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CONTRACTING ORGANIZATION: The Trustees of Columbia University in the City of New York

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<b>14. ABSTRACT</b> Given the multi-organ manifestation of TS lesions, and the mesenchymal lineage of tumors recovered from TS patients, it has been postulated that TS is a neurocristopathy in which tumorigenesis is initiated by neural crest cells, but direct supporting experimental evidence has not been produced. We hypothesize that <u>by tracking neurocristogenesis in a TSC2<sup>+/-</sup> mouse model, we will delineate the molecular mechanisms underlying temporal ontogenesis and progression of benign neoplasms characterizing TS and LAM. Furthermore, we hypothesize that HMGA2 misexpression defines tumorigenesis in TS and LAM caused by differentiating neural crest progenitor cells. This will subsequently lead to novel therapeutic approaches to the disease. <b>Aim 1: To delineate the biochemical signaling that determines the temporal sequence of neural crest cell-induced initiation and progression of tumors using a TSC2<sup>+/-</sup> mouse model. Aim 2: To determine the role of HMGA2 in tumor pathogenicity driven by TSC2-haploinsufficient iPSC-derived neural crest precursors.</b></u>					
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## INTRODUCTION:

Tuberous Sclerosis (TS) is a genetic disorder caused by inactivating mutations in either *Tsc1/2* tumor suppressor genes leading to dysregulation of cellular differentiation, migration, proliferation during tissue development causing hamartomatous lesions, cysts, and tubers in almost every organ in the body. The origin of tumorigenic cells in TS is not known, and the biochemical signals initiating onset and progression of tumors has not been delineated. Given the prevalence of TS at 1 in 6,000 births, and the potential that the disorder is under-diagnosed with significant phenotype under-recognition and no known cure, the need for comprehensive understanding of the pathophysiology of the disease cannot be overemphasized. The marked variability and multi-systemic occurrence of TS symptoms, and the heterogeneity in tissue composition of TS neoplasms biased towards a mesenchymal phenotype, informed our hypothesis that tuberous sclerosis is a neurocristopathy. Proposed studies test this hypothesis by tracking and analyzing tumor development in a *Tsc2*<sup>+/-</sup> reporter mouse model whose neural crest cells are genetically labeled with a fluorophore, and by recapitulating germline and somatic mutations during neurocristogenesis using induced pluripotent stem cells (iPSCs). This report summarizes our progress and findings thus far.

## KEYWORDS:

mTOR, Neurocristogenesis, High Mobility Group AT-Hook 2 (HMGA2), Induced Pluripotent Stem Cells (iPSCs), Tumorigenesis

## ACCOMPLISHMENTS:

### What were the major goals of the project?

There are two major goals for this project. The first goal was to determine the molecular signals governing the onset and progression of tumors whose development is initiated by neural crest cells using a *Tsc2*<sup>+/-</sup> reporter mouse model. To achieve this goal, the appropriate reporter mouse genotypes have to be bred, tumors allowed to develop, extracted at defined timepoints, and analyzed by single cell RNA sequencing. We have completed the crossing and generation of progeny, which was 30% of the aim and identified the onset of tumor progression in the *TSC2*<sup>+/-</sup>-*MPZ*-*Cre*<sup>26R</sup> mice to give us a 50% complete aim. We are on target for these studies to be completed in the final year of the proposal.

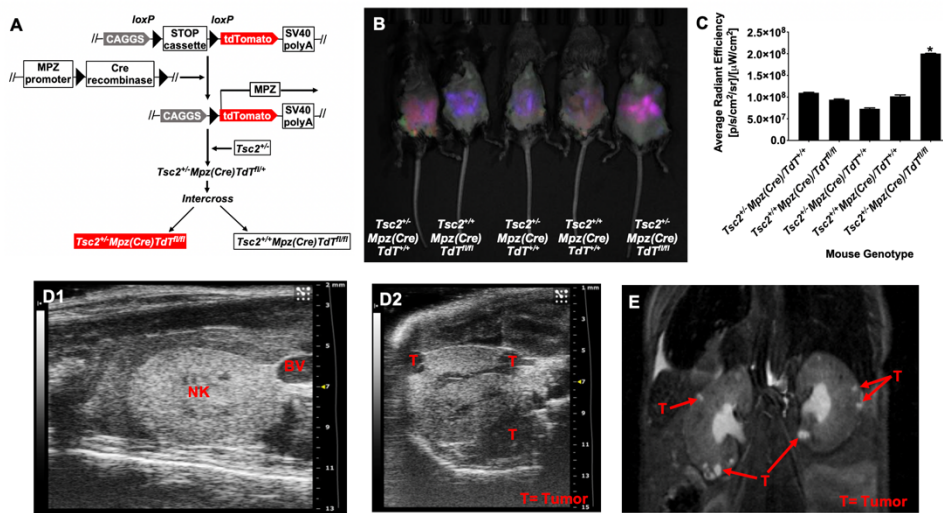
Our second goal was to recapitulate a tuberous sclerosis disease state by differentiating induced pluripotent stem cells (iPSCs) into neural crest cells and engineering inactivating and overexpressing genetic mutations in *Hmga2* and *Tsc2* to assess the role of the interaction between these two genotypes in tumorigenesis using a cell culture-based system. We are generated and characterized iPSC colonies from peripheral blood mononuclear cells (PBMCs) of TSC patients and assessed the pluripotency of the line (40% of the milestone has been completed). In the last year we will perform the final differentiation studies into neural crest progenitor cells and identify if the HMGA2 pathway alters growth of these cells. We will assess proliferation and cell invasion as described in the aims. We fully expect to complete the aims of the study.

### 1. What was accomplished under these goals?

In specific Aim 1, we intend to track temporal ontogenesis of tumors in *Tsc2*<sup>+/-</sup> reporter mice and perform single cell RNA sequencing of renal and hepatic tumor extracts at onset of tumorigenesis and at defined timepoints through the course of tumor growth. In doing so, we can illuminate genetic and biochemical changes that initiate tumor incidence and growth thereby recapitulating the pathophysiology of tumors in TS. To generate the *Mpz-Cre*<sup>R26R</sup>*Tsc2*<sup>+/-</sup> mouse reporter model required to commence investigations in this aim, we have successfully bred *Gt(ROSA)26Sor*<sup>tm9(CAG-tdTomato)Hze/J</sup> (JAX #007909) and *Tg(MPZ-Cre)26Mes/J* (JAX #017927) mice and facilitated the mating of these two mouse models. We have also

backcrossed progeny from this cross with ROSA mice to obtain progeny homozygous for this floxed mice and commenced mating with *Tsc2*<sup>+/-</sup> mice. We continued this mating regimen and genotyping mouse progeny into comparative genotypes used for tumor tracking and analyses - *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup>, *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>+/+</sup>, *Tsc2*<sup>+/+</sup>*Mpz(Cre)*<sup>fl/fl</sup>, and *Tsc2*<sup>+/+</sup>*Mpz(Cre)*<sup>+/+</sup>.

These genetic crossings enables an animal model that allows for systemic epifluorescent detection of neural crest lineage cells and their role in developing TS tumors by Cre-recombinase mediated insertion of the *Mpz* promoter to drive tdTomato expression. Tumor tracking in this *Tsc2*<sup>+/-</sup> reporter mice was performed beginning at 2 months of age at weekly intervals using sequential IVIS spectral imaging detecting tdTomato excitation/emission spectra at 570nm/620nm, and small animal ultrasound. Small animal MRI imaging was used to confirm sites of tumor development prior to excision for subsequent analysis. **Figure 1B** displays results of IVIS spectral imaging of an array of age-matched littermate progeny of the selected genotypes used in this study showing tdTomato epifluorescence to be perceptibly brightest in the abdominal region of only the *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice. Spectral quantification of tdTomato epifluorescence revealed significantly higher average radiant efficiency measurements (p=0.0001\*) in *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice compared to other mouse genotypes in two different sets of mice imaging studies (**Figure 1C**). TS tumorigenesis in this select mouse group was further verified by ultrasound imaging revealing the occurrence of tumors in the kidneys of *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice (**Figure D2**) compared to tumor-less renal tissue in the *Tsc2*<sup>+/+</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice (**Figure D1**) and *Tsc2*<sup>+/+</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice (not shown). High-resolution magnetic resonance imaging further confirms the occurrence of bilateral cortical tumor growth in both kidneys of *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice (**Figure 1E**). *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice spontaneously developed renal tumors which could not be detected by tdTomato epifluorescence.

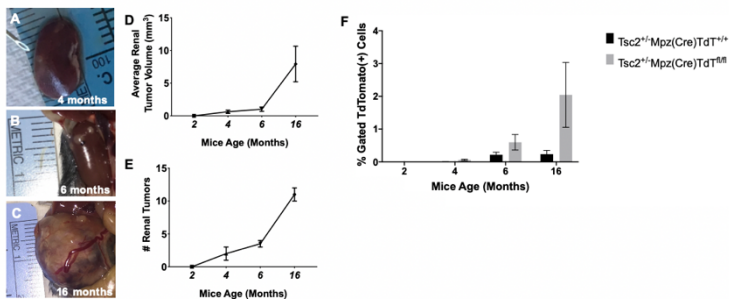


**Figure 1: Generation of Tuberosclerosis Reporter Mouse Model and Imaging Techniques for Visualizing TS tumors – A) Schematic of genetic crosses to generate *Tsc2*<sup>+/-</sup> reporter mouse models show floxed *R26Sor<sup>tm9</sup>(CAG-tdTomato)<sup>Hze</sup>* mice (n=10 animals) were crossed with *Tg(Mpz-Cre)26Mes* mice (n=10 animals) expressing Cre recombinase under the control of myelin protein zero (MPZ) promoter. *Mpz(Cre)*<sup>tm1.1JDar</sup> mouse progeny (n=8 animals) were then**

mated with *Tsc2*<sup>+/-</sup> mice (n=10 animals) to source *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> tuberosclerosis reporter mouse models (shown in red) that spontaneously develop renal tumors driven by neural crest cells labeled with tdTomato. **B)** IVIS spectral images of abdominopelvic cavities of 1.3yr old *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> tuberosclerosis mouse model compared to age-matched *Tsc2*<sup>+/+</sup>*Mpz(Cre)*<sup>fl/fl</sup>, *Tsc2*<sup>+/+</sup>*Mpz(Cre)*<sup>fl/fl</sup>, and *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice showing epifluorescence of tdTomato-expressing regions (n=4 mice/genotype). **C)** Average radiant efficiency was used to compare tdTomato epifluorescence in these mouse models by one-way ANOVA and post-hoc Dunnett's test (p=0.0001\*). Small animal ultrasound scan of normal kidney (NK) parenchyma of adult mice wildtype for the *Tsc2* gene (**D1**) in comparison to the kidneys in *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice (**D2**) with highlighted sites of tumors (T). BV denotes blood vessels. Rapid magnetic resonance images of kidneys (**E**) in *Tsc2*<sup>+/-</sup>*Mpz(Cre)*<sup>fl/fl</sup> mice identifying tumors (T), shown with arrows and dashes.

## Renal Tumorigenesis in *Tsc2*<sup>+/-</sup> mice Correlate with Onset and Proliferation of Neural Crest Precursor Populations

Prior to our study, the earliest reported detection of renal neoplasms in tuberous sclerosis *Tsc2*<sup>+/-</sup> mouse models was 6 months of age, and earlier polycystic lesions occurring in the *Dermo1*<sup>Cre</sup>;*Tsc2*<sup>fl/fl</sup> mice led to mouse lethality by 3 weeks of age. We report the earliest known detection of ectopic fluid-filled renal cysts in *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> by 4 months of age (**Figure 2A**). These tumors were detectable by both IVIS spectral imaging and ultrasound. By 6 months of age, multiple solid tumors and/or cysts containing denser fluid can be observed (**B**), and by 16 months, enlarged, dysmorphic, inflamed renal tissue coalescing with multiple cysts can be observed (**C**). *Tsc2*<sup>+/-</sup> mice renal tumors were excised at 4, 6 and 16 months and dissociated into single cell populations for flow cytometric sorting of tdTomato<sup>+</sup> neural crest cells in these tumors. Results indicate that the significant increase in renal tumor volume (**D**) and number of tumors (**E**) in *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> mice over the 16-month period correlated with a marked increase in the fraction of gated tdTomato<sup>+</sup> cells from an average of 0.22% beginning at 6 months to 2% at 16 months (**Figure 1F**) compared to sorted cells from *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>+/+</sup> and *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> mice after gating protocols for live cells (DAPI) and Texas Red (TR). Representative dot plots comparing gating results of flow cytometric separation of renal tissue and tumor cells between *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> and *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> obtained at 4 months (**G,H**), 6 months (**I,J**) and 16 months (**K,L**) respectively are displayed. Single cell suspensions of cranial neural crest cells (CNCCs) (**M**) and trunk neural crest cells (TNCCs) (**N**) excised from 9.5dpc *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> mice embryos were used as positive controls to assess the emergence of tdTomato<sup>+</sup> neural crest cells in tuberous sclerosis mice. The resolution of tdTomato<sup>+</sup> cells in renal tumor cell dissociates indicates active *Mpz* promoter activity in cells of a neural crest lineage. We believe this unique cell population are migrants from the neural crest that retain their proliferative and stem cell character and differentiate to source pathogenic tumor cells of TS as phenotypic manifestations of mono-allelic and bi-allelic mutations at the *Tsc2* gene locus. Although the fraction of these tdTomato-expressing cells in both the gated and whole cell populations analyzed by flow cytometry is quite low, they are proportional in quantity to the neural crest cell precursors obtained from 9.5dpc embryonic cranial tissue and mesodermal somites used to obtain our cranial and trunk neural crest cell controls respectively.



**Figure 2: Identification of Neural Crest Lineage Cells in Ectopic Renal Tumor Onset of the *Tsc2*<sup>+/-</sup> mice – A) Earliest reported detection of ectopic renal tumors in the *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> mice begins at 4 months of age (**A**) then pictured at 6 months (**B**) and at 16 months (**C**). Tumor volume (**D**) and number (**E**) increased proportionally during the same period. Tumors from *Tsc2*<sup>+/-</sup> *Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> mice were**

dissociated, gated for live/dead staining (Sytox Green/DAPI) and sorted for tdTomato expression in comparison with renal cortical cells obtained from age-matched *Tsc2*<sup>+/-</sup> *Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> (**F**). Results showed proportional increase in tdTomato(+) cells with increase in mice age, tumor volume and number of tumors. Representative flow cytometric data for comparing single cell events obtained between renal cortical tissue and TS reporter mice at 4 months (**G,H**), 6 months of age (**I,J**), and 16 months (**K,L**) are displayed respectively. Single cell suspensions of cranial neural crest cells (CNCCs) (**M**) and trunk neural crest cells (TNCCs) (**N**) excised from the 9.5dpc *Tsc2*<sup>+/-</sup>/*Mpz*(*Cre*)/*TdT*<sup>fl/fl</sup> mice embryos were used as positive controls to assess the emergence of tdTomato<sup>+</sup> neural crest cells in tuberous sclerosis mice. Flow cytometric data is one representative experiment of doublet studies performed at different times using N=2 mice/genotype/study. Acquisition and analyses of flow cytometric data was performed on a Bio-Rad S3e cell sorter and analyzed using FCS Express 7 Plus software.

For specific aim 2, we have isolated peripheral blood mononuclear cells (PBMCs) obtained from six patients with clinical diagnosis of TSC and reprogrammed them into iPSC clones (N=4 clones per patient). We have confirmed their pluripotency using marker expression biology and performed G-band karyotype analysis to assess the presence of chromosomal abnormalities. We also intend to employ the same iPSC reprogramming paradigm using fibroblasts dissociated from biopsies of cutaneous angiofibromas similarly diagnostic of TSC biopsies. To characterize the genotypic identity of iPSC clones, we have determined loss of heterozygosity (LOH) status for each clone in the TSC locus and selected three iPSC clones per patient sample for use in further experimentation based on the presence or absence of LOH. We also sequenced the DNA from these clones for identification of single nucleotide polymorphisms (SNP) mutations in the TSC coding region (42 exons). In the last year we have been able to identify TSC mutations in these cells even when DNA from the PBMCs did not demonstrate a mutation. When utilizing iPSC clones from healthy patients and patients with diseases not associated with LAM no TSC mutations were identified. In the next year we will utilize these cells to determine if the HMGA2 pathway alters growth of the neural crest cells isolated from these iPSCs as described in the proposal.

## 2. **What opportunities for training and professional development has the project provided?**

This project has enabled the training of an Associate Research Scientist (ARS), Uchenna Unachukwu, under mentorship of the Principal Investigator, Dr. Jeanine D'Armiento, in skills including iPSC reprogramming, sanger sequencing and SNP mutational analysis, mouse breeding, mating and genotyping protocols, and in the reporting of experiments for presentations. Due to the limitations on traveling this year due to COVID the ARS was unable to attend the ATS where his work was initially selected for an oral presentation. Upon restructuring of the meeting due to COVID this presentation was cancelled by the organization. Dr. Unachukwu will submit the work for presentation at virtual meetings this coming fall.

### ○ **How were the results disseminated to communities of interest?**

Nothing to Report since our work was not presented at the American Thoracic Society. However, we will be submitting a manuscript in the next few weeks based on the first portion of work from Aim 1.

### ○ **What do you plan to do during the next reporting period to accomplish the goals?**

The prior funding period has allowed us to generate our mouse study population and identify the onset of tumor progression in our transgenic mice and identified the stem cell origin of the tumors as described above. We will now isolate the cells and perform RNA sequencing to dissect the molecular pathway in the neural crest derived tumors.

We have generated iPSCs from our patients as described above and in the last year we will differentiate these cells into neural crest cells and identify the molecular signaling governing the HMGA2-mediated tumorigenesis.

## 3. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

### ○ **What was the impact on the development of the principal discipline(s) of the project?**

The studies in the last year have allowed us to validate the initial studies that demonstrate the stem cell nature of the tumors in the TSC heterozygous mice. This finding is critical because it allows us to target the LAM cell with agents directed at the unique stem cell and will open up the treatment options.

The DNA sequencing and SNP mutational analysis of iPSC clones to date from our patients revealed a lack of isogenicity between clones, a very surprising results that could inform future reprogramming practices in the field of Tuberous Sclerosis and in pathophysiological investigations as a whole. We intend to determine whether this lack of genetic stability is unique to the TSC genotype or occurs at the nucleotide level in other disorders, and if the lack of isogenicity between clones is the result of viral reprogramming or integration/non-integration artifacts or induced by cell culture expansion. Our findings will lend evidential support to the phenomena which has been previously summarized to occur in other pathologies: *Turinetto et al. Int J Mol Sci. 2017 Sep 13;18(9)*. Furthermore, the presence of TSC mutations in the iPSC cells strengthens the literature demonstrating the importance of TSC in this disease.

- **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

4. **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**

There have been no changes to our project.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

No issues in the last funding period.

- **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

- **Significant changes in use or care of human subjects**

Nothing to Report

- **Significant changes in use or care of vertebrate animals.**

Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

Nothing to Report

**5. PRODUCTS:**

**Publications, conference papers, and presentations**

- **Journal publications.**

Nothing to Report.

- **Books or other non-periodical, one-time publications.**

*Nothing to Report.*

- **Other publications, conference papers, and presentations.**

Unachukwu, U., Shiomi, T., Goldklang, M., Damoci, C., Chada, K., and D’Armiento, J. The Neural Crest Origin of Tuberous Sclerosis American Thoracic Society International Meeting (2020)

- **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.*

**6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name	Jeanine D’Armiento, MD, PhD
Project Role:	Principal Investigator
Researcher Identifier	
Contribution to Project:	1 person Month
Funding Support:	No Change

Name	Uchenna Unachukwu
Project Role:	Associate Research Scientist
Researcher Identifier	
Contribution to Project:	6 person Months
Funding Support:	No Change

Name	Tina Zelonina
Project Role:	Technician
Researcher Identifier	
Contribution to Project:	5 person months
Funding Support:	No Change

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

*Nothing to Report*

- **What other organizations were involved as partners?**

*Nothing to Report*

**7. SPECIAL REPORTING REQUIREMENTS  
COLLABORATIVE AWARDS:**

Not applicable

**QUAD CHARTS:**

Not applicable

8. **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. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***