if needed to increase productivity. The first is to breed CD1 mice to increase the average amount of pups/litter. The second is to use conditional Tsc2 mice without other genetic backgrounds and to electroporate with transposon targeted fluorescent proteins. If these measures are needed, we will request approval. Currently there does not appear to be a need for these changes.

6. Products

Poster and Abstract Submitted and Presented: “A Mouse Model of Subependymal Giant Cell Astrocytomas” *International TSC Research Conference* 2021

7. Participants & Other Collaborating Organizations

Name: David M. Feliciano, Ph.D.
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0001-5985-9109
Nearest person month worked: 12
Contribution to Project: Dr. Feliciano has performed experiments for Aim 1, Major task 1, Subtask 1. Dr. Feliciano has directed all aspects of this project.

Name: Aidan M. Sokolov
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 9
Contribution to Project: Aidan Sokolov was assigned to perform experiments for electroporations for Aim 1, Major task 1, Subtask 1. This individual graduated with a Ph.D. in May.

Name: Victoria A Riley
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12
Contribution to Project: Ms. Riley has performed tamoxifen injections for Aim 1, Major task 1, Subtask 2. Ms. Riley has assisted with harvesting, sectioning, staining, and imaging tissue.

Name: Victoria N. Neckles
Project Role: Graduate Student
Researcher Identifier (e.g., ORCID ID):
Nearest person month worked: 3
Contribution to Project: Ms. Neckles has performed tamoxifen injections for Aim 1, Major task 1, Subtask 2 and assisted with harvesting, sectioning, staining, and imaging tissue.

Name: Jennie C. Holmberg
Project Role: Research Scientist
Researcher Identifier (e.g., ORCID ID):
Nearest person month worked: 1
Contribution to Project: Jennie Holmberg's roles include operations support, data acquisition, and analysis as well as cell isolations and culture.

Three doctoral students were supported during this period. Aidan M. Sokolov completed his doctoral degree during this period. Victoria (Tori) Riley performed experiments with Dr. Sokolov. Ms. Riley has assisted with breeding mice, genotyping, electroporation, tamoxifen injections, harvesting brains, slicing, and imaging. Tori presented work at the International TSC Research Conference in June 2021. A third graduate student, Victoria N. Neckles has taken over for Dr. Sokolov and is performing experiments with Tori. During this period, all students were able to receive additional one-on-one training with Dr. Feliciano. Tori Riley also received training in bioinformatics to assist in executing Aim 2 sequencing on polysomes. Tori has also been working on optimizing polysome fractionations during this reporting period.

8. Special Reporting Requirements

Award Chart: See Appendix.

9. Appendices

W81XWH-19 -TSCRP-IDA: A Subependymal Giant Cell Astrocytoma (SEGA) Mouse Model

PI: David M. Feliciano, Ph.D., Clemson University, South Carolina **Budget:** $667,968

Topic Area: TSCRP **Mechanism:** Idea Development Award for New Investigator

Research Area(s): SCS Coding **Award Status:** 06/01/202005/31/2021

Study Goals:

The overall goal of the research proposed here is to test the hypothesis that Tsc2 deletion in SVZ NSCs hyperactivates mTOR and elevates translation of mRNAs that generates a stochastic unstable stem-like state which can be stabilized with mTOR inhibitors or retinoic acid. The following aims will achieve this goal.

Specific Aims:

Aim 1. To characterize the cellular composition of a novel SEGA model.
Aim 2. To characterize molecular alterations in a SEGA mouse model.
Aim 3. To measure efficacy of two compounds in a SEGA mouse model.

Key Accomplishments and Outcomes:

Publications: none to date
Patents: none to date
Funding Obtained: none to date