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14. ABSTRACT

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 by tracking neurocristogenesis in a $TSC2^{+/-}$ 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. ***Aim 1:*** *To delineate the biochemical signaling that determines the temporal sequence of neural crest cell-induced initiation and progression of tumors using a $TSC2^{+/-}$ mouse model*. **Aim 2:** *To determine the role of HMGA2 in tumor pathogenicity driven by TSC2-haploinsufficient iPSC-derived neural crest precursors.*

15. SUBJECT TERMS
None listed.

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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 harmatomatous 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+/- 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 $Tsc2^{+/-}$ reporter mouse model. This aim was completed, and we have published the studies this year.

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). We have performed differentiation studies into neural crest progenitor cells and assessed proliferation and cell invasion in these cells. We also were able to perform RNA Sequencing analysis to further define the mechanism of tumor formation from the neural crest derived tumors.

1. What was accomplished under these goals?

In specific Aim 1, we intend to track temporal ontogenesis of tumors in $Tsc2^{+/-}$ 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-$Cre^{R26R}Tsc2^{+/-}$ mouse reporter model required to commence investigations in this aim, we have successfully bred Gt(ROSA)26$Sor^{tm9(CAG-tdTomato)Hze}$/J (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 $Tsc2^{+/-}$ mice. We continued this mating regimen and genotyping mouse progeny into comparative genotypes used for tumor tracking and analyses - $Tsc2^{+/-}Mpz(Cre)^{fl/fl}$, $Tsc2^{+/-}Mpz(Cre)^{+/+}$, $Tsc2^{+/+}Mpz(Cre)^{fl/fl}$, and $Tsc2^{+/+}Mpz(Cre)^{+/+}$.

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 $Tsc2^{+/-}$ 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 $Tsc2^{+/-}$/*Mpz(Cre)*/$TdT^{fl/fl}$ mice. Spectral quantification of tdTomato epifluorescence revealed significantly higher average radiant efficiency measurements (p=0.0001*) in $Tsc2^{+/-}$/*Mpz(Cre)*/$TdT^{fl/fl}$ 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 $Tsc2^{+/-}$/*Mpz(Cre)*/$TdT^{fl/fl}$ mice **(Figure D2)** compared to tumor-less renal tissue in the $Tsc2^{+/+}$/*Mpz(Cre)*/$TdT^{fl/fl}$ mice **(Figure D1)** and $Tsc2^{+/+}$/*Mpz(Cre)*/$TdT^{+/+}$ mice (not shown). High-resolution magnetic resonance imaging further confirms the occurrence of bilateral cortical tumor growth in both kidneys of $Tsc2^{+/-}$/*Mpz(Cre)*/$TdT^{fl/fl}$ mice **(Figure 1E)**. $Tsc2^{+/-}$/*Mpz(Cre)*/$TdT^{+/+}$ mice spontaneously developed renal tumors which could not be detected by tdTomato epifluorescence.

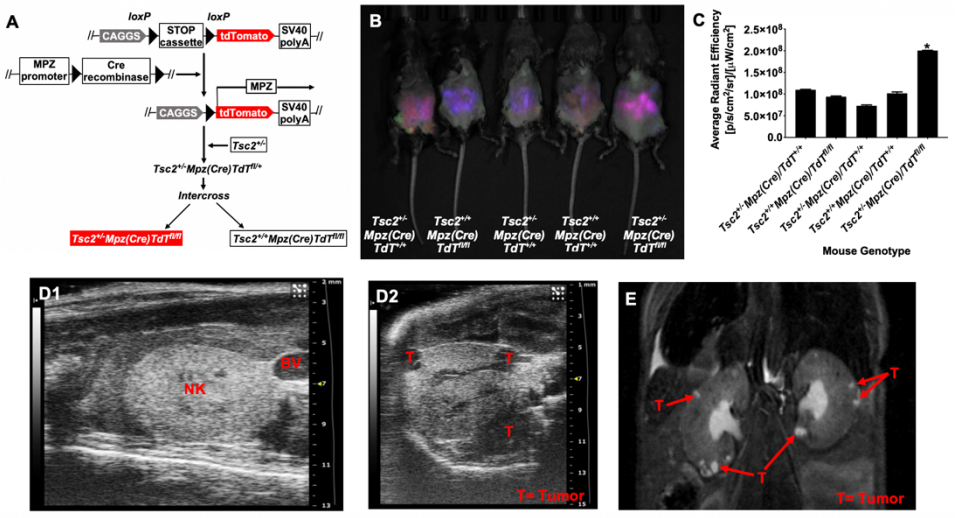


Figure 1: Generation of Tuberous Sclerosis Reporter Mouse Model and Imaging Techniques for Visualizing TS tumors – A) Schematic of genetic crosses to generate $Tsc2^{+/-}$ reporter mouse models show floxed *R26Sor*$^{tm9(CAG-tdTomato)Hze}$ mice (n=10 animals) were crossed with Tg(Mpz-Cre)26Mes mice (n=10 animals) expressing Cre recombinase under the control of myelin protein zero (MPZ) promoter. *Mpz(Cre)* $^{tm1.1JDar}$ mouse progeny (n=8 animals) were then mated with $Tsc2^{+/-}$ mice (n=10 animals) to source $Tsc2^{+/-}$*Mpz(Cre)*$^{fl/fl}$ tuberous sclerosis 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 $Tsc2^{+/-}$*Mpz(Cre)*$^{fl/fl}$ tuberous sclerosis mouse model compared to age-matched $Tsc2^{+/+}$*Mpz(Cre)*$TdT^{+/+}$, $Tsc2^{+/+}$*Mpz(Cre)*$TdT^{fl/fl}$, and $Tsc2^{+/-}$*Mpz(Cre)*$TdT^{+/+}$ 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 $Tsc2^{+/-}$*Mpz(Cre)*$^{fl/fl}$ mice **(D2)** with highlighted sites of tumors (T). BV denotes blood vessels. Rapid magnetic resonance images of kidneys **(E)** in $Tsc2^{+/-}$*Mpz(Cre)*$TdT^{fl/fl}$ mice identifying tumors (T), shown with arrows and dashes.

Renal Tumorigenesis in $Tsc2^{+/-}$ 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 $Tsc2^{+/-}$ mouse models was 6 months of age, and earlier polycystic lesions occurring in the $Dermo1^{Cre}$;$Tsc2^{fl/fl}$ mice led to mouse

lethality by 3 weeks of age. We report the earliest known detection of ectopic fluid-filled renal cysts in *Tsc2*$^{+/-}$*/Mpz(Cre)*/TdT$^{fl/fl}$ 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*$^{+/-}$ mice renal tumors were excised at 4, 6 and 16 months and dissociated into single cell populations for flow cytometric sorting of tdTomato^{+} neural crest cells in these tumors. Results indicate that the significant increase in renal tumor volume **(D)** and number of tumors **(E)** in *Tsc2*$^{+/-}$*/Mpz(Cre)*/TdT$^{fl/fl}$ mice over the 16-month period correlated with a marked increase in the fraction of gated tdTomato^{+} cells from an average of 0.22% beginning at 6 months to 2% at 16 months **(Figure 1F)** compared to sorted cells from *Tsc2*$^{+/-}$*/Mpz(Cre)*/TdT$^{+/+}$ and *Tsc2*$^{+/+}$*/Mpz(Cre)*/TdT$^{fl/fl}$ 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*$^{+/+}$*/Mpz(Cre)*/TdT$^{fl/fl}$ and *Tsc2*$^{+/-}$*/Mpz(Cre)*/TdT$^{fl/fl}$ 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*$^{+/-}$*Mpz(Cre)TdT*$^{fl/fl}$ mice embryos were used as positive controls to assess the emergence of tdTomato^{+} neural crest cells in tuberous sclerosis mice. The resolution of tdTomato^{+} 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.

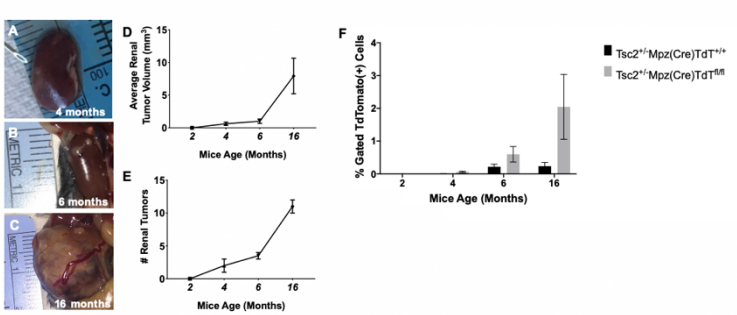
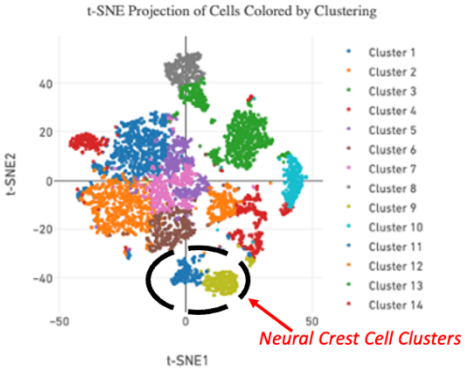


Figure 2: Identification of Neural Crest Lineage Cells in Ectopic Renal Tumor Onset of the *Tsc2*$^{+/-}$ mice – A) Earliest reported detection of ectopic renal tumors in the *Tsc2*$^{+/-}$*Mpz(Cre)TdT*$^{fl/fl}$ mice begins at 4 months of age **(A)** then pictured at 6 months **(B)** and at 16 months **(C).** Tumor volume **(D)** and number **(E)** increased proportionally during the same period. Tumors from *Tsc2*$^{+/-}$ *Mpz(Cre)TdT*$^{fl/fl}$ 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*$^{+/+}$ *Mpz(Cre)TdT*$^{fl/fl}$ **(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*$^{+/-}$*Mpz(Cre)TdT*$^{fl/fl}$ mice embryos were used as positive controls to assess the emergence of tdTomato+ 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.

Single Cell Transcriptomic Analysis annotates neural crest cells in *Tsc2*$^{+/-}$ Mice Renal Tumors. A total of 6708 *Tsc2*$^{-/+}$*Mpz(Cre)TdT*$^{fl/fl}$ mouse renal tumor cells were analyzed using the 10x Genomics Cell Ranger pipeline with 52,657 mean reads per cell. Fourteen cell clusters were identified by significant log2Fold Change and q-values (<0.05). scRNA-seq alignment and quantification was performed by comparison with: GRCm38 mm10 mouse reference genome and publicly available E8.5 & E20.5 stage embryonic neural tissue from C57BL/6J mice. Canonical neural crest markers including *Tfap2*, *Hoxa7*, *Hoxb9* were used to annotate neural crest cell clusters seen as 9 & 11 **(Figure 3)**.



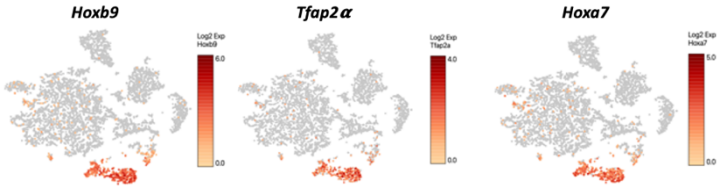


Figure 3. Single Cell Transcriptomic Analysis of TSC tumors in *Tsc2*$^{-/+}$*Mpz(Cre)TdT*$^{fl/fl}$ mice.

Post-hoc sctransform analysis using Seurat in R was used to identify top 5 differentially expressed genes in clusters 9 and 11. Select novel genes including Sox***9***, ***Lcn2***, ***Wfdc2***, ***Clu***, ***Ndrg1*** were expressed at 2-8-fold higher magnitude than canonical neural crest markers. Expression of the select neural crest markers significantly increased in *Tsc2*$^{+/-}$ mice kidney tumors (KT) compared to normal kidney cortical tissues (NK) with mice age **(Figure 4).**

Gene Name	Gene	Cluster	Fold Change	p-Adj
Keratin 18	Krt18	9	3.17	0
Polymeric immunoglobulin receptor	Pigr	9	3.04	0
Lipocalin-2	Lcn2	9	3.54	8.80E-291
WAP Four-Disulfide Core Domain	Wfdc2	9	3.41	2.91E-266
Clusterin	Clu	9	3.43	1.98E-215
Glutathione Peroxidase 3	Gpx3	11	1.40	2.55E-80
FXYD Domain Containing Ion Transport Regulator 2	Fxyd2	11	1.50	9.38E-71
SRY-Box Transcription Factor 9	Sox9	11	2.29	7.69E-240
N-Myc Downstream Regulated 1	Ndrg1	11	2.98	3.27E-163
Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 10	Chchd10	11	2.00	1.76E-151

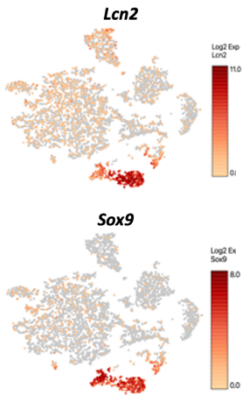


Figure 4. Differentially expressed neural crest markers in *Tsc2*$^{+/-}$ mice kidney tumors, include Lcn2 and Sox9.

This study provides evidence for an ontogenetic neural crest cellular lineage for *Tsc2*$^{+/-}$ mice renal tumors with implications for the TS and LAM human disorders. Sub-populations of neural crest cells exist in masses within *Tsc2*$^{+/-}$ mice renal neoplasms (and also in hepatic tumors). Multi-organ loci of tumorigenic

neural crest cells and their selective gene expression states confirms that these tumor cells are post-migratory cranial neural crest relics of neurocristogenesis that co-opt tumorigenesis in their domiciled organs due to germline, somatic *Tsc2* mutations or environmental stimuli. Neural crest cells exhibit an increase in proliferation with tumor volume, multiplicity, and mice age akin to reported multi-potent self-renewal capacity of neural crest cells even in adulthood.

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 with continued COVID precautions he was unable to present at the National meetings. However, he was accepted as a speaker at the 2021 International TSC and LAM Research Conference to present the described work.

- **How were the results disseminated to communities of interest?**

The first portion of the work was published in iScience (iScience. 2021 Jun 4;24(7):102684) and the second portion of the studies are in preparation for submission.

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

We are in a no cost extension and are finalizing the work for publication on the second Aim.

3. **IMPACT:**
   - **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, but conclusively demonstrated the presence of TSC mutations in the iPSC cells of all patients including those with sporadic LAM. These findings not only strengthen the literature demonstrating the importance of TSC in this disease but suggest that the cells may be more susceptible to mutations during the process of reprogramming similar to what occurs during development in the patients. Future studies will examine this hypothesis.

   - **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:**
   - **Changes in approach and reasons for change**

     There have been no changes to the 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 (2021)

   - **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:**