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CONTRACTING ORGANIZATION: University of Illinois at Chicago
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14. ABSTRACT Breast cancer is a complex disease that is dependent on both genetic and environmental factors. Although the genetic factors have been explored for a long time, the nutritional dependence, in particular the effect of the gut microbiome in the development and prevention of breast cancer is still poorly understood. The metabolism of gut microbiome releases contents from microbial cells that may affect many aspects of breast cancer. One chemical released by the gut bacteria is the micronutrient queuine derived from a de novo synthesized metabolite in bacteria; it is taken up through the intestine and circulated in the blood. All human cells then use queuine and incorporate it into the wobble anticodon position of specific transfer RNAs (tRNA) by two protein enzymes encoded in our genome. We are working on elucidating the function and importance of dynamic queuine-dependent tRNA modifications and tRNA biology on breast cancer growth, prevention, and biomarker determination using both cultured cells and animal models. The ultimate goal is to understand the molecular, cellular and organismal mechanisms of this microbiome-dependent micronutrient, and to derive countermeasures for breast cancer prevention, diagnosis and therapy.					
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1. INTRODUCTION:

tRNAs are small non-coding RNAs that read the genetic code and provide amino acids to growing polypeptide chains during translation. tRNAs are essential for the proliferation, fitness, and adaptation of the cell. Emerging roles of tRNA in many human diseases have been implicated through genetic mutations, post-transcriptional modifications, overexpression and RNA-protein interactions. We were the first to show that breast cancer cells and tumors overexpress tRNA at high levels compared to non-tumorigenic cells and breast tissues (1). tRNA derived small RNA fragments (tRFs) have been shown to be associated with breast cancer in the extracellular vehicles and in the blood, indicating that tRFs may be useful as biomarkers for breast cancer progression and treatment (2-5). Specific tRFs have been shown to strongly associate with breast cancer metastasis (2), and cell proliferation in estrogen-receptor positive (ER+) breast cancer cells (3), indicating that tRFs can be a strong driver for breast cancer development and metastasis. Both tRNA and tRF function in cells, and in turn their effect on breast cancer tumor development and progression may be modulated by a microbiome-dependent, queuosine (Q)-tRNA modifications. We aim to (i) identify the mechanisms of tRNA Q-modification dependent cellular phenotypes and on gene expression; (ii) examine microbiome dependency and tRNA Q-modification on breast tumor growth and biomarker potential.

2. KEYWORDS:

tRNA modification, queuosine, microbiome, breast cancer.

3. ACCOMPLISHMENTS:

(a) What were the major goals of the project?

Task 1: Confirm that tRNA Q-modification has cellular and molecular phenotypes in two breast cancer cell lines. Characterize the molecular mechanisms of tRNA Q-modification.

Month 1-36: 67% completed.

Task 1 is to be performed at the University of Chicago.

Task 2: Examine the relationship of tRNA Q-modifications, microbiome and breast cancer in mice.

Month 1-36: 67% completed.

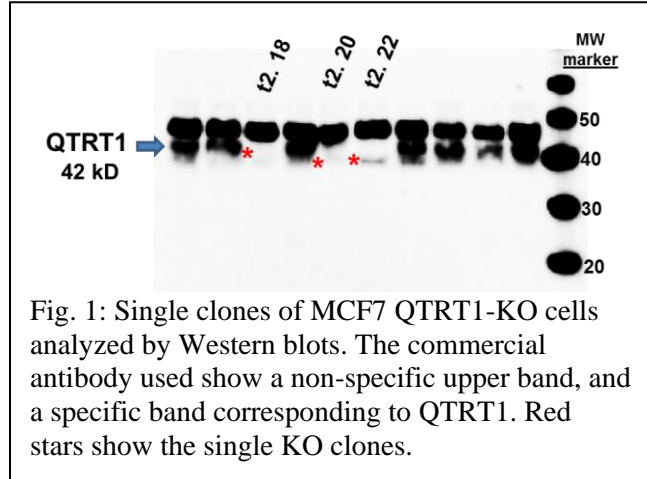
Task 2 is to be performed at the University of Illinois at Chicago.

(b) What was accomplished under these goals?

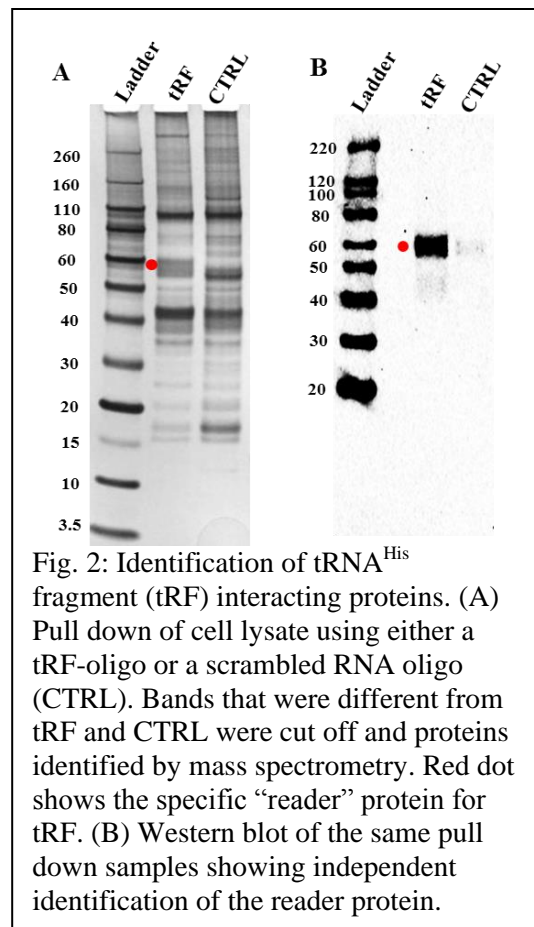
At U. Chicago: In the last report from February 2018, we generated tRNA Q-modification free (0Q) breast cancer cell lines MCF7 and MDA-MB-231, and the non-tumorigenic control cell line of MCF10. We tested the phenotype of cell proliferation rate of 0Q versus 100Q cells. For MDA-MB-231 and MCF10 cells, no difference of proliferation was observed. For MCF7 cells, however, proliferation differences were readily observed, and 0Q cells have a faster proliferation rate both under normoxic and hypoxic conditions.

For xenograft tumor studies, however, we cannot use the same set up of 0Q/100Q cells as the animals cannot be maintained at these 0Q/100Q conditions. We therefore focused on generating the Crispr/Cas9 knockout MCF7 and MDA-MB-231 cells for the two genes that form the enzyme complex required for Q-modifications, QTRT1 and QTRTD1. We found that in contrast to HEK293T cells that we have routinely used for Crispr/Cas9 KO genes in our lab, it

was much more difficult to generate MCF7 and MDA-MB-231 KO cells. After considerable efforts, we were able to produce single clones of complete QTRT1-KO in MCF7 cells (Fig. 1), and partial QTRT1-KO in MDA-MB-231 cells (data not shown). These clones were then sent to Dr. Sun's lab at UIC for *in vivo* mouse tumor studies described below. For QTRTD1-KO, we obtained a mixture of MCF7 and MDA-MB-231 KO cells from a commercial source which were also sent to Dr. Sun's lab for future mouse tumor studies.



At the mechanistic front, we published a paper on Q-modification protecting its cognate tRNAs from ribonuclease cleavage, thus affecting the small RNA pools in cells (6). For breast tumor cells, we focused on differential tRNA fragment generation in 0Q and 100Q MCF7 cells in the previous February 2018 report which were shown to have markedly different cell proliferation rates. This difference could be restored by transfecting a single tRNA^{His} fragment whose generation depends on the Q-modification. Since then we identified a specific “reader” protein that binds to this specific tRNA^{His} fragment (Fig. 2). This reader protein is part of a cellular complex that performs two different functions in (i) catalyzing ribosomal RNA pseudouridine modification and (ii) assisting telomere maintenance, both are crucial for tumor development and progression. We are currently investigating the cellular effects of this tRNA fragment-reader protein interaction to reveal the molecular mechanism of Q-tRNA modification function.

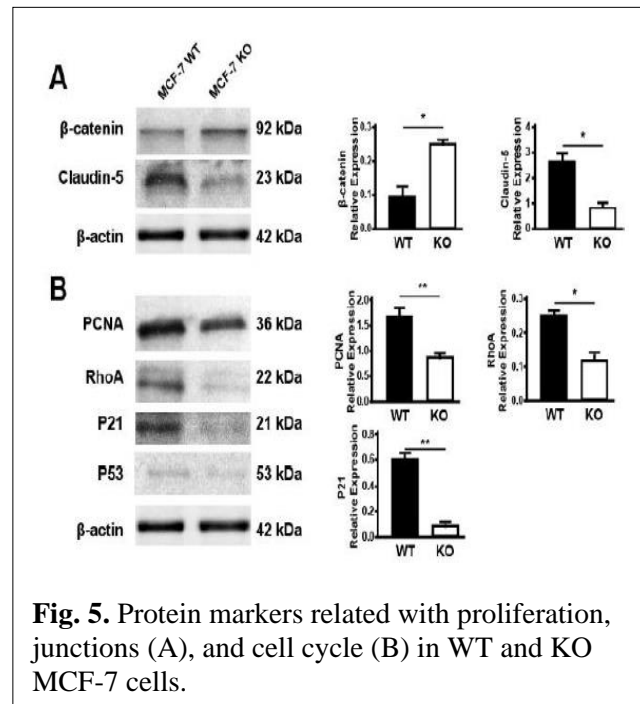
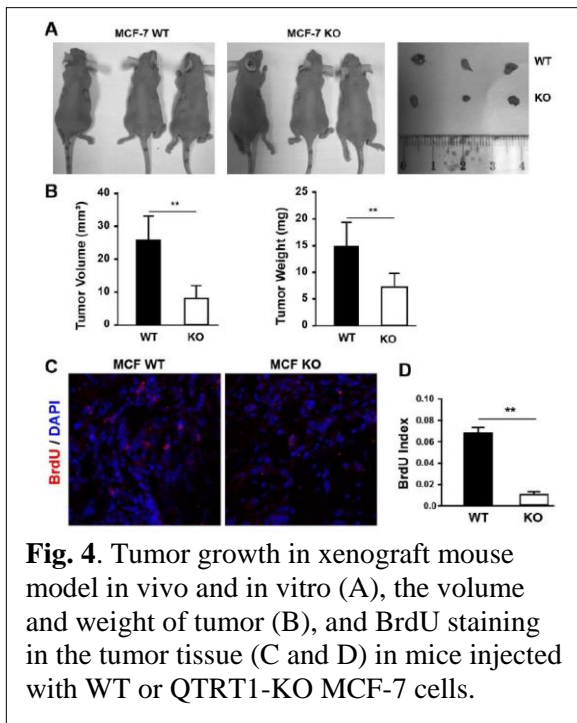
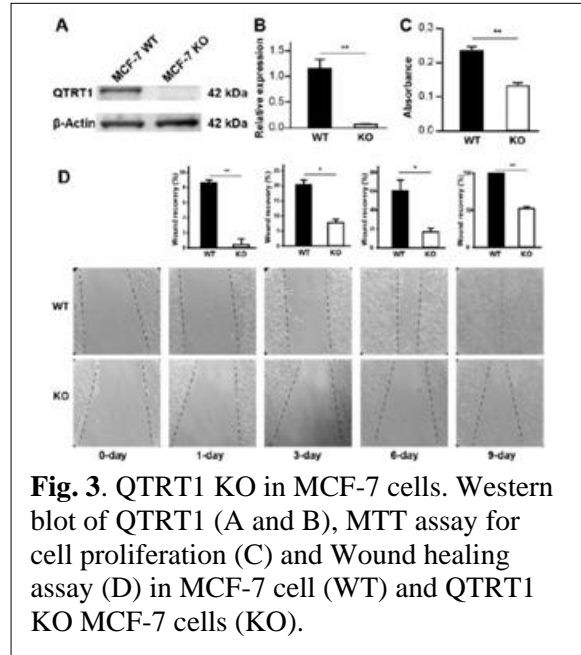


At UIC: we are working on the Subtask 2: cellular effects of QTRT1 KO cells *in vivo*. We used the QTRT1 gene knockout MCF7 cells. First, we tested the protein levels of QTRT1 in the KO cells (Fig. 3A and B). Then, we measured the cell proliferation by MMT and wound healing in the cells to demonstrate that deletion of QTRT1 significantly reduced the cell proliferation and migration ability (Fig. 3C and D).

We then developed a xenograft tumor model and tested tumor growth and cellular effects of QTRT1 KO cells *in vivo*. In the xenograft models, we characterized the changes of tumor growth in breast cancer cells with deficiency in tRNA Q-modification (Fig. 4). Our data showed less tumors in the mice injected with the KO cells (Fig. 4A-C).

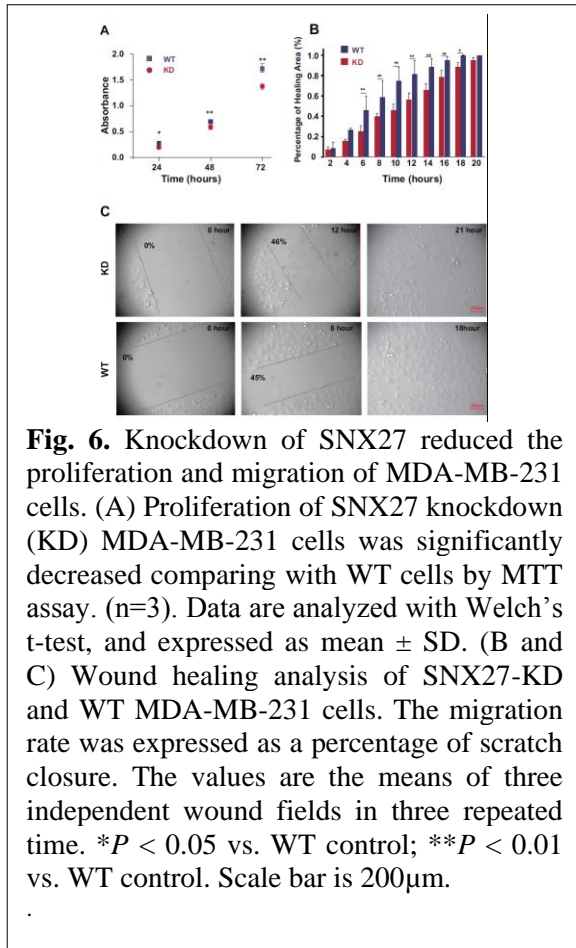
We further tested the biological response of tRNA Q-modifications and their correlation with breast tumor growth, tumor size, and proliferation. We showed that QTRT1 KO cells had reduced BrdU staining, suggesting less cell proliferation (Fig. 4D). At the protein level, we investigated the expression of several key regulators in cell proliferation and junctions. Our data showed that increased beta-catenin and reduced Claudin 5 in the KO cells (Fig. 5A).

Furthermore, we found PCNA, the proliferation marker was reduced in the KO cells. RhoA, the

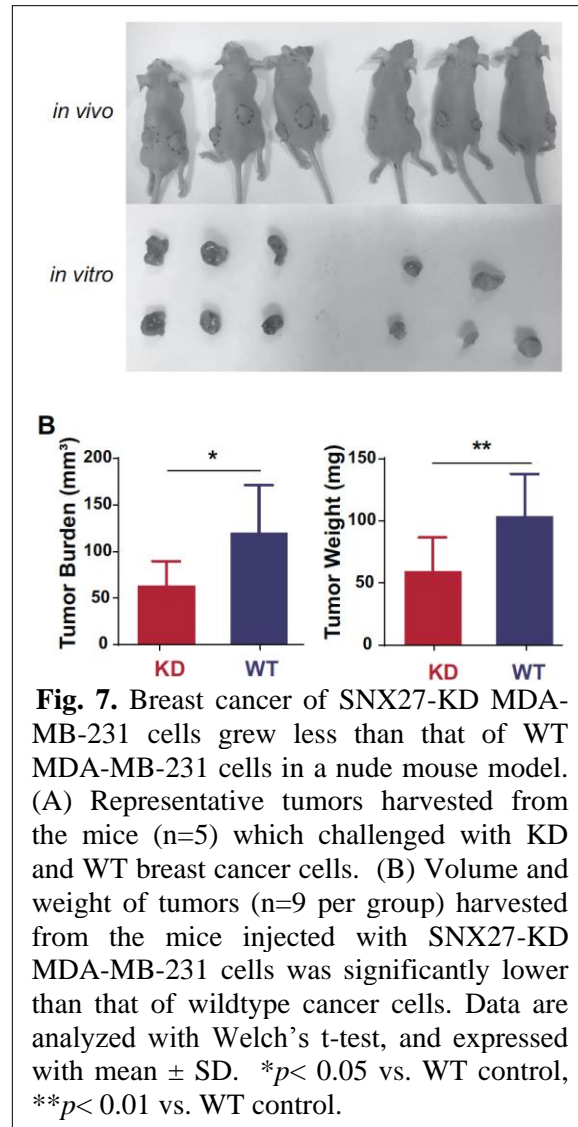


upstream regulator of junction, was also decreased due to the QTRT1 KO. In the meantime, we showed the reduction of P21 and P53, both are critical for cell cycle (Fig. 5B).

We have collected blood, feces and tissue samples. Dr. Pan's lab is testing the changes of Q modification levels in these tumors.



One of the interesting findings was that QTRT1 or QTRTD1 KO cells led to reduction of SNX27, a critical sortin nexin protein mediating protein-protein interaction in intracellular trafficking, membrane remodeling, organelle motility, and tight junctions. We further knocked down the SNX 27 in the highly aggressive breast cancer MDA-MB-231 Cells. KD of SNX 27 suppressed proliferation and migration *in vitro* (Fig. 6). The WT MDA-MB-231 cancer cells recovered ~50% of the wound area in 8 hours and healed the whole area in 18 hours, but KD cells took 3 hours longer than WT cancer cells to cover the whole wound area. Meanwhile, the percentages of healing area were different with different time points. *In vivo* deletion of SNX27 also reduced the tumor growth (Fig. 7). Our study demonstrated a novel function of QTRT1 in regulating the downstream target SNX27 in breast cancer growth.



In Subtask 3, we propose to study VDR^{lox} and $VDR^{\Delta IEC}$ mice in a DMBA-induced breast cancer model.

- (i) We have developed the DMBA tumor models (Fig. 8).
- (ii) We have collected blood, fecal and tissue samples for the first set of experiments. We will repeat our studies and add more animals in each group.
- (iii) We are performing pathological and cellular analysis of the tumor samples, including apoptosis and proliferation, ROS and ATP synthase activity.

We determined the change of microbiome *in vivo*. By analyzing the 16S profile from fecal samples, we showed that intestinal epithelial VDR status changed the profile of microbiome (Fig. 9). Lacking VDR increased the trend of cancers and cell division (Fig. 10). Using the novel 3D organoid culture system developed from VDR^{lox} and $VDR^{\Delta IEC}$ mice, we also demonstrated that the regulatory role of VDR on apoptosis/autophagy balance.

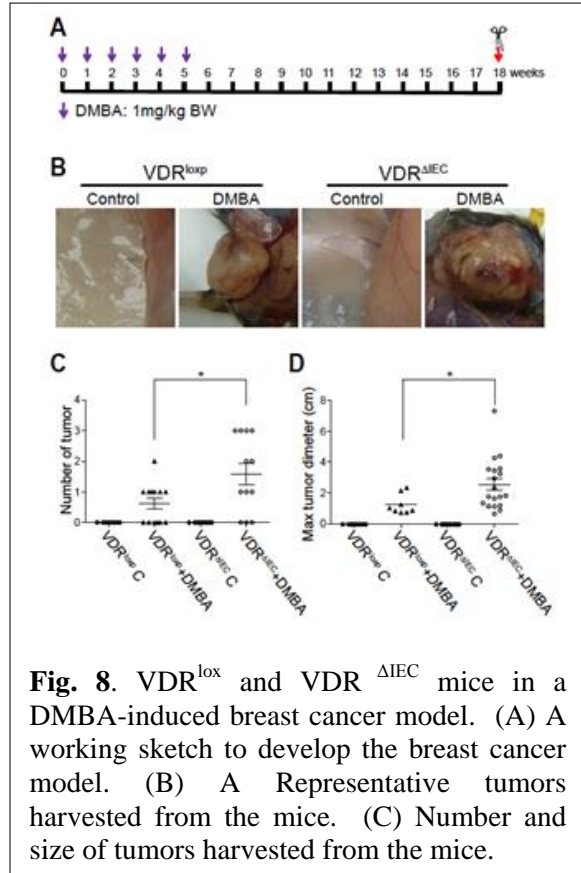


Fig. 8. VDR^{lox} and $VDR^{\Delta IEC}$ mice in a DMBA-induced breast cancer model. (A) A working sketch to develop the breast cancer model. (B) A Representative tumors harvested from the mice. (C) Number and size of tumors harvested from the mice.

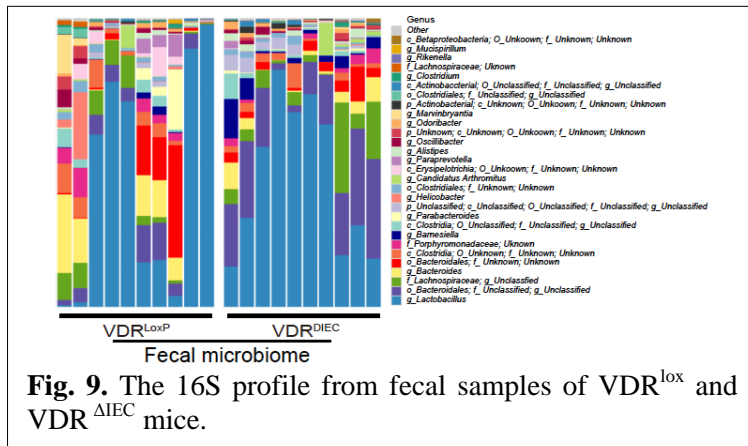


Fig. 9. The 16S profile from fecal samples of VDR^{lox} and $VDR^{\Delta IEC}$ mice.

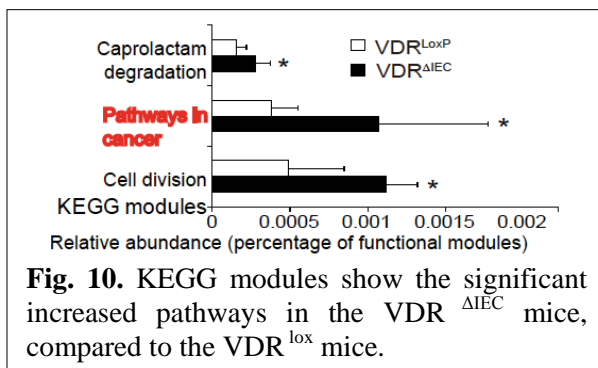


Fig. 10. KEGG modules show the significant increased pathways in the $VDR^{\Delta IEC}$ mice, compared to the VDR^{lox} mice.

Loss of this balance leads to hyperproliferation and high risk of cancer (data not shown).

In summary, tRNA Q-modifications occur specifically in 4 cellular tRNAs at the position that is directly involved in decoding of the genetic information. tRNA Q-modifications also affect the cellular small RNA pools that affect cell physiological through RNA-protein interactions. Our results have

demonstrated the cellular and molecular phenotypes of altered tRNA Q-modifications *in vivo* and *in vitro*. We identify the functions of tRNA Q-modification in the regulation of gene expression critical in cell proliferation and migration in human breast cancer cells and in the breast tumor mouse models.

(c) What opportunities for training and professional development has the project provided?

Personnel involved in the year 2 of this project include:

Xiaoyun Wang, Ph.D.: Dr. Wang has been working on tRNA biology for several years. He produced some of the preliminary results for the application of this proposal. His continued involvement in this project has been crucial for his professional development to become a principal investigator on cancer research. These include proposal writing, hypothesis derivation and testing, and technical skills, especially in the area of microbiome studies.

Zaneta Matuszek: Ms. Matuszek was a post baccalaureate technician in the Pan lab until July 2018 when she left the lab to attend graduate school. She generated all 0Q and 100Q cells for this project, performed functional assays, and generated QTRT1-KO MCF7 cells for the mouse study. Her involvement in this project has been crucial for her professional development to become a graduate student in cancer research. These include hypothesis derivation, testing, and technical skills.

Wen Zhang: Mr. Zhang is a graduate student who has been working on the molecular and cellular mechanistic studies of Q-modification. He has identified the “reader” protein for the Q-modified dependent tRNA^{His} fragments, and is continuing to delineate the mechanism of this RNA-protein interaction on cell physiology. His involvement in this project has been crucial for his professional development to become a research scientist in cancer research. These include hypothesis derivation, testing the hypothesis, and other technical skills.

Yong-guo Zhang: Dr. Zhang, Research Assistant Professor, is a well-trained Microbiologist and cell biologist with significant experience in cell culture and animal models. He has worked on the VDR regulation of intestinal inflammation since he joined Sun lab at the University of Rochester in 2008. He is involved in the organoid culture and all aspects of animal models for Western blot, PCR, and immunohistochemical (IHC) work in the proposal. He helped with getting the animal protocol approved. The skills for lab management, proposal writing, hypothesis derivation and testing, and technical skills, are critical for his professional development, especially in the area of microbiome studies. He is recognized for his outstanding research with an Early Career GI Researcher Award. He is awarded a travel Award to present his study on VDR and microbiome in liver-gut axis in the 2018 James W. Freston Single Topic Conference, *Obesity and Metabolic Disease*, AGA. He is planning a NIH R21 as PI in the coming months.

Rong Lu: Dr. Lu is an Instructor in Dr. Sun’s laboratory. He has had training in animal experimental models, cancer research, bioinformatics, and molecular biology. He is helping with organoids and breeding the mice and establishing the breast cancer models. In 2017, he has established the 3D organoid culture system from VDR and VDR^{ΔIEC} mice. He will orally presented his studies using the organoids from VDR and VDR^{ΔIEC} mice at the Digestive Diseases Week 2019. He is recognized for his outstanding research with an Early Career GI Researcher Award.

Jilei Zhang: Dr. Zhang is a postdoctoral fellow joined Dr. Sun’s lab at UIC in 2018. He has contributed to testing Cellular effects of QTRT1 and/or QTRTD1 KO cells *in vivo* for the

xenograft models. He also contributes to the novel role of SNX27 in breast cancer. A manuscript is under minor revision in the *BMC Cancer*.

(d) How were the results disseminated to communities of interest?

We published a paper on Q-modification effect on tRNA fragment generation and cellular small RNA pool (*RNA* 24, 1305-1313, 2018).

(e) What do you plan to do during the next reporting period to accomplish the goals?

Since our new results on breast cancer cells indicate a new hypothesis on the tRNA Q-modification effects, we will continue to perform studies to elucidate the biological mechanisms on tRNA Q-modifications on breast cancer in cell cultures. These will include characterization of Q-modification dependent RNA-protein interaction on the cellular pathways of ribosome assembly, maturation, and efficiency in translation, and of telomere maintenance.

We will also analyze the tumor samples already collected in the mouse studies on Q-modification levels and the expression levels of selected mRNA genes.

The ongoing experiments include breeding more VDR^{ΔIEC} mice for co-housing of VDR and VDR^{ΔIEC} mice in the DMBA-induced breast cancer model, testing microbiota isolated from mice, and further testing tumors and Cellular effects of QTRT1 and/or QTRTD1 KO cells *in vivo* for the xenograft models.

We will continue pathological and cellular analysis of the tumor samples, including apoptosis and proliferation, ROS and ATP synthase activity (proposed in Subtask 3). We will determine the change of microbiome and Q-modification *in vivo* (proposed in Subtask 4). We will work on Subtask 5: molecular mechanisms and potential biomarkers: (i) Determine the change of Q-modification on tRNA fragment formation (precise and quantitative tRF-seq using our DM-tRNA-seq method) *in vivo*; and (ii) Dissect the molecular mechanisms of VDR regulation of microbiome, Q-tRNA modifications and cell proliferation in tumorigenesis.

4. IMPACT:

(a) What was the impact on the development of the principal discipline(s) of the project?

A major impact of our project would be to associate breast cancer tumorigenesis with gut microbiome-dependent chemical modifications in a major cellular class of RNA. tRNA Q-modifications occur specifically in 4 cellular tRNAs at the position that is directly involved in decoding of the genetic information. Q-modification also affect cell physiological through affecting small RNA-protein interactions. Our results would identify the function of tRNA Q-modifications in the regulation of gene expression in human breast cancer cells and in the breast tumor mouse models. Our results would also identify the potential of using tRNA Q-modified dependent gene expression in breast tumors as biomarkers, and of targeting these specific genes as a new route for breast cancer treatment. Ultimately, we aim to demonstrate the suitability of gut microbial dependent activities as a new route for the diagnosis and treatment of breast tumors.

(b) What was the impact on other disciplines?

Gut microbes have become increasingly important in the association of diet, life styles, and cancer biology. Although we are focusing on specific tRNA modifications, our studies fall into the overall umbrella of microbiome-host interactions which currently are still poorly

understood at the cellular and molecular levels. Therefore, our results could have broad implications beyond the breast cancer studies proposed here.

(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:

(a) Changes in approach and reasons for change.

Nothing to report.

(b) Actual or anticipated problems or delays and actions or plans to resolve them.

We made significant progress in generating the specific Crispr/Cas9 QTRT1 breast tumor cells of MCF7 and MDA-MB-231. As reported here, these cells are being used for in vivo breast tumor studies to enable our proposed studies to move forward.

(c) Changes that had a significant impact on expenditures.

We proposed 300 mice in the 3-year studies. The cost for animal studies has been higher than our budget due to 1) the increased cost of mouse purchasing and 2) low breeding successful rate of the VDR^{ΔIEC} mice. We have to spend more money to breed the mice.

(d) Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report.

6. PRODUCTS:

(a) Publications, conference papers, and presentations.

Wang, X-W., Z. Matuszek, Y. Huang, M. Parisien, Q. Dai, W.C. Clark, M.H. Schwartz, T. Pan: Queuosine modification protects cognate tRNAs against ribonuclease cleavage, *RNA* 24, 1305-1313 (2018).

Presentations

- Chicago Cancer Biology Retreat, Oct. 15, 2018

Jilei Zhang, Kendy Li, Yongguo Zhang, Rong Lu, Shaoping Wu, Jingrong Tang, Yinglin Xia, **Jun Sun**. Deletion of Sorting Nexin 27 Suppresses Proliferation and Migration in Highly Aggressive Breast Cancer Cells in vitro and in vivo.

- University of Chicago, Molecular Biosciences Retreat, November 9-10, 2018.

Wang, X-W., Z. Matuszek, Y. Huang, M. Parisien, Q. Dai, W.C. Clark, M.H. Schwartz, **Tao Pan**: Queuosine modification protects cognate tRNAs against ribonuclease cleavage.

- 2019 11th AACR-JCA Joint Conference on Breakthroughs in Cancer Research: Biology to Precision, Maui, HI, Feb. 9-12, 2019.

Jilei Zhang, Kendy Li, Yongguo Zhang, Rong Lu, Shaoping Wu, Jingrong Tang, Yinglin Xia, **Jun Sun**. Deletion of Sorting Nexin 27 Suppresses Proliferation and Migration in Highly Aggressive Breast Cancer Cells in vitro and in vivo.

(b) Website(s) or other Internet site(s).

Nothing to report.

(c) Technologies or techniques.

Nothing to report.

(d) Inventions, patent applications, and/or licenses.

Nothing to report.

(e) Other Products.

We generated Crispr/Cas9 KO cell lines for research use including MDA-MB-231 and MCF7.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

(a) What individuals have worked on the project?

Name:	<i>Xiaoyun Wang</i>
Project Role:	<i>Instructor</i>
Researcher Identifier (e.g. ORCID ID):	<i>xwang13</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Wang worked on the high throughput sequencing aspects of the project.</i>
Funding Support:	<i>NIDDK (12 months)</i>
Name:	<i>Zaneta Matuszek</i>
Project Role:	<i>Technician</i>
Researcher Identifier (e.g. ORCID ID):	<i>zmatuszek</i>
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>Ms. Matuszek worked on the cell and molecular biology aspects of the project. She left the lab to attend graduate school.</i>
Funding Support:	<i>This grant (6 months)</i>

Name:	<i>Wen Zhang</i>
Project Role:	<i>Graduate student</i>
Researcher Identifier (e.g. ORCID ID):	<i>article</i>
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>Mr. Zhang worked on the cell and molecular biology aspects of the project.</i>
Funding Support:	<i>This grant (6 months), NIGMS (6 months)</i>

At UIC

Name:	<i>Rong Lu</i>
Project Role:	<i>Instructor</i>
Researcher Identifier (e.g. ORCID ID):	<i>ORCID ID 0000-0003-4564-8890</i>
Nearest person month worked:	<i>9.6</i>
Contribution to Project:	<i>Dr. Lu worked on animal models of the project.</i>
Funding Support:	<i>This grant (9.6 months), NIDDK (2.4 months)</i>
Name:	<i>Yongguo Zhang</i>
Project Role:	<i>Research Assistant professor</i>
Researcher Identifier (e.g. ORCID ID):	<i>ORCID ID 0000-0002-8299-0233</i>
Nearest person month worked:	<i>1.2</i>
Contribution to Project:	<i>Dr. Zhang worked on the cell and molecular biology aspects of the project in cells and animal models.</i>
Funding Support:	<i>This grant (1.2 months), NIDDK (3.6 months), UIC Cancer grant (7.2 months)</i>
Name:	<i>Jilei Zhang</i>
Project Role:	<i>Research Associate/Postdoc. fellow</i>
Researcher Identifier (e.g. ORCID ID):	<i>ORCID ID: 0000-0002-9589-1162</i>
Nearest person month worked:	<i>3.6</i>

Contribution to Project:	<i>Dr. Zhang worked on the cell and molecular biology aspects of the project in cells and animal models.</i>
Funding Support:	<i>This grant (3.6 months), Jun Sun's UIC start-up (8.4 months).</i>

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

Jun Sun and Tao Pan have updated other support that do not overlap with this project.

(c) What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS:

(a) Collaborative awards

We submit duplicative reports.

Task 1 is to be performed at the University of Chicago.

Task 2 is to be performed at the University of Illinois at Chicago.

(b) Quad charts

N/A

9. APPENDICES:

References

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6. Wang, X., Matuszek, Z., Huang, Y., Parisien, M., Dai, Q., Clark, W., Schwartz, M.H. and Pan, T. (2018) Queuosine modification protects cognate tRNAs against ribonuclease cleavage. *RNA*, **24**, 1305-1313.