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14. ABSTRACT

This project is aimed to investigate the molecular mechanisms underlying mTORC1-LARP1-mediated ribosome biogenesis and cell growth, and the roles of LARP1 in the development of TSC tumors. During the last funding period, we further investigated the molecular mechanisms by which LARP1 scaffolds mTORC1 to LARP1-interacting mRNAs. Upon LARP1 phosphorylation by Akt and mTORC1, LARP1 dissociates from the 5'UTR of RP mRNAs and strongly interacts with the 3'UTR of these mRNAs with mTORC1 (related to Aim1). We have also initiated the characterizations of podocyte-specific LARP1 knockout mice and the generation of LARP1/TSC1 double knockout mice (related to Aim2). It has been well documented that both hyper- and lack of mTORC1 activation in podocytes cause their injury and glomerular dysfunctions. Our preliminary data suggest that LARP1 is required for normal podocyte and glomerular functions as seem in podocyte-specific Raptor knockout mice. Although the pathological phenotypes in the podo-LARP1 KO mice is weaker than those in podo-Raptor KO mice, the results are consistent with our proposed model that LARP1 is a downstream effector of mTORC1 to stimulate or maintain podocyte growth and function. We will continue to study whether LARP1 plays an important role in aberrant activation of mTORC1-induced podocyte growth and dysfunctions.

15. SUBJECT TERMS
La-related protein 1, mTORC1, TSC complex, ribosome, mRNA, translation, podocyte, glomerular function

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Table of Contents

Page

Technical Report

1. INTRODUCTION

The TSC-mTORC1 signaling pathway plays essential roles in the regulation of cell growth and proliferation. Loss of functional Tuberous Sclerosis Complex (TSC) gene products causes hyperactivation of mTORC1 and causes the development of TSC-associated diseases. Hyper-activation of mTORC1 leads to aberrant ribosome biogenesis by stimulating the translation of an essential class of mRNAs encoding translational machinery such as ribosome proteins, thereby enhancing global protein synthesis. We have identified that La-related protein 1 (LARP1) is a direct substrate of mTORC1 and plays essential roles in stimulating ribosome biogenesis and cell proliferation in response to mTORC1 activation. In this proposal, we will explore the molecular mechanisms underlying mTORC1-LARP1-dependent RP mRNA translation and ribosome biogenesis, and functional importance of LARP1 for aberrant ribosome biogenesis, cell proliferation/growth control in TSC both in vitro and in vivo.

2. KEYWORDS

La-related protein 1, Akt, S6K, mTOR, mTORC1, TSC complex, TSC1, TSC2, ribosomal protein, ribosome, mRNA, translation, podocyte, glomerular function, rapamycin

3. ACCOMPLISHMENTS

A) What were the major goals of the project?
The major goal of this project is to investigate the role of LARP1, a substrate of mTORC1, in the regulation of ribosome biogenesis and protein synthesis in wild-type and TSC cells both in vitro and in vivo.

B) What was accomplished under these goals? Publications supported by this grant are listed.

1) LARP1 functions as a molecular switch for mTORC1-mediated translation of an essential class of mRNAs.
Hong S, Freeberg MA, Han T, Kamath A, Yao Y, Fukuda T, Suzuki T, Kim JK, Inoki K. (2017), *Elife*. pii: e25237 doi: 10.7554/eLife.25237.
2) Evaluating the mTOR Pathway in Physiological and Pharmacological Settings.
Hong S, Inoki K. (2017), *Methods Enzymol*. 587:405-428. doi: 10.1016/bs.mie.2016.09.068.
3) Lysosomal Regulation of mTORC1 by Amino Acids in Mammalian Cells.
Yao Y, Jones E, Inoki K. (2017), *Biomolecules*. pii: e51. doi: 10.3390/biom7030051.
4) Macropinocytosis, mTORC1 and cellular growth control.
Yoshida S, Pacitto R, Inoki K, Swanson J. (2018), *Cell Mol Life Sci.* 75: 1227-1239. doi: 10.1007/s00018-017-2710-y.

1) Major activities
a) Measurement of the rate of ribosome biogenesis in TSC cells by using the established ribosome protein (RP) mRNA reporters.
b) Determination of LARP1 interacting sites on the RP mRNAs by PAR-CLIP analyses.
c) Measurement of cell size of podocytes in podocyte-specific LARP1 KO, TSC1 KO, LARP1/TSC1 double KO mice.
d) Monitoring glomerular histological phenotypes and glomerular function in podocyte-specific LARP1, TSC1, and LARP1/TSC1 KO mice.

2) Specific objective:

Aim 1: Determine the roles of LARP1 in the regulation of mTORC1-mediated ribosome biogenesis and cell growth/proliferation in TSC cells.

Aim 2: Determine the in vivo roles for LARP1 in ribosome biogenesis and cell growth control in a tissue-specific TSC animal model.

3) Significant results:

A. In the previous funding period, we have developed multiple reporter mRNAs to examine the role of LARP1 in the regulation of RP mRNA translation under the supervision of Dr. Kim, an expert in RNA research field. We demonstrated that LARP1 preferentially interacted with both the 5'UTR and 3'UTR of RP mRNA (RpL32) under low mTORC1 activity conditions while it dissociated from the 5'UTR of RP mRNA upon mTORC1 activation. These biochemical observations are consistent with our previous observation seen in the PAR-CLIP analyses (Figure 1). To determine if ribosome biogenesis is indeed enhanced in cells lacking functional TSC complex, we measured rate of ribosome subunit translation using the reporters we developed in TSC1 KO HEK293T or MEF cells. As expected, the rate of RpL32 mRNA translation was significantly increased in both cells lacking TSC1 compared to that in their control cells (Figure 2). To test if mTORC1-dependent LARP1 phosphorylation plays an important role in RpL32 mRNA translation in cells lacking functional TSC complex, we expressed the wild-type and phosphor-defective LARP1 mutant in TSC1 deficient HEK293T cells and found that the rate of RpL32 mRNA translation was significantly decreased in the cells expressing the mutant LARP1 compared to wild-type LARP1 expressing cells (Figure 3). These results suggest that the phosphorylation of LARP1 plays an essential role in RpL32 mRNA translation in TSC cells. To test whether LARP1 positively regulates cell proliferation in TSC1 deficient MEF cells, LARP1 was ablated, and cell proliferation was monitored. As LARP1 showed an essential role in cell proliferation in multiple cancer cells, the ablation of LARP1 also reduced the rate of cell proliferation in cells lacking functional TSC complex. Together these observations suggest that LARP1 plays a key role in the proliferation of cells

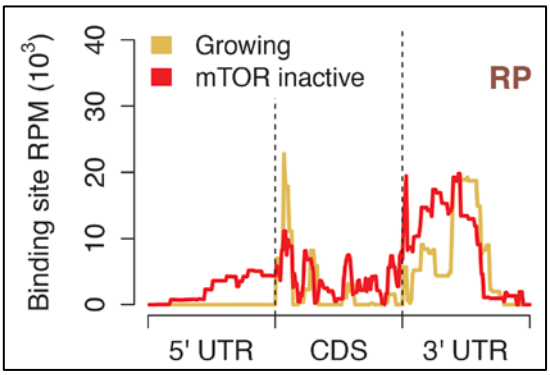

Figure 1. LARP1 binding regions on RP mRNAs (PAR-CLIP). LARP1 mainly interacts with the 3'UTR of RP mRNAs under growing conditions, while it also binds to the 5UTR upon mTORC1 inhibition.

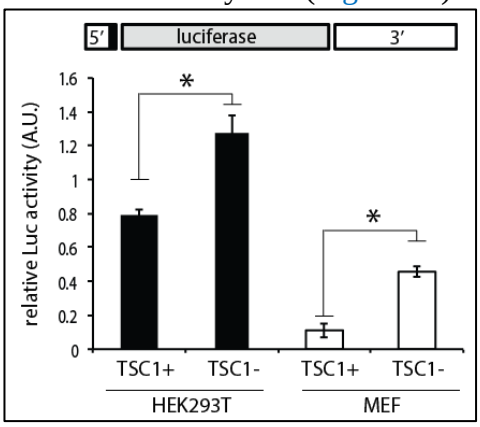

Figure 2. RpL32 mRNA translation in TSC1 deficient cells. Levels of RpL32 mRNA reporter activity were monitored in control and TSC1 deficient cells.

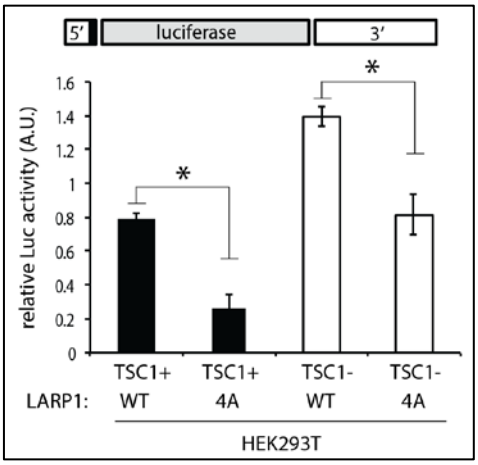

Figure 3. LARP1 4A mutant blocks RpL32 mRNA translation. The effect of wild-type and the 4A LARP1 mutant on RpL32 mRNA reporter activity were monitored in control and TSC1 deficient HEK293T cells.

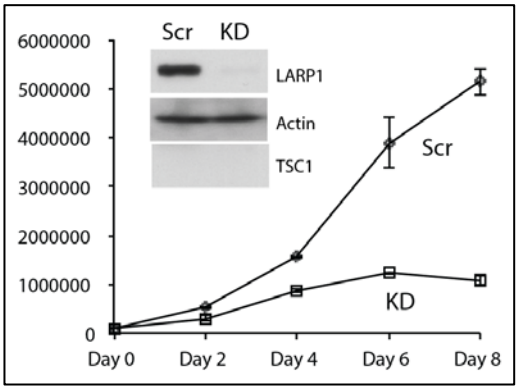

Figure 4. LARP1 knockdown (KD) inhibits cell proliferation in TSC deficient MEF cells. LARP1 was ablated in TSC1 -/- MEF cells and their cell proliferation was monitored. Scr (control shRNA).

lacking the functional TSC complex likely though its positive regulation of ribosome biogenesis under high mTORC1 activity conditions.

B. In the previous reporting period, we generated podocyte-specific LARP1/TSC1 double knockout mice to determine if additional ablation of LARP1 could prevented aberrant cell growth and glomerular dysfunction seen in the podocytes lacking TSC1 expression. Our data demonstrated that levels of proteinuria, a critical indicator of glomerular dysfunction, were not attenuated by the additional ablation of LARP1 in the podocytes of podocyte-specific TSC1 KO mice. Glomerular podocytes are highly differentiated epithelial cells and lack their capacity to proliferate. We have shown that mTORC1 activity in podocytes play a crucial role in their growth. To investigate whether the ablation of LARP1 can attenuate aberrant podocyte cell growth in podocyte-specific TSC1 KO mice, we directly monitor glomerular podocytes in the podocyte-specific TSC1/LARP1 double KO mice. Consistent with the observations that podocyte-specific TSC1/LARP1 DKO mice show sever glomerular dysfunction as seen in the podocyte-specific TSC1 KO mice, the size of podocytes (cell body) in the DKO mice also did not change compared to that in the TSC1 KO mice (Figure 5). These results indicate that although LARP1 exerts a key role in the ribosome biogenesis and cell growth/proliferation in cells lacking functional TSC complex in vitro, it has little role in the hyper-mTORC1-induced cell growth in podocytes in this mouse model. We will further characterize levels of ribosome biogenesis in wild-type, podocyte-specific LARP1 KO, and LARP1/TSC1 DKO by analyzing the amount of ribosome subunits in their glomeruli.

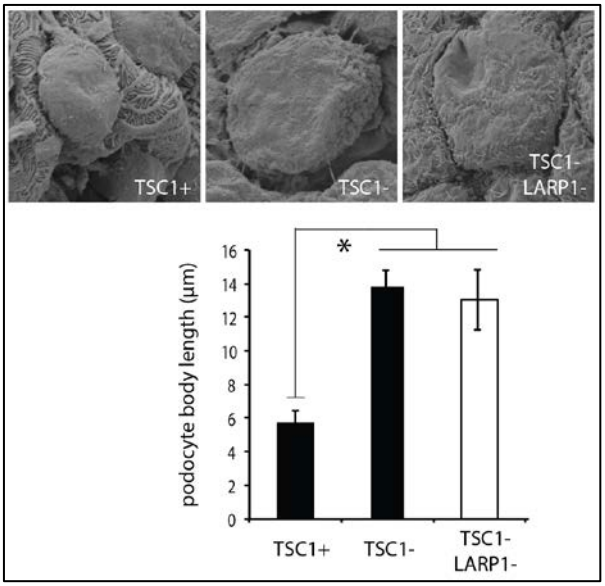

Figure 4. Podocyte cell body size in wild-type, TSC1 KO, and TSC1/LARP1 DKO mice (8 weeks old). The maximum diameter of podocyte cell body in the indicated podocyte-specific KO mice was measured by Scanning electron microscopy.

4) Other achievements:
To understand further regulations and roles of LARP1, we have performed mass spectrometry analyses to identify LARP1 interacting proteins. We have a plan to further study whether these interacting proteins

C) What opportunities for training and professional development has the project provided?
Nothing to report.

D) How were the results disseminated to communities of interest?
At the LARP society meeting, we presented a part of the data generated under this proposal. In the publication, we demonstrated the regulation of LARP1 by mTORC1 activity.

E) What do you plan to do during the next reporting period to accomplish the goals?
For Aim1, we will measure the amount of LARP1 that binds to the 3'UTR of RP mRNAs in cells lacking functional TSC complex. We anticipate that LARP1 more interacts with the 3'UTR of RP mRNAs in TSC deficient cells compared to that in wild-type cells. In Aim2, we will continue to investigate the effect of LARP1 ablation on ribosome biogenesis in podocytes of wild-type, podocyte-specific LARP1 KO, and the LARP1/TSC1 KO mice. If the ablation of LARP1 decreases levels of ribosomal proteins in podocyte-specific LARP1 KO mice compared to the wild-type mice, or in podocyte-specific LARP1/TSC1 DKO mice compared to the TSC1 KO mice, these results would indicate that LARP1 also plays a critical role in ribosome biogenesis in vivo. However, these observations would also suggest that aberrant mTORC1 activation-induced podocyte growth and glomerular dysfunction may not correlate with the number of ribosomes as the lower amount of ribosome in the LARP1/TSC1

KO mice may suffice for inducing cell growth in podocytes under high mTORC1 activation conditions. In contrast to the observations in cell culture system, if the amount of ribosomes does not decrease by ablation of LARP1 in podocytes, these observations strongly suggest that other family of LARP proteins such as LARP1b (LARP2) may compensate a loss of functional LARP1 in podocytes. As we discussed this possibility in the original proposal, we will study the role of LARP1b (LARP2) using both in vitro (podocyte cell culture) and in vivo (mice).

4. IMPACT

A) What was the impact on the development of the principal discipline(s) of the project?
As we described in the previous reports, the completion of this project will provide valuable information about LARP1, an atypical mTORC1 substrate/regulator that stimulates RP mRNA translation in response to mTORC1 activity. It is a consensus that hyperactivation of the mTORC1 pathway exerts pathological roles in the development of TSC diseases. However, the molecular mechanisms underlying the development of TSC diseases by mTORC1 activity remain elusive. We believe that the completion of this project would provide crucial information for understanding the molecular mechanism of TSC cell growth/proliferation and reveal the functional importance of LARP family proteins in cell growth/proliferation control.

B) What was the impact on other disciplines?

Nothing to report.

C) What was the impact on technology transfer?

Nothing to report.

D) What was the impact on society beyond science and technology?
Nothing to report.

5. CHANGES/PROBLEMS

Nothing to report. No significant changes to the aims are envisioned in the next funding year.

6. PRODUCTS

Other Products:
We have developed LARP1 flox and TSC1/LARP1 double flox animals.
We have generated reporter mRNA systems related to RpL32 mRNAs.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

Name	Ken Inoki
Project Role	PI
Researcher Identifier	
Month worked	2.4
Contribution to project	Dr. Inoki is responsible for in vivo experiments and all the design and analyses.
Funding Support	NIH RO1
Name	Sungki Hong
Project Role	Research Assistant Scientist
Researcher Identifier	
Month worked	2.4
Contribution to project	Dr. Hong is responsible for in vitro experiments using cultured cells.
Funding Support	NIH RO1
Name	Junying Wang
Project Role	Research technician
Researcher Identifier	
Month worked	1.2
Contribution to project	Mrs. Wang is responsible for in vivo experiments using animal models.
Funding Support	NIH RO1
Name	John Kim
Project Role	Collaborator
Researcher Identifier	
Month worked	1.2
Contribution to project	Dr. Kim is responsible for the designs and analyses of mRNA experiments.
Funding Support	NIH RO1

A) 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 regarding a change of support.

B) What other organizations were involved as partners?

No other organizations were involved.

8. SPECIAL REPORTING REQUIRMENTS

Nothing to report.

9. APPNDICES

Nothing to report.