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13. SUPPLEMENTARY NOTES

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

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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. 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. In this cycle we had not yet been approved for animal studies but fortunately the mouse mating and breeding studies can continue under our internal funding since they are necessary for the experiments. Therefore, we are on target for these studies to commence when the approval for animal work has been achieved.

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 currently generating and characterizing iPSC colonies from peripheral blood mononuclear cells (PBMCs) of TSC patients and are ahead of schedule given that 6-10 months was allotted to complete this phase of the goal. In general, we have accomplished 25% of tasks specified for this goal.

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

We are still awaiting ACURO approval for work with mice. 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. We require these matings for other studies within our laboratory therefore we were able to perform the breeding and mating to prepare for our studies in the DOD proposal and not fall behind in our schedule. To generate the Mpz-Cre^{R26R}Tsc2$^{+/-}$ mouse reporter model required to commence investigations in this aim, we have successfully bred Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/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 are continuing this mating regimen and genotyping mouse progeny into comparative genotypes to be used for tumor tracking and analyses - Tsc2$^{+/-}$Mpz(Cre)$^{fl/fl}$, Tsc2$^{+/-}$Mpz(Cre)$^{+/+}$, Tsc2$^{+/+}$Mpz(Cre)$^{fl/fl}$, and Tsc2$^{+/+}$Mpz(Cre)$^{+/+}$.

We have successfully obtained HRPO approval for work with human biological samples. For specific aim 2, we have isolated peripheral blood mononuclear cells (PBMCs) obtained from two patients with clinical diagnosis of TSC and reprogrammed them into iPSC clones (N=12 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). Surprising, we resolved differences in SNPs between clones reprogrammed from cells of the same patient and we are currently investigating this phenomenon prior to commencing genetic engineering protocols manipulating Hmga2 and Tsc2 expression in these iPSCs. We have summarized differences in SNP mutations resolved between iPSC clones reprogrammed from TSC patient 211 (Appendix 1) and patient 025 (Appendix 2), in comparison to whole blood from each individual patient and iPSC controls from healthy individuals.

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. The ARS also attended the Tuberous Sclerosis Conference (Toronto 2019) and participated in an iPSC breakout session where techniques in tumor/tissue dissociation, culture, and iPSC reprogramming was discussed and good laboratory practices for these procedures examined.

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

     Nothing to Report

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

     The last funding period has allowed us to set the stage for the pertinent studies in this proposal. After we receive approval from ACURO for our work we will set up matings to generate the study population and identify the onset of tumor progression in our transgenic reporter mice. These

tumors will then be characterized with RNA sequencing to achieve our goal of dissecting the molecular pathway in the neural crest derived tumors.

Now that we have generated iPSCs from our patients as described above we will differentiate these cells into neural crest cells and identify the molecular signaling governing the HMGA2-mediated tumorigenesis. In the next funding period we will perform the differentiation and cloning of HMGA2 into the cells.

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?**

      DNA sequencing and SNP mutational analysis of iPSC clones 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).*

    - **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**

      We have generated iPSCs not only from fibroblasts to be excised from cutaneous angiofibromas of diagnosed TSC patients but to also include PBMCs as starting material for reprogramming. In doing so, we determine whether the patient has a germline or somatic TSC mutation, whether mutations in different organs of the body and between patients are isogenic, and whether changes in TSC genes correlate to disease severity.

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

In performing tasks to satisfy objectives outlined in specific aim 2, we determined that iPSC clones obtained from the same patient samples are not isogenic and we are currently investigating the phenomena to determine whether the heterogeneity in clonal genotypes is the result of viral reprogramming protocols, associated with inherent variability in TSC genotype-phenotype correlations, or other factors related specifically to the disease that are yet to be ascertained. This investigation has slightly delayed progression of the project towards the genetic engineering phase of this aim. We however do anticipate initiating the genetic engineering studies in select samples within the next month.

- **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**

  We included an additional sample type – Blood – as starting material for iPSC reprogramming and this change is IRB approved AAAR7738
- **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.**

  Nothing to Report.

- **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.***