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14. ABSTRACT

Tuberous sclerosis complex (TSC) is a rare genetic disease that causes benign tumors to grow in many different organs of the body. Although TSC tumors are not metastatic, these tumors can still cause problems, some critical, when the growth is located in vital organs such as the brain, kidney, and lung. Currently, there is no cure for TSC and treatment is largely devoted to surgical removal of tumors. Sometimes drugs such as rapamycin or related drugs are used to keep tumors from growing. Unfortunately, rapamycin only stops the tumor growth, does not make the tumors go away, and tumors enlarge further as soon as rapamycin is discontinued. In addition, long-term treatment can lead to side effects, such as diabetes, and resistance. In other words, TSC is an incurable lifelong disease, and the current treatments available only stop disease progression. They are not a cure. It is imperative to develop a new therapeutic approach that can only eliminate tumor rather just block growth but also do so without major side effects.

15. SUBJECT TERMS
None listed.

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1. **Introduction.** Tuberous sclerosis complex (TSC) is a rare genetic disease that causes benign tumors to grow in many different organs of the body. Although TSC tumors are not metastatic, these tumors can still cause problems, some critical, when the growth is located in vital organs such as the brain, kidney, and lung. Currently, there is no cure for TSC and treatment is largely devoted to surgical removal of tumors. Sometimes drugs such as rapamycin or related drugs are used to keep tumors from growing. Unfortunately, rapamycin only stops the tumor growth, does not make the tumors go away, and tumors enlarge further as soon as rapamycin is discontinued. In addition, long-term treatment can lead to side effects, such as diabetes, and resistance. In other words, TSC is an incurable lifelong disease, and the current treatments available only stop disease progression. They are not a cure. It is imperative to develop a new therapeutic approach that can only eliminate tumor rather just block growth but also do so without major side effects.

2. **Keywords**

   Tuberous Sclerosis Complex (TSC), mTORC1 inhibitors, rapamycin, RNAseq

3. **Accomplishments**

   Aim 1. Determine the molecular basis for rapamycin-induced survival of TSC2-deficient cells in vivo. Our hypothesis is that prolonged rapamycin treatment leads to resistance by triggering pro-survival genes that confer survival.

   Major Task 1. Establish xenograft tumors of 621-101 in response to rapamycin.

   Subtask 1: We have established xenograft tumors at 4 tumor stages (Tumor-1 [vehicle], 2 [shrinking], 3 [refractory], & 4 [regrowth]) (**Figure 1**).

   Subtask 2: We have harvested xenograft tumors at 4 tumor stages and isolated RNA.

   Major Task 2: Identify gene expression signature responsible for tumor refractoriness.

   Subtask 1: Perform two-step gene selection to identify genes responsible for resistance and regrowth by comparing with **ELT3 xenograft data**.

   We have previously employed RNAseq on ELT3 tumor samples. We collaborated with Dr. Medvedovic to perform data mining to identify genes responsible for refractoriness. Step-1: distinguish gene signatures associated with rapamycin-induced regrowth by comparing transcriptome of Tumor-3 with Tumor-2. Step-2: eliminate common genes to cytostatic tumor-2. Step-3: perform secondary pathway analysis to identify pro-

survival and cell death pathways **(Figure 2**). Among the significant differentially expression genes, we identified the epidermal growth factor like domain multiple 6 (*EGFL6*), a secreted protein classically known to promote endothelial cell angiogenesis during wound healing and in cancer growth. *EFGL6* expression was increased by 119.4-fold in rapamycin refractory tumor compared to rapamycin sensitive tumor. We further validated RNA-seq findings using Real-time PCR analyses. The transcript level of *EGFL6* is significantly elevated in rapamycin refractory ELT3 tumors relative to that in rapamycin sensitive tumors (**Figure 3**).

Moreover, we have discovered an Mitogen-activated protein kinase (MAPK)-evoked positive feedback loop that dampens the efficacy of mTORC1 inhibition. Mechanistically, mTORC1 inhibition increased MEK1-dependent activation of MAPK in TSC-deficient cells. Pharmacological inhibition of MAPK abrogated this feedback loop activation. Importantly, the combinatorial inhibition of mTORC1 and MAPK induces the death of TSC2-deficient cells. Our results provide a rationale for dual targeting of mTORC1 and MAPK pathways in TSC and other mTORC1 hyperactive neoplasm. This work was published in August 2020 [1].

In collaboration with Dr. Medvedovic, we developed the complete protocol for performing connectivity analysis using scRNA-seq data, including signatures construction and connectivity analysis with individual drug signatures as well as the whole classes of drugs with the same mechanism of action. The methods are described in the context of CMaP of LAM scRNA-seq signatures. Our analyses successfully predict therapeutic effects of currently used drugs inhibiting mTORC1 signaling. Importantly, we demonstrate that these results are contingent on use of scRNA-seq data and our methods for constructing single-cell disease signature and would not be possible by connectivity analysis of standard bulk RNA-seq disease signatures. This work was published in April 2021 [2].

To assess tumor cell viability in response to drug treatments, we performed a pilot study of noninvasive bioluminescent imaging and fluorescent imaging to detect cell proliferation and apoptosis in vivo, respectively. Mice inoculated with LAM patient-derived 621-101 cells expressing luciferase reporter gene (mouse ID 2, 3, 4) showed strong bioluminescent signals at flanks whereas cells were inoculated, relative

to the mouse inoculated with cells without luciferase gene (Mouse ID 1), indicative of proliferating cells (**Figure 4**, upper panel). In a pilot experiment, we also used IVISense Annexin-V 750 Fluorescent Probe (Annexin-Vivo) (PerkinElmer) to detect tumor cells undergoing apoptosis. Annexin-Vivo™ enables in vivo visualization and quantification of membrane-bound phosphatidylserine (PS) during the early stages of apoptosis in vivo. In mice bearing xenograft tumors, the Annexin V signals were concentrated in tumor cores, whereas luciferase activity was absent, indicative of apoptotic regions in tumors (**Figure 4,** lower panel). We will use this approach to assess the therapeutic efficacy in xenograft tumor models.

During the fun ding period, we also reported in two review articles that mTORC1 hyperactivation has been identified in TSC and pulmonary LAM [3, 4].

4. **Impact.** Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder with multi-system manifestations including development of benign neoplasms in the brain, heart, lung, and kidneys. TSC is caused by TSC1 or TSC2 gene mutations, resulting in constitutive activation of the mechanistic target of rapamycin complex 1 (mTORC1). mTOR is a protein kinase that regulates gene transcription, protein synthesis, ribosome biogenesis, autophagy, cell metabolism, and cell growth. Recent clinical trials in TSC demonstrate that mTORC1 inhibitors (rapamycin or everolimus) decrease the volume of TSC tumors, subependymal giant cell astrocytomas and renal angiomyolipomas, and stabilize pulmonary function decline. However, tumors regrow, and symptoms resume when treatment is discontinued. This cytostatic rather than a cytocidal effect accounts for need for continuous therapy. Furthermore, despite the efficacy in most patients, a subset of TSC patients experience rapamycin resistance and progress. Therefore, there is a critical need to identify the molecular drivers of distinct therapeutic responses in TSC. We will utilize tow innovative, state-of-the-art technologies: RNASeq to identify rapamycin-induced gene expression in xenograft tumors of rat ELT3 and patient angiomyolipoma-derived cells, and non-invasive imaging for evidence of tumor growth and cell death. Our proposal directly addresses the following FY18 focus areas: 1) Understanding phenotypic heterogeneity in TSC. Our proposal aims to identify phenotypic heterogeneity of TSC by performing RNAseq gene expression analysis of TSC tumors under informative conditions; (1) no treatment control condition, (2) chronic rapamycin treated cytostatic condition, 3) rapamycin-resistant refractory condition, and 4) rapamycin cessation. 2) Gaining a deeper knowledge of TSC signaling pathways and the cellular consequences of TSC deficiency: Our study primarily focuses on identifying the

signaling pathways that, under long-term rapamycin treatment, protect TSC tumor cells from cell death induction. We will elucidate the molecular mechanisms by which cells acquire rapamycin-resistance. The RNAseq gene analysis is powerful and informative, more so than any other gene analysis, with regard elucidation of cell signaling analysis. This technique can clearly detail the cellular responses of tumor cells in each response stage. Therefore, we can identify distinct and extraordinarily relevant signal transduction pathways in response to the treatment in all the cells in a tumor, but not the possibility of the tumor response to the treatment. 3) Improving TSC disease models. Our study will investigate the biology of TSC tumor cells under rapamycin treatment. Our approach using RNA-seq can provide us information to understand how rapamycin treatment affects the heterogeneous cell populations and their response to the treatment in the TSC tumors. These studies will refine an existing rodent model of TSC. 4) Facilitating therapeutics and clinical trials research. The effect of mTOR inhibitors on TSC tumors in these experiments has been consistently cytostatic rather than cytotoxic, tumors typically regrow upon the cessation of treatment, and resistance can evolve. This proposal aims to identify novel signaling pathways that account for these responses to inhibitor therapy by targeted RNAseq analysis of the discrete tumor response.

These basic studies will ultimately lead to transformative clinical trials with new combination therapies with rapamycin and/or its analogs. In summary, understanding the mechanism of mTOR inhibitor-induced cytostasis and resistance will lead to improved therapy for patients with TSC and other neoplasms.

5. **Changes/Problems.** This award began in October 2019. We started xenograft tumor experiments proposed in Aim 1 in October 2019. Tumors were harvested and RNA was isolated. Research operation was shutdown at University of Cincinnati due to the COVID-19 pandemic outbreak in March - May 2020. Our experiments were halted for three months. Consequently, we were unable to continue some wet bench studies. Because Dr. Medvedovic could continue bioinformatic analysis in remote settings, we started to work on Major Task 2 focusing on analyzing previous ELT3 xenograft datasets for comparison work. Moreover, the graduate student Naim Mahi who performed bioinformatic analysis of RNAseq under the Supervision of Dr. Medvedovic (Co-I) completed his PhD study in June 2020, and Yiyang Lu who was Under Dr. Yu supervision finished her PhD dissertation and left the lab in September 2020. The recruitment of new lab members took much longer than we anticipated. Dr. Astreinidis joined Yu Lab on 8/30/2021 and he started to work on studies funded by this award. This award has been extended by one year enabling us to complete additional proposed studies.

6. Products.

1. Yiyang Lu, Erik Y. Zhang, Jie Liu, Jane J. Yu. Inhibition of the mechanistic target of rapamycin induces cell survival via MAPK in tuberous sclerosis complex. ***Orphanet J Rare Dis***. 2020 Aug 17;15(1):209. PMID: 32807195 PMCID: PMC7433150

2. Naim Al Mahi, Erik Y. Zhang, S Sherman, Jane J. Yu and Mario Medvedovic. Connectivity analysis of single cell RNA-sequencing derived transcriptional signature of lymphangioleiomyomatosis (LAM). ***Int. J. Mol. Sci.*** 2021 April 22, 4371. PMCID: PMC8122562. PMID: 33922083

3. Cormac McCarthy, Nishant Gupta, Simon R. Johnson, Jane J. Yu, Francis X. McCormack. Lymphangioleiomyomatosis: Pathogenesis, Translation and Clinical Management. ***Lancet Respir Med***. 2021 Nov;9(11):1313-1327. PMID: 34461049

4. Jane J. Yu and Elena A. Goncharova. mTOR Signaling Network in Cell Biology and Human Disease. ***Int. J. Mol. Sci***. 2022, 23(24), 16142; https://doi.org/10.3390/ijms232416142

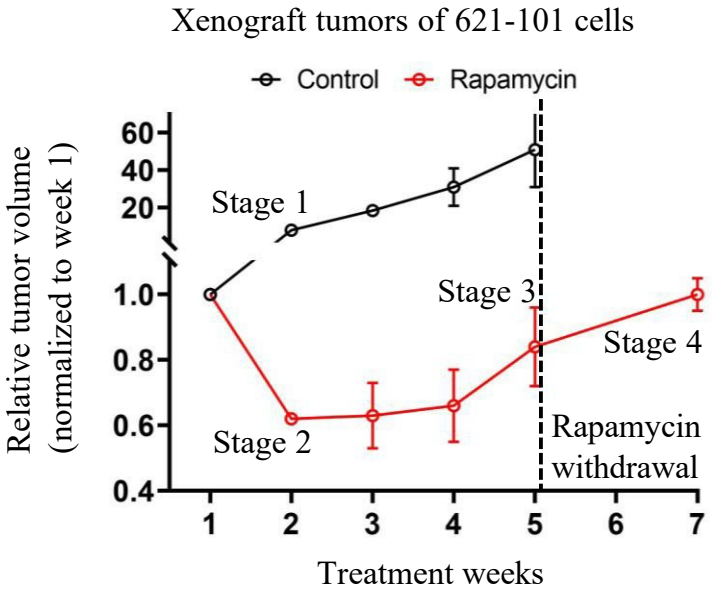
7. Participants & Other Collaborating Organizations

Naim Mahi, Yiyang Lu, Jane Yu, Erik Zhang, Aristotelis Astreinidis, Mario Medvedovic

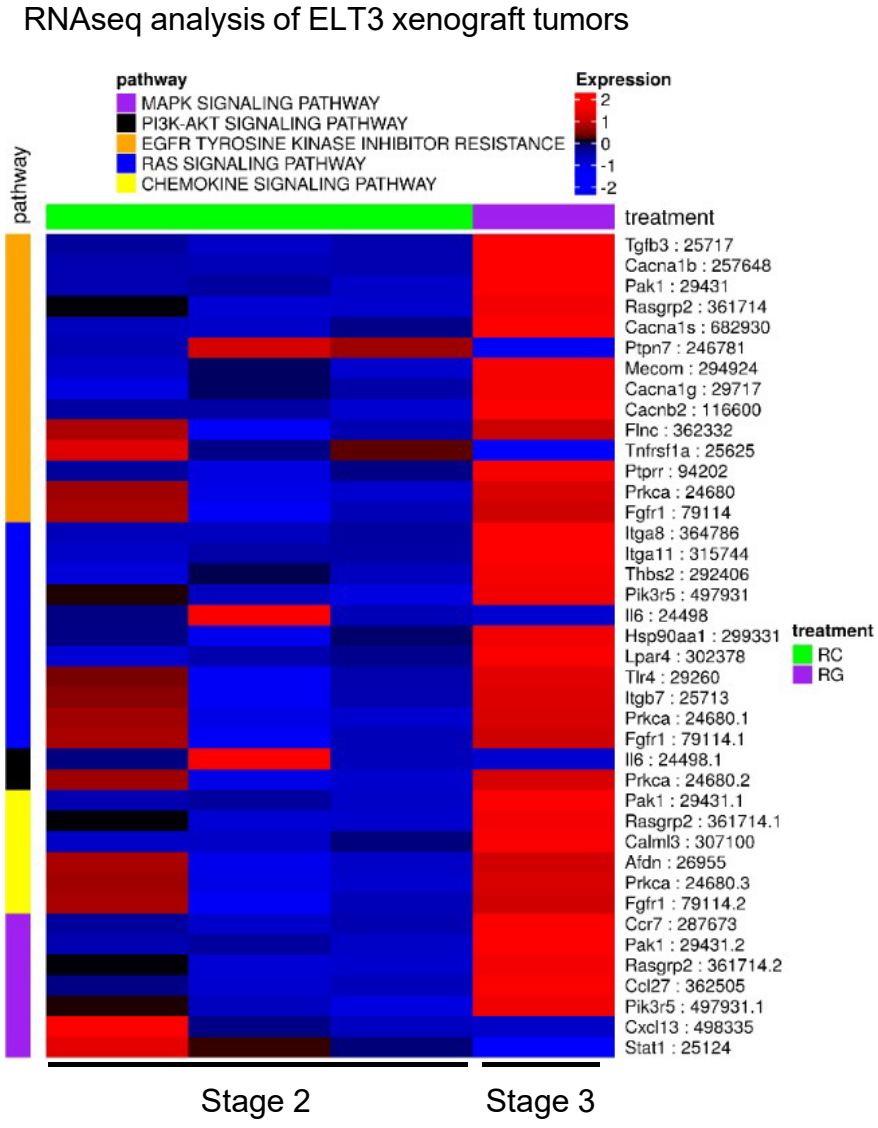
8. Special Reporting Requirements. N/A

9. Appendices.

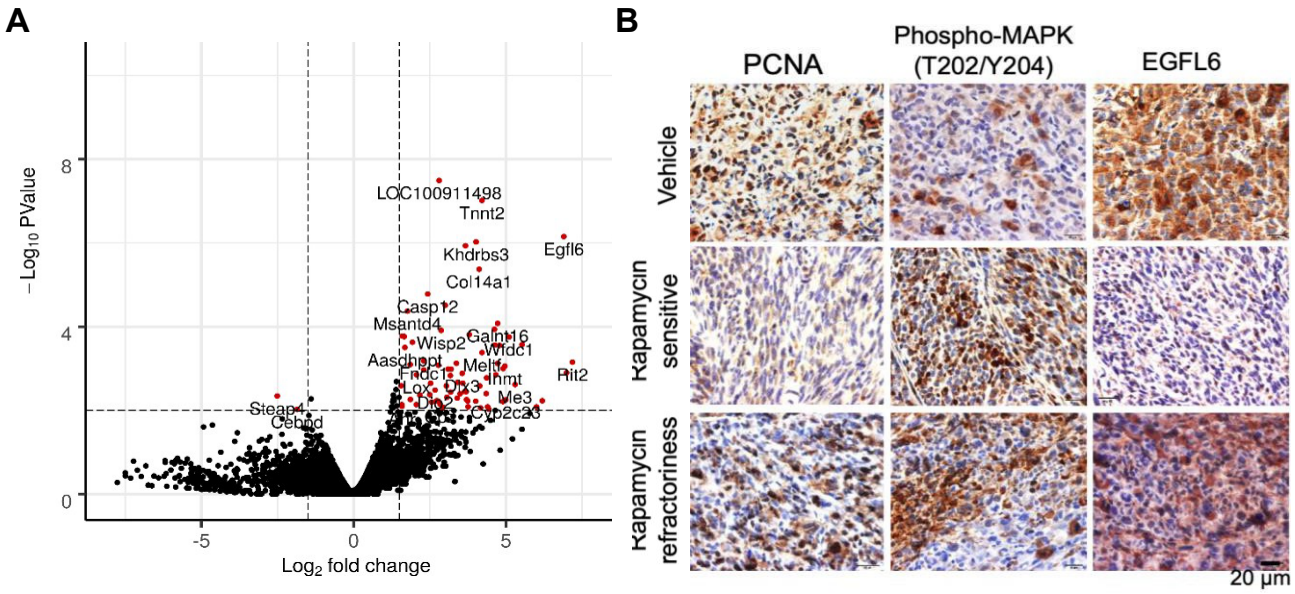
9a. Figure 1. Development of xenograft tumors of 621-101 cells in response to rapamycin treatment. TSC2-null angiomyolipoma-derived 621-101 cells were subcutaneously inoculated into NSG mice. Mice bearing xenograft tumors were treated with rapamycin (2 mg/kg/day, i.p.) for four weeks (Week 1 to 5). Tumor regrowth was observed for two additional weeks (Week 6-7) upon rapamycin withdrawal. Tumor volume was measured using digital calipers and normalized to those prior to rapamycin treatment. Tumor Stage 1=rapamycin shrinking tumor; Tumor Stage 2=rapamycin sensitive shrinking tumor; Tumor stage 3=rapamycin growing tumor; and Tumor Stage 4=rapamycin withdrawn growing tumor.


Xenograft tumors of 621-101 cells
Control
Rapamycin
Relative tumor volume
(normalized to week 1)
60
40
20
1.0
0.8
0.6
0.4
Stage 1
Stage 2
Stage 3
Stage 4
Rapamycin
withdrawal
1
2
3
4
5
6
7
Treatment weeks

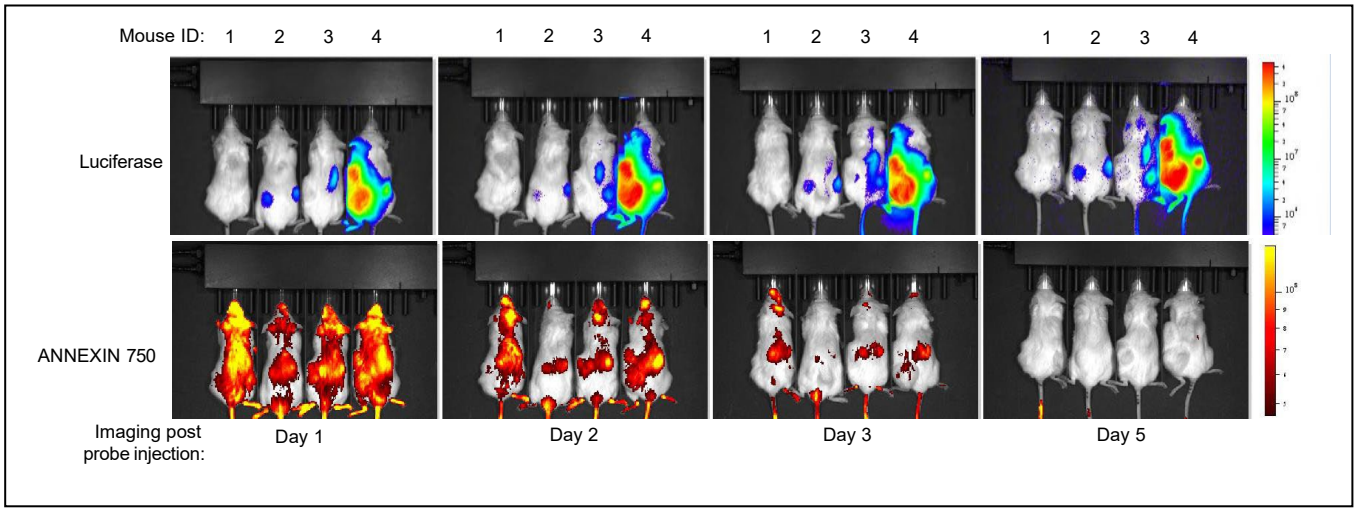
9b. Figure 2. RNAseq analysis of ELT3 xenograft tumors in response to prolonged rapamycin treatment. RNA samples were isolated from xenograft tumors of Tsc2-null ELT3 cells at Tumor Stage-2 rapamycin sensitive and Tumor Stage-3 rapamycin refractory. RNAseq analysis was performed. The Heatmap shows representative genes differentially expressed and enriched in signaling pathways in response to prolonged rapamycin treatment.



9c. Figure 3. EGFL6 expression is prominent in xenograft tumors of ELT3 in response to prolonged rapamycin treatment. (A) RNAseq was performed in xenograft tumors of ELT3 cells. Volcano plot shows significant gene (red, P value<0.01) between rapamycin-sensitive tumors vs rapamycin-refractory tumors. Red dots represent genes whose expression was significantly altered (P <0.01), and black dots represent genes without significant changes. (B) Immunohistochemistry staining of xenograft tumors from vehicle, rapamycin-sensitive and rapamycin-refractory groups. Tumor tissues were stained with antibodies against PCNA, phospho-MAPK (T202/Y204), and EGFL6. Representative images are shown.



9d. Figure 4. Detection of cell proliferation and cell apoptosis in xenograft tumors of LAM patient-derived cells in vivo. Female NSG mice (8 weeks of age) were subcutaneously inoculated with $2x10^6$ 621-101 (ID 1) and 621-101-luciferase (621L9) (ID 2-4). Bioluminescent imaging was performed 12 weeks post cell inoculation (upper panel). Fluorescent imaging was performed within 5 days post probe injection (Day 1, 2, 3 and 5). Annexin V signals were concentrated in focal regions of xenograft tumor by Day 3, indicative of apoptosis in tumor cells (lower panel).



9e. SOW and highlight progress

Specific Aim 1: Determine the molecular basis for rapamycin-induced survival of TSC2-deficient cells in vivo.	**Timeline (Months)**	**Site 1** (PI)
Obtain HRPO and ACURO approval	1-3	Dr. Yu
Major Task 1: Establish xenograft tumors of 621-101 in response to rapamycin.		
Subtask 1: Collect xenograft tumors at 4 tumor stages (Tumor-1 [vehicle], 2 [shrinking], 3 [refractory], & 4 [regrowth]) and characterize these tumors for mTORC1 suppression and growth. (n= 8 per stage, for a total of 32 animals). Step-1: subcutaneously inoculate NOD/SCID mice with 621-101-luciferase expressing cells and monitor xenograft tumor development using bioluminescent imaging. Step-2: When tumors become well established based on stable bioluminescent imaging, drug treatment will be given 5 times/week: 1) rapamycin (2 mg/kg/day, i.p.), and 2) vehicle (0.5% hydroxypropylmethylcellulose, 0.2% Tween 80 in diH2O). Step-3: collect xenograft tumors at 4 tumor stages as described above. Step-4: validate each tumor stage for rapamycin responsiveness using immunohistochemistry of phospho-S6 (S235/236).	4-6	Dr. Yu
Subtask 2: Harvest tumors, isolate RNA, and perform RNAseq.	7-9	UC Core
Subtask 3: Perform RNAseq analysis, data processing, and data mining in collaboration with Dr. Medvedovic.	10-15	Drs. Yu and Medvedovic
Major Task 2: Identify gene expression signature responsible for tumor refractoriness	Months	
Subtask 1: Perform two-step gene selection to identify genes responsible for resistance and regrowth by comparing with ELT3 xenograft data.	16-17	Dr. Yu
Subtask 2: Identify gene sets responsible for rapamycin-resistance and/or tumor refractoriness. Step-1: distinguish gene signatures associated with rapamycin-induced regrowth by comparing transcriptome of Tumor-3 with Tumor-2. Step-2: eliminate common genes to cytostatic tumor-2. Step-3: perform secondary pathway analysis to identify pro-survival and cell death pathways.	18-19	Dr. Yu
Subtask 3: Validate gene expression using real-time quantitative PCR and immunoblot analysis with RNAs and proteins from four tumor stages, as biological triplicates, in each condition.	20-24	Dr. Yu
Milestone #1: Manuscript preparation on rapamycin-induced gene signatures responsible for tumor refractoriness in TSC.		
Specific Aim 2: Determine the effect of pro-survival signaling blockade on rapamycin resistant TSC2-deficient cells in vivo.	Months	
Obtain HRPO and ACURO approval	1-3	Dr. Yu

Major Task 3: Develop mesenchymal tumor xenograft model of patient-associated renal angiomyolipoma-derived cells carrying bi-allelic TSC2 mutations		
Subtask 1: Establish tumors for testing agents in xenograft models. (n=8 per group, for a total of 32 animals)	4-6	Dr. Yu
Subtask 2: When tumors become well established based on stable bioluminescent imaging, drug treatment will be given 5 times/week for four weeks: 3) rapamycin (2 mg/kg/day, i.p.) 4) trametinib (2 mg/kg/day, oral gavage) 5) rapamycin (2 mg/kg/day) plus trametinib (2 mg/kg/day), and 6) vehicle (0.5% hydroxypropylmethylcellulose, 0.2% Tween 80 in diH2O).		
Subtask 2: Test drug effects on tumor progression using bioluminescent imaging.	7-12	Dr. Yu
Major Task 4: Testing the effect of pharmacologic suppression of MAPK activation using MEK1/2 inhibitor trametinib, singly or in combination with the mTORC1 inhibitor rapamycin, on the progression of 621-101 cells using non-invasive bioluminescent imaging technology.	Months	
Subtask 1: Using animals from Major Task 3, test drug treatment effects on 621-101 tumor growth using non-invasive bioluminescent imaging. Efficacy of downregulation of mTORC1 and MEK1/2 will be examined using immunoblotting analyses and immunohistochemistry of phospho-S6 and phospho-MAPK	13-15	Dr. Yu
Subtask 2: Test drug treatment effects on tumor regrowth after drug discontinuation.	16-18	Dr. Yu
Major Task 5: Testing the effect of molecular depletion of pro-survival genes identified in Aim 1 on the progression of 621-101 xenograft tumors	Months	
Subtask 1: Establish stable knockdown of gene of interest in 621-101 cells using shRNA. Stable cell lines will be injected subcutaneously into NOD/SCID mice. (n=8 per shRNA group, two independent shRNAs/gene of interest, two control shRNAs, for a total of 32 animals per gene of interest)	19-21	Dr. Yu
Subtask 2: Examine the effect of depletion of pro-survival genes identified in Aim 1 on the progression of xenograft tumors of 621-101 cells. Step-1: deplete gene "X" using two independent shRNA-X or shRNA-control vector in 621-101 cells. Stable clones will be selected, validated for gene depletion using qPCR and immunoblotting analysis. Step-2: shRNA-X and shRNA-control cells will be subcutaneously inoculated into NOD/SCID mice (n=8 per group, for a total of 32 animals per gene of interest). Step-3: tumor development and progression will be monitored using bioluminescent imaging very two weeks. Bioluminescent intensity in xenograft tumors shRNA-X mice will be compared with shRNA-control mice.	22-24	Dr. Yu
Subtask 3: To assess tumor cell viability, perform noninvasive fluorescent imaging to detect cell proliferation and apoptosis, in vivo and ex vivo. Fluorescence intensity in xenograft tumors from drug-treated mice will be compared with vehicle treatment. Mouse plasma will be prepared and analyzed for levels of rapamycin.	22-24	Dr. Yu

Milestone #2: Manuscript preparation on identification of novel pro- survival regulators induced by rapamycin treatment in TSC		

Publication

1. Yiyang Lu, Erik Zhang, Jie Liu and Jane J. Yu. Inhibition of the mechanistic target of rapamycin induces cell survival via MAPK in tuberous sclerosis complex. ***Orphanet J Rare Dis***. 2020 Aug 17;15(1):209. PMID: 32807195

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