AWARD NUMBER: W81XWH-19-1-0031

TITLE: Notch3 as a tumor suppressor in the postpartum mammary gland

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CONTRACTING ORGANIZATION: University of Mississippi Medical Center

REPORT DATE: MAY 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MAY 2020	Annual	04/01/2019-03/31/2020

4. TITLE AND SUBTITLE

Notch3 as a Tumor Suppressor in the Postpartum Mammary Gland

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0031

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to determine the functions of Notch3 in the pathogenesis of postpartum breast cancer, with a focus on the regulation of brown adipose tissue repopulation in the postpartum mammary gland and its potential role in modulating tumor microenvironment, as well as the regulation of parity-induced mammary epithelial cells in the postpartum mammary gland. To our surprise, we found that the post-lactational involuting mammary microenvironment of Notch3 knockout mice suppressed tumor growth and metastasis. Moreover, the nonparous mammary microenvironment of Notch3 knockout mice showed a similar tumor-suppressive effect. We are currently determining whether Notch3 functions in the mammary epithelium or the stroma in this context. Our preliminary results suggest that Notch3 may regulate tumor-infiltrating lymphocytes.

15. SUBJECT TERMS

Postpartum breast cancer, Tumor microenvironment, Brown adipocyte differentiation, Parity-induced mammary epithelial cells, Notch signaling

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

We propose to test the hypothesis that Notch3 functions as a tumor suppressor in the postpartum mammary gland by promoting brown adipocyte differentiation during post-lactational involution, which may ameliorate the pro-tumorigenic inflammatory microenvironment, and by restricting mammary stem/progenitor cell proliferation in the postpartum mammary gland. One objective is to determine the mechanisms underlying brown adipose tissue repopulation in the postpartum mammary gland as well as its potential role in modulating postpartum mammary microenvironment, which may link obesity-associated metabolic changes to the progression of postpartum breast cancer. The specific aims of this project are (1) to determine whether the post-lactational involuting mammary microenvironment of Notch3 knockout mice accelerates tumor growth and metastasis compared to that of wild-type mice and whether Notch3 functions in the mammary epithelium or the stroma in this context, (2) to determine how Notch3 regulates brown adipocyte differentiation during involution and whether brown adipocytes have an impact on the postpartum mammary microenvironment, and (3) to determine the regulation of parity-induced mammary epithelial cells or other stem/progenitor populations by Notch3 in the postpartum mammary gland.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Postpartum breast cancer, Tumor microenvironment, Brown adipocyte differentiation, Parity-induced mammary epithelial cells, Notch signaling, Tumor-infiltrating lymphocytes

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Task 1: Determine whether post-lactational involuting mammary microenvironment of Notch3 knockout mice accelerates tumor growth and metastasis compared to that of wildtype mice
Milestone/target date: 01/31/2020
Actual completion date: 12/01/2019

Major Task 2: Determine the compartment in which Notch3 regulates pro-tumorigenic mammary microenvironment.
Milestone/target date: 09/30/2020
Ongoing (60% completion)

Major Task 3: Determine the mechanisms by which Notch3 regulates brown adipocyte differentiation in the involuting mammary gland
Milestone/target date: 04/30/2021
Ongoing (20% completion)

Major Task 4: Determine whether brown adipocytes have an impact on pro-tumorigenic microenvironment in the postpartum mammary gland
Milestone/target date: 12/31/2021
Not started

Major Task 5: Determine alterations in the self-renewal and/or differentiation of PI-MECs or other stem/progenitor populations in parous Notch3 knockout mice
Milestone/target date: 03/31/2022
Not started

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major activities:

1) Performed injection of basal-like (B5725) and claudin-low (C0321) breast cancer cell lines into the mammary fat pad of isogenic *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ and wildtype mice (littermates, FVB background) during post-lactational involution, and compared mammary tumor growth and dissemination between the two hosts.
2) Performed injection of basal-like (B5725) and claudin-low (C0321) breast cancer cell lines into the mammary fat pad of isogenic *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ and wildtype mice (littermates, FVB background) at 4-6 weeks of age (non-parous), and compared cancer cell proliferation and dissemination between the two hosts.
3) Performed injection of basal-like (B5725) and claudin-low (C0321) breast cancer cell lines at a posterior dorsolateral subcutaneous site in addition to the mammary fat pad of isogenic *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ and wildtype mice (littermates, FVB background), and compared cancer cell proliferation and dissemination between the two hosts.
4) Tested the cytotoxic effects of splenocytes isolated from *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ or wildtype hosts on breast cancer cells *in vitro*.
5) Performed X-gal staining in spontaneous mammary tumors from the *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice.
6) Performed X-gal staining in orthotopic xenografts from the *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ and *Notch3*$^{\beta\text{-}Geo/+}$ hosts, as well as the spleens of the host mice.

7) Examined brown adipocyte differentiation in the involuting mammary gland of the *Prdm16$^{flox/flox}$;MMTV-Cre* and *Prdm16$^{flox/flox}$;K14-CreER* mice (in comparison with the *Prdm16$^{flox/flox}$* mice).

Specific objectives:

1) Determine whether post-lactational involuting mammary microenvironment of Notch3 knockout mice accelerates tumor growth and metastasis compared to that of wildtype mice.
2) Determine the compartment in which Notch3 regulates mammary tumor microenvironment.
3) Determine the mechanisms by which Notch3 regulates brown adipocyte differentiation in the involuting mammary gland.

Significant results:

1) Xenografts of both basal-like and claudin-low breast cancer cells were drastically suppressed in the post-lactational involuting mammary microenvironment of *Notch3$^{-/-}$* mice compared to that of wildtype mice. Interestingly, xenografts were partially suppressed in the *Notch3$^{+/-}$* mice.
2) Xenografts of both basal-like and claudin-low breast cancer cells were drastically suppressed in the non-parous mammary microenvironment of *Notch3$^{-/-}$* mice compared to that of wildtype mice. Xenografts were partially suppressed in the *Notch3$^{+/-}$* mice as well.
3) The tumor-suppressive effect of the *Notch3$^{-/-}$* hosts was observed even when breast cancer cells were ectopically injected at a posterior dorsolateral subcutaneous site.
4) Splenocytes isolated from the *Notch3$^{-/-}$* mice (following inoculation of breast cancer cells) showed cytotoxic effect on breast cancer cells *in vitro*, whereas splenocytes isolated from the wildtype host mice had no effect.
5) In spontaneous mammary tumors, Notch3 expression was readily detected in the blood vessels but not in tumor cells.
6) In xenograft experiments, Notch3 was expressed in a small subset of splenocytes of the host mice as well as in the tumor microenvironment.
7) Deletion of Prdm16 in the mammary epithelium abolished brown adipocyte differentiation during post-lactational involution.

Stated goals not met:

Our plan was to perform xenograft experiments using cell lines of three breast cancer subtypes (ER-positive, basal-like, and claudin-low). We have performed xenograft experiments with the basal-like (B5725) and claudin-low (C0321) cell lines, and found that deletion of Notch3 in the host mice caused eradication of injected breast cancer cells of both basal-like and claudin-low subtypes. It is important to determine whether deletion of Notch3 in the host mice also suppresses ER-positive tumor. However, we have not been able to establish an ER^{+} breast cancer cell line from the *Notch3$^{-/-}$* mice on FVB background. This is due to the very low incidence of spontaneous mammary tumors in these mice. We will increase the number of *Notch3$^{-/-}$* mice for tumor harvesting.

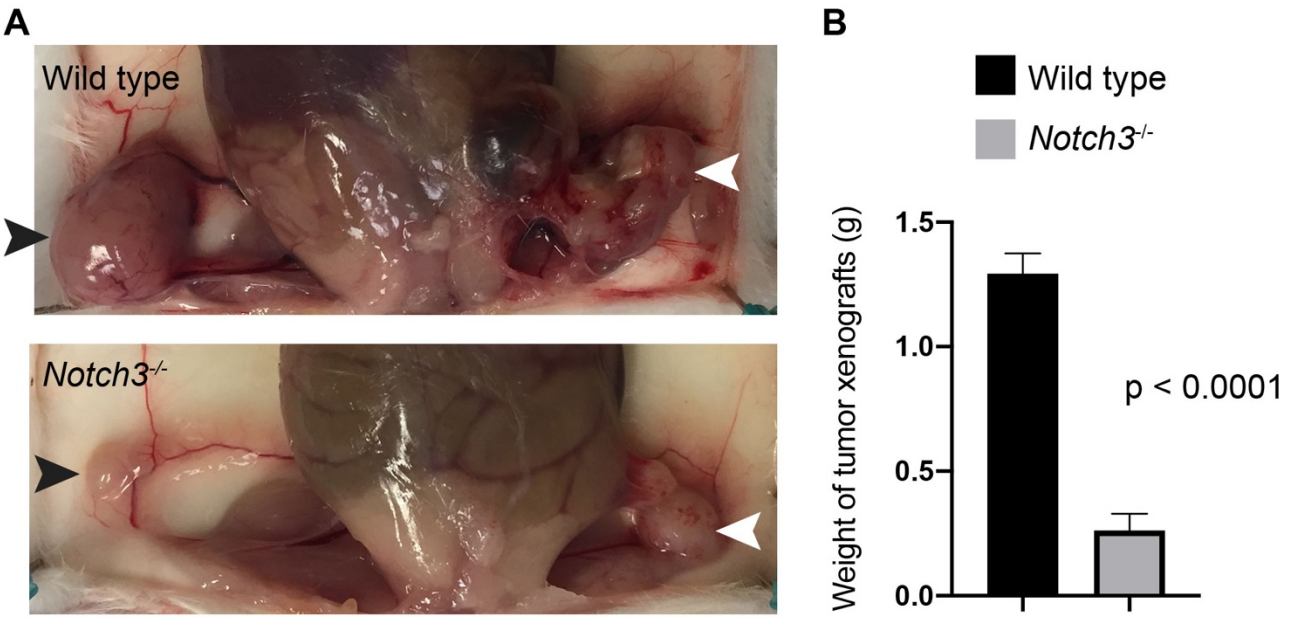


Fig. 1. Reduced growth of tumor xenografts in the Notch3 knockout mice as compared to the wildtype mice. (A) Representative images of tumors at 3-4 wks post-injection of breast cancer cells (1 x 10^5 cells on each side of #4 mammary fat pad). (B) Average of tumor weights in the wild type and *Notch3*$^{-/-}$ host mice.

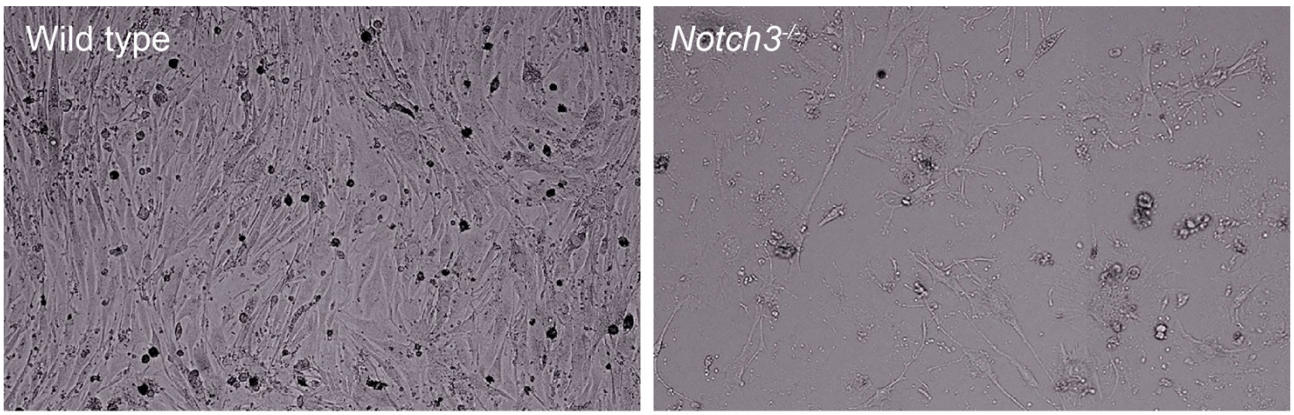
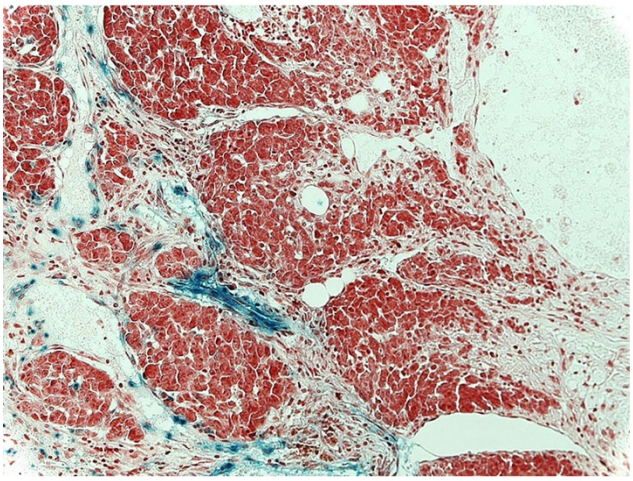


Fig. 2. Cytotoxic effect of splenocytes isolated from the *Notch3*$^{-/-}$ host mice. Shown are representative images of breast cancer cells (C0321) after 2 weeks of co-culture with splenocytes isolated from the wild type or *Notch3*$^{-/-}$ host mice.

Notch3$^{\beta\text{-}Geo/\beta\text{-}Geo}$



Notch3$^{\beta\text{-}Geo/+}$*;MMTV-Wnt1*

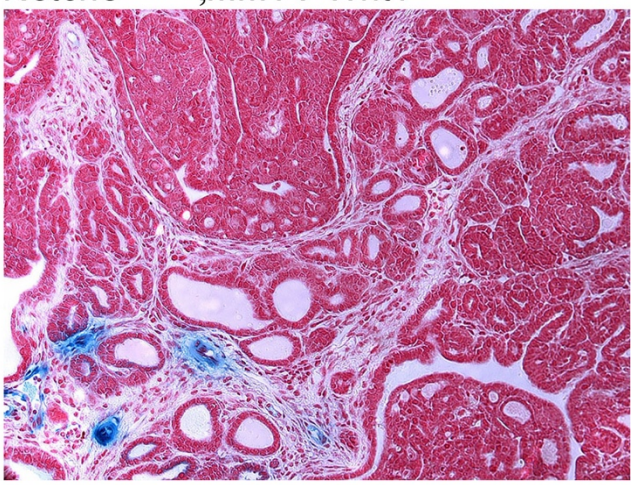


Fig. 3. Notch3-expressing cells in spontaneous mammary tumors. Shown are representative images of X-gal staining in the mammary tumors from *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ and *Notch3*$^{\beta\text{-}Geo/+}$*;MMTV-Wnt1* mice.

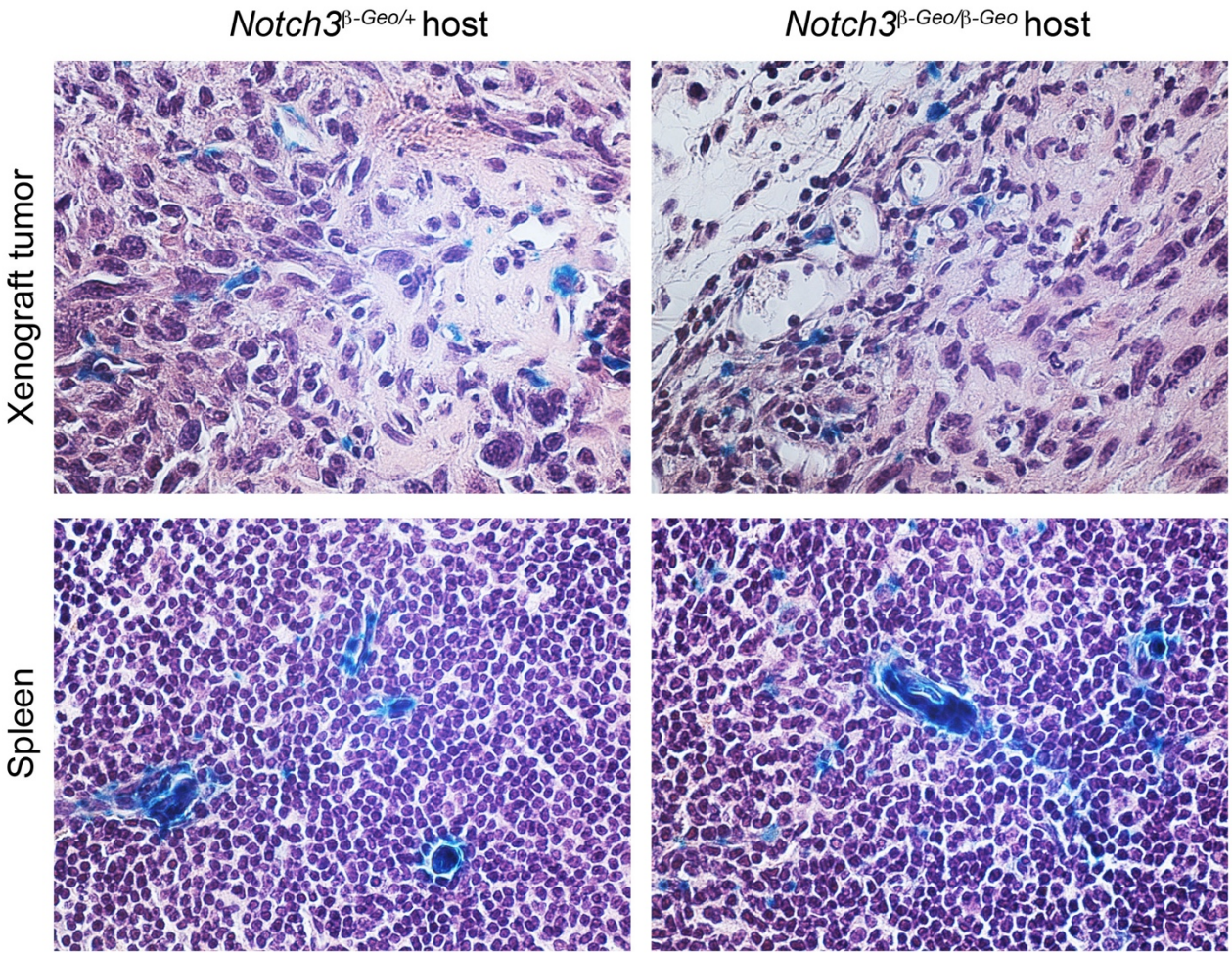


Fig. 4. Infiltration of Notch3^{+} cells in xenograft mammary tumors. Shown are representative images of X-gal staining in the *Notch3*$^{\beta\text{-}Geo/+}$ and *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ host mice. Note: Injected breast cancer cells do not carry the *Notch3*$^{\beta\text{-}Geo}$ reporter. All LacZ^{+} cells inside the tumor originated from the host mice.

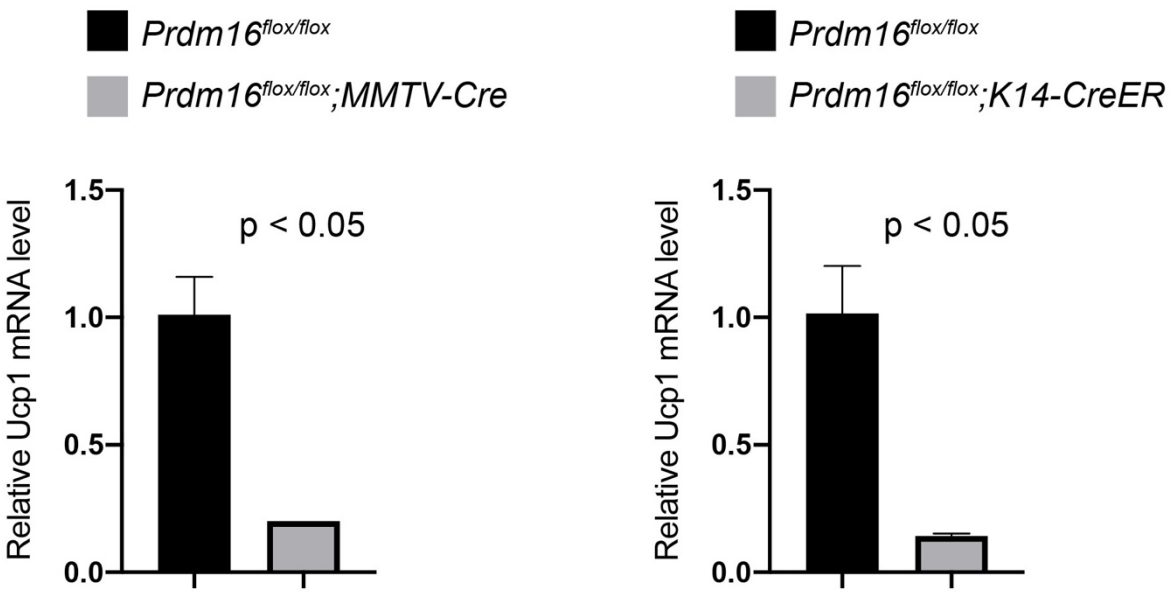


Fig. 5. Deletion of Prdm16 in the mammary epithelial cells caused defective brown adipocyte differentiation. Relative Ucp1 mRNA levels were determined by quantitative RT-PCR in mammary tissues at postlactational involution day 1.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Our plan is to complete Major Task 2 (determine the compartment in which Notch3 regulates pro-tumorigenic mammary microenvironment) and Major Task 3 (determine the mechanisms by which Notch3 regulates brown adipocyte differentiation in the involuting mammary gland) in the next reporting period. We will also take on Major Task 4 (determine whether brown adipocytes have an impact on pro-tumorigenic microenvironment in the postpartum mammary gland). Results from the first reporting period suggest that immune cells play an important role in the Notch3 regulation of the mammary microenvironment. Therefore, we will need to determine whether Notch3 regulates immune cells directly or affects immune cells through its regulation of brown adipocytes.

4. **IMPACT:** D*escribe 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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:*

There were no significant changes in the project or its direction during this reporting period. However, we anticipate a minor change in the project direction for the next reporting period based on the current results. Specifically, we discovered that Notch3 knockout mice suppressed, rather than promoted, mammary tumor xenografts. Moreover, suppression of xenografts occurs independent of the post-lactational mammary microenvironment. Our preliminary results support the hypothesis that Notch3 may exert an oncogenic role through its regulation of tumor-infiltrating lymphocytes.

It remains to be determined whether Notch3 exerts a tumor-suppressive function in the postpartum mammary microenvironment through the promotion of brown adipocyte differentiation thereby ameliorating the protumorigenic inflammatory microenvironment. Therefore, we will continue to perform all experiments outlined in SOW to achieve the goal of this project. Meanwhile, we intend to perform additional experiments and analyses, including RNA-seq analysis using xenograft tissues as well as splenocytes to determine the mechanisms by which Notch3 regulates tumor immune microenvironment.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We have not been able to establish an ERα-positive breast cancer cell line from the *Notch3*$^{-/-}$ mice on FVB background. This is due to the very low incidence of spontaneous mammary tumors in *Notch3*$^{-/-}$ mice. We will increase the number of *Notch3*$^{-/-}$ mice for tumor harvesting.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

No significant changes in use or care of vertebrate animals.

Significant changes in use of biohazards and/or select agents

No significant changes in use of biohazards and/or select agents.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report.

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

  Nothing to Report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  Nothing to Report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of

compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Keli Xu
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0001-5535-1076
Nearest person month worked:	1.8
Contribution to Project:	Dr. Xu supervised and participated in all studies, performed data analysis, and wrote the report.
Name:	Wen-Cheng Chung
Project Role:	Scientist I
Researcher Identifier (ORCID ID):	
Nearest person month worked:	9.0
Contribution to Project:	Dr. Chung has been responsible for key duties of this project. He conducted mouse experiments, cell culture, and performed techniques of molecular biology and biochemistry, immunohistochemistry, flow cytometry, etc. He also took part in data analysis.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Dr. Keli Xu, the PI of this project received a grant from The Joe W. and Dorothy Dorsett Brown Foundation
Title: Lunatic Fringe-modulated Notch signaling in pancreatic cancer
Dates of Funding: 09/01/2019 - 08/31/2020
Amount: $30,000
Role: PI

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. **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.*