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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to determine the functions of Notch3 in the pathogenesis of postpartum breast cancer, focusing on the regulation of parity-induced mammary epithelial cells, as we as regulation of brown adipose tissue repopulation in the postpartum mammary gland and its potential role in modulating tumor microenvironment. We showed that parous Notch3 mutant glands exhibited an expansion of CD24HiCD49fLo cells, a subpopulation known to be enriched in bipotent lobule progenitors and multipotent stem cells, associated with upregulation of Gata3. Unexpectedly, we found that Notch3 knockout mice suppressed mammary tumor xenografts. We identified candidates of Notch3 target genes in immune cells that may regulate tumor-immune interaction. Our results suggest that Notch3 functions as a tumor suppressor in the mammary epithelium and brown adipocytes in the postpartum mammary gland while exerting an oncogenic role in the immune system.

15. SUBJECT TERMS

Postpartum breast cancer, Notch signaling, Parity-induced mammary epithelial cell, Tumor microenvironment, Brown adipocyte, Immune cell

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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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/or by restricting expansion of parity-induced mammary stem-like cells. 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. Our objectives are: (1) to determine the mechanism underlying brown adipose tissue repopulation in the postpartum mammary gland and its potential role in modulating postpartum mammary microenvironment, which may link obesity-associated metabolic changes to the progression of postpartum breast cancer; and (2) to determine the contribution of parity-induced mammary epithelial progenitors to postpartum breast cancer and its regulation by Notch signaling.

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 immune cells

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
Actual completion date: 11/30/2020

Major Task 3: Determine the mechanisms by which Notch3 regulates brown adipocyte differentiation in the involuting mammary gland
Milestone/target date: 04/30/2021
Actual completion date: 04/12/2021

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
Ongoing (20% completion)

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
Ongoing (50% completion)

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 RNASeq analysis in the splenocytes isolated from wild-type and *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ host mice transplanted with isogenic mammary tumor cells (Major Task 2)
2) Performed transplantation experiments of nulliparous *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ epithelial cells into nulliparous wild-type hosts, or parous *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ epithelial cells into parous wild-type hosts (Major Tasks 2, 3)
3) Determined the effects of mammary epithelium-specific deletion of *Prdm16* on mammary development during pregnancy, lactation and post-weaning involution (Major Tasks 3, 4)
4) Determined alterations in mammary stem/progenitor populations in the parous Notch3 knockout mice (Major Task 5)

Specific objectives:

1) Identify Notch3-regulated genes and pathways involved in the regulation of tumor-infiltrating immune cells.
2) Determine the compartment in which Notch3 functions as a tumor suppressor in the mammary gland.
3) Determine whether ablation of Prdm16 in the mammary epithelium leads to mammary epithelial hyperplasia and/or alters microenvironment of postpartum mammary gland.
4) Determine the mechanisms by which loss-of-Notch3 contributes to the initiation and progression of postpartum breast cancer.

Significant results:

1) RNASeq analysis in the splenocytes identified differentially expressed genes between the wild-type and *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ host mice transplanted with mammary tumor cells. Enrichment analysis indicated that these genes may regulate immune processes including neutrophil degranulation, degradation of the extracellular matrix, regulation of TLR, complement cascade, and immunoregulatory interactions between a lymphoid and a non-lymphoid cell (Fig. 1-3). In particular, histocompatibility 2 genes *H2-Ea* and *H2-Eb1* were two of the top differentially expressed genes in the *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ splenocytes (see table below). Importantly, these two genes were also identified from our previous microarray gene expression analysis using wild-type and *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary tissues during post-lactational involution. Thus, H2-Ea and H2-Eb1 represent top candidates of Notch3 effectors in the immune system, and their potential roles in impeding tumor growth warrant further investigation.

gene_id	log2foldchange	pvalue	padjust	gene_name
ENSMUSG00000036322	-6.811490542	1.79E-30	2.65E-26	H2-Ea-ps
ENSMUSG00000113061	-3.131815465	8.47E-24	6.25E-20	Gm11361
ENSMUSG00000060586	4.341980059	2.29E-15	1.13E-11	H2-Eb1
ENSMUSG00000092571	-4.956331381	5.97E-15	2.20E-11	Gm4134

Table 1. List of top differentially expressed genes in the *Notch3* mutant splenocytes

2) Transplantation of mammary epithelial cells from postpartum *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice into postpartum wild-type mammary glands showed that *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary epithelial cells gave rise to mammary ductal end buds in the wild-type host, suggesting stem-like capability (Fig. 4). We also performed transplantation of postpartum *Notch3*$^{\beta\text{-}Geo/+}$ mammary epithelial cells, in comparison with the postpartum *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary epithelial cells. Our preliminary results suggest that homozygous deletion (compared with heterozygous deletion) of *Notch3* in mammary epithelium caused an increase in the activity or number of mammary stem cells in the postpartum mammary gland. We are in progress of transplanting *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ cells from postpartum donor into virgin wild-type host to determine whether postpartum microenvironment is indispensable for enhanced stem cell activity of *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ cells.

3) Whole-mount preparation of mammary glands showed that *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice underwent post-lactational involution normally. However, parous *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice developed mammary ductal hyperplasia by 10 months of age, some of which progressed to ductal carcinoma in situ, ultimately leading to invasive and metastatic tumors (Fig. 5A and data not shown). Interestingly, parous *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ glands exhibited an expansion of $CD24^{Hi}CD49f^{Lo}$ cells, a subpopulation known to be enriched in bipotent lobule progenitors and multipotent stem cells (Fig. 5B). These results suggest that Notch3 may prevent postpartum breast cancer through suppression of $CD24^{Hi}CD49f^{Lo}$ and/or parity-induced mammary stem-like cells (Matulka et al., 2007). We are currently generating *Rosa*$^{LSL\text{-}YFP}$*;WAP-Cre* and *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$*;Rosa*$^{LSL\text{-}YFP}$*;WAP-Cre* mice to determine whether deletion of Notch3 increases parity-induced stem-like cells by lineage tracing experiments.

4) Western blot analysis in multiparous mammary glands showed drastically increased levels of Cyclin D1 in *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice compared to wild-type mice (Fig. 6), suggesting a role for Cyclin D1 in the pathogenesis of postpartum breast cancer in these mice. Previous study using breast cancer cell lines suggested that Notch3 suppressed tumorigenesis and metastasis of breast cancer via trans-activating estrogen receptor-alpha (ERα) (Dou et al., 2017). However, we found that protein levels of ERα were similar in the multiparous *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ and wild-type mammary glands (Fig 6), suggesting a different Notch3 function may be involved in suppression of postpartum breast cancer. Interestingly, *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary glands expressed much higher levels of Gata3 (Fig. 6), a transcription factor that is essential for mammary-gland morphogenesis and luminal-cell differentiation and up-regulated in parous breast tissues (Asselin-Labat et al., 2007; Peri et al., 2012). These results suggest that Notch3 may regulates postpartum mammary epithelial differentiation and proliferation through Gata3.

5) Although deletion of *Prdm16* in mammary epithelium caused defective brown adipocyte differentiation at the initiation of post-lactational involution (reported in the previous Annual Report), whole-mount preparation of mammary glands showed that *Prdm16*$^{flox/flox}$*;MMTV-Cre* mice underwent similar involution compared with the control mice (Fig. 7). We are currently monitoring postpartum *Prdm16*$^{flox/flox}$*;MMTV-Cre* mice for mammary hyperplasia. We will also perform flow cytometry analysis to determine whether deletion of *Prdm16* in the mammary epithelial cells has an impact on parity-induced mammary stem-like cells.

Stated goals not met:

For Major Tasks 2 and 3, our original plan was to transplant *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary epithelial cells into epithelium-divested wildtype mammary fat pad (Epithelium^{N3}Stroma^{Wt}), and wildtype epithelial cells into epithelium-divested *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ fat pad (Epithelium^{Wt}Stroma^{N3}) using 4-week-old donor and host mice. These mice would undergo pregnancy, lactation, post-lactational involution, and be used for tumor cell injection during involution. However, lactation in transplants was not possible because the transplanted epithelium was not attached to the nipple. In fact, Jackson-Fisher and colleagues reported that apoptosis and remodeling of the transplanted mammary tissues commenced immediately after pups birth (Jackson-Fisher et al., 2004). As an alternative, we transplanted mammary epithelial cells isolated from postpartum *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice into postpartum wild-type mammary glands. Our results indicated that mammary epithelial cells from the postpartum *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ donor contributed extensively to the wild-type host, in particular, to the end buds of mammary ductal system.

Although we could not determine by transplantation as to in which compartment Notch3 regulates postpartum mammary microenvironment, other results suggest that Notch3 may function in the immune system to regulate tumor-immune interaction (Significant Result #1), while exerting its tumor-suppressive function in the mammary epithelium (Significant Result #2, 3, 4). In addition, decreased Ucp1 expression in *Prdm16*$^{flox/flox}$*;MMTV-Cre* and *Prdm16*$^{flox/flox}$*;K14-CreER* mammary tissues at the beginning of involution suggest that Notch3 may promote transdifferentiation of alveolar epithelial cells to brown adipocytes (reported in the Annual Report last year).

References:

Asselin-Labat, M.L., Sutherland, K.D., Barker, H., Thomas, R., Shackleton, M., Forrest, N.C., Hartley, L., Robb, L., Grosveld, F.G., van der Wees, J., *et al.* (2007). Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. Nature cell biology *9*, 201-209.

Dou, X.W., Liang, Y.K., Lin, H.Y., Wei, X.L., Zhang, Y.Q., Bai, J.W., Chen, C.F., Chen, M., Du, C.W., Li, Y.C., *et al.* (2017). Notch3 Maintains Luminal Phenotype and Suppresses Tumorigenesis and Metastasis of Breast Cancer via Trans-Activating Estrogen Receptor-alpha. Theranostics *7*, 4041-4056.

Jackson-Fisher, A.J., Bellinger, G., Ramabhadran, R., Morris, J.K., Lee, K.F., and Stern, D.F. (2004). ErbB2 is required for ductal morphogenesis of the mammary gland. Proceedings of the National Academy of Sciences of the United States of America *101*, 17138-17143.

Matulka, L.A., Triplett, A.A., and Wagner, K.U. (2007). Parity-induced mammary epithelial cells are multipotent and express cell surface markers associated with stem cells. Dev Biol *303*, 29-44.

Peri, S., de Cicco, R.L., Santucci-Pereira, J., Slifker, M., Ross, E.A., Russo, I.H., Russo, P.A., Arslan, A.A., Belitskaya-Levy, I., Zeleniuch-Jacquotte, A., *et al.* (2012). Defining the genomic signature of the parous breast. BMC Med Genomics *5*, 46.

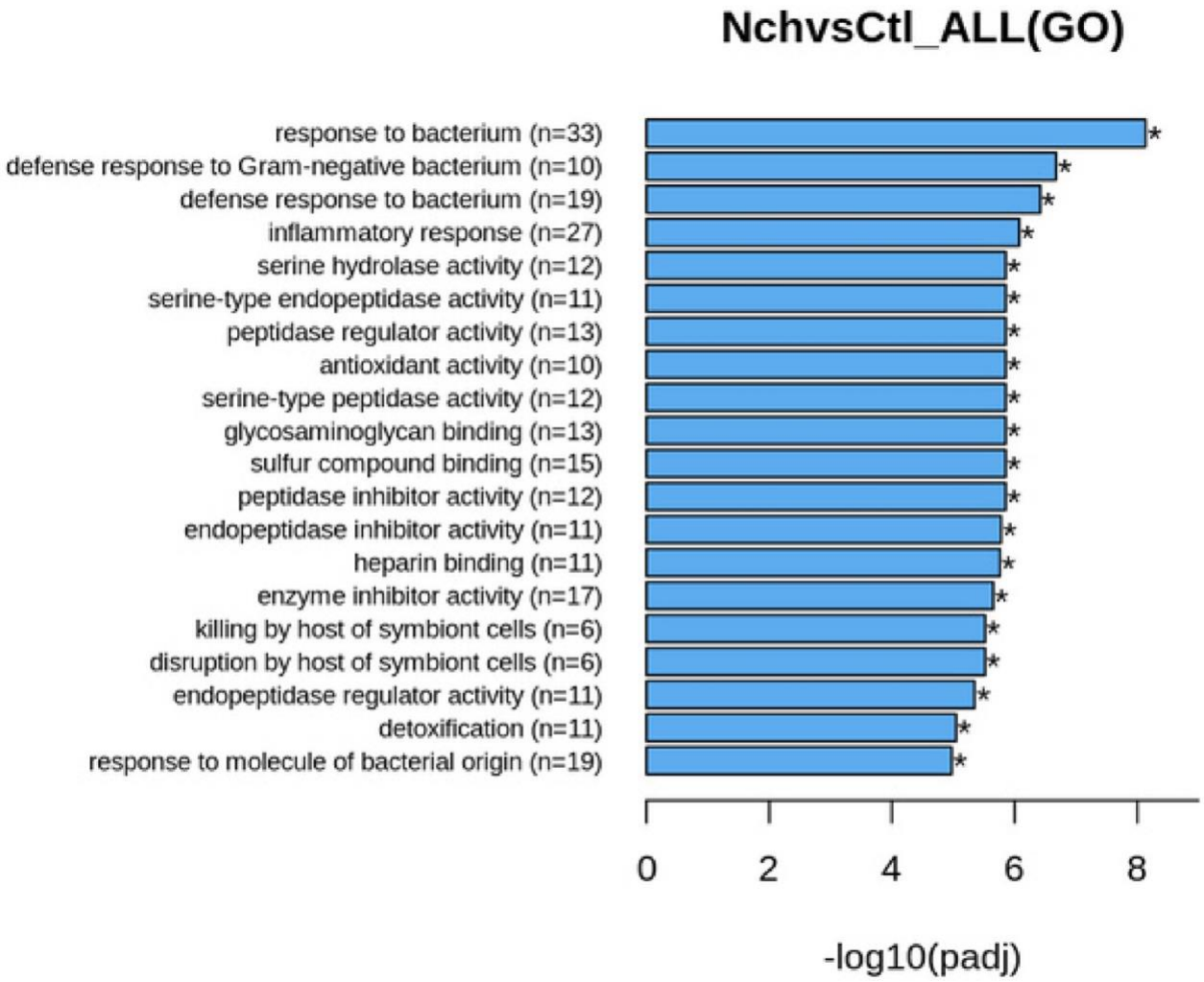

Fig. 1. GO enrichment analysis of the differentially expressed genes in the splenocytes of wild-type and *Notch3*$^{\beta\text{-Geo}/\beta\text{-Geo}}$ mice transplanted with isogenic mammary tumor cells.

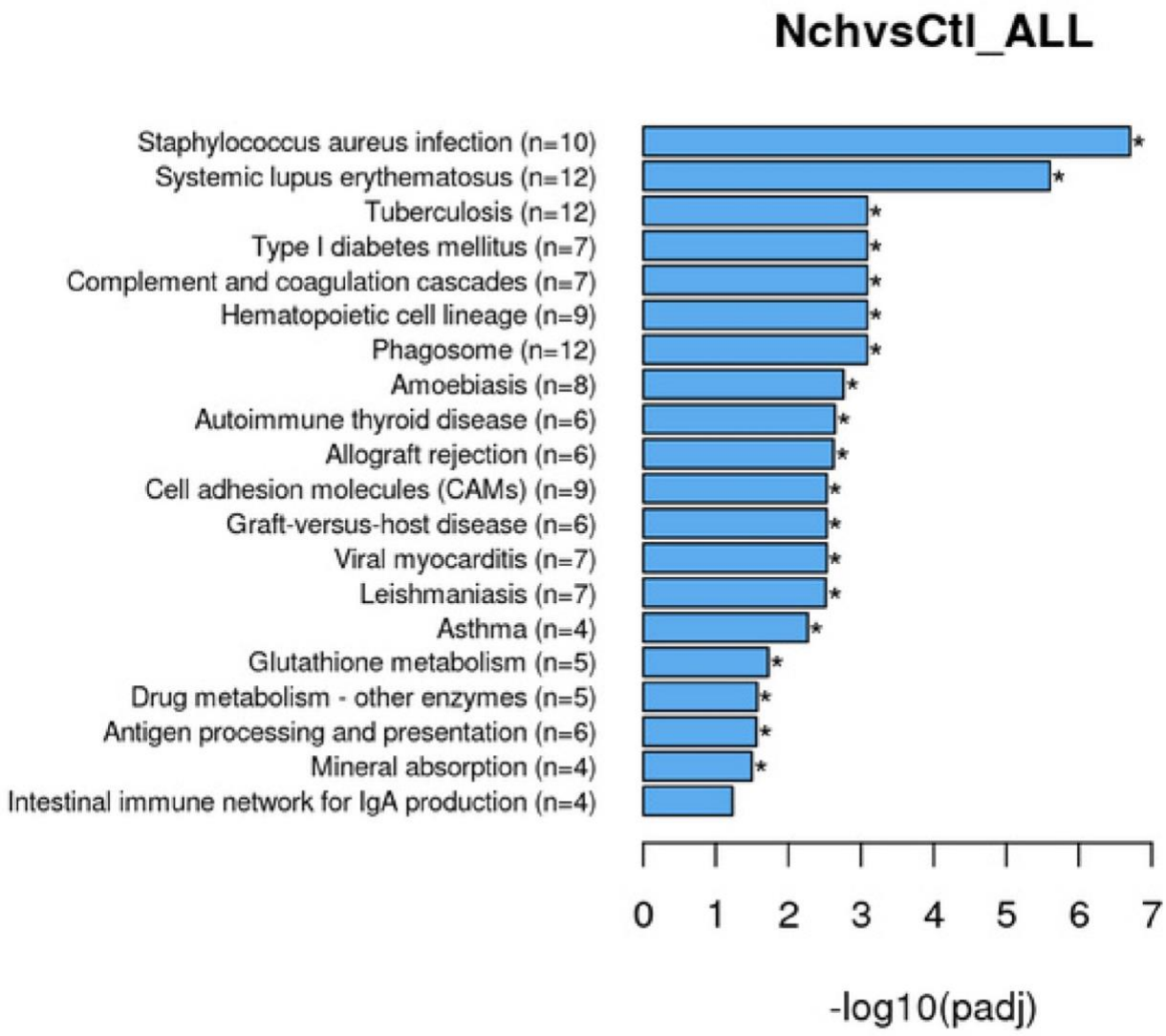

Fig. 2. KEGG enrichment analysis of the differentially expressed genes in the splenocytes of wild-type and *Notch3*$^{\beta\text{-Geo}/\beta\text{-Geo}}$ mice transplanted with isogenic mammary tumor cells.

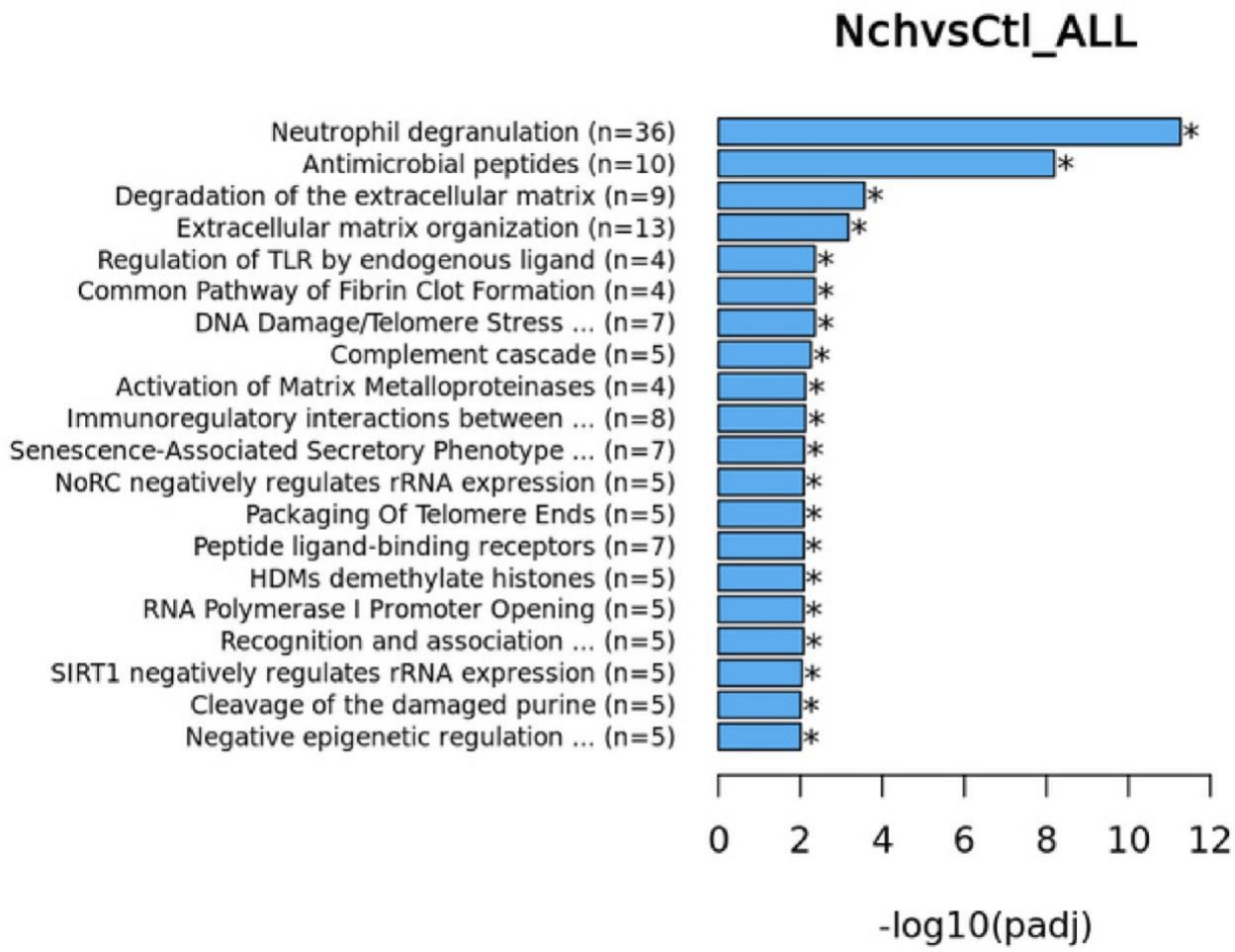

Fig. 3. Reactome enrichment analysis of the differentially expressed genes in the splenocytes of wild-type and *Notch3*$^{\beta\text{-Geo}/\beta\text{-Geo}}$ mice transplanted with isogenic mammary tumor cells.

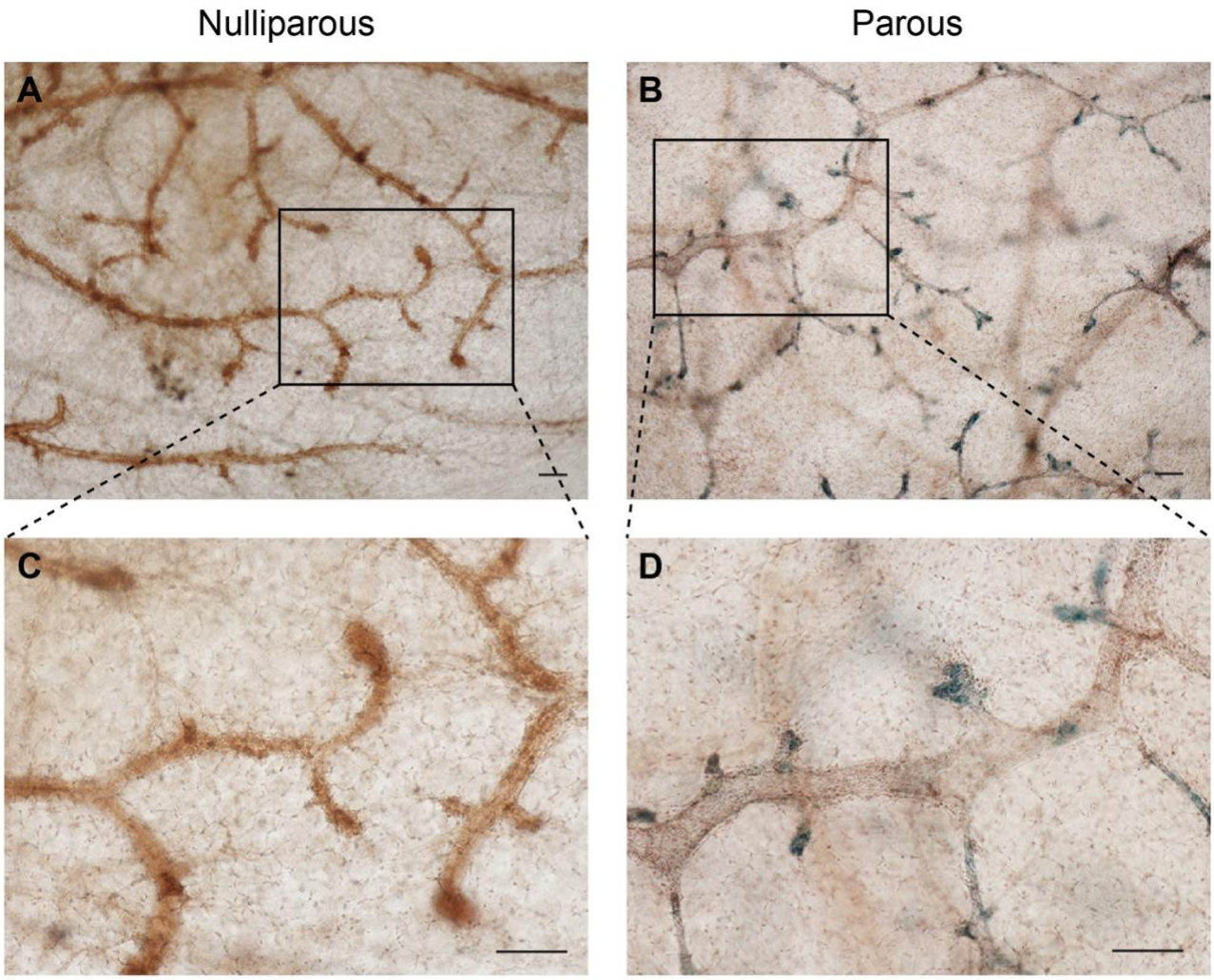

Fig. 4. Contribution of parous *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary epithelial cells to the mammary ductal buds in parous wild-type host. (A, C) Whole-mount X-gal staining of a nulliparous wild-type mamary gland harvested two months after transplantation of *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary epithelial cells isolated from a nulliparous donor. (B, D) Whole-mount X-gal staining of a parous wild-type mamary gland harvested two months after transplantation of *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary epithelial cells isolated from a parous donor. Scale bars: 50 μm.

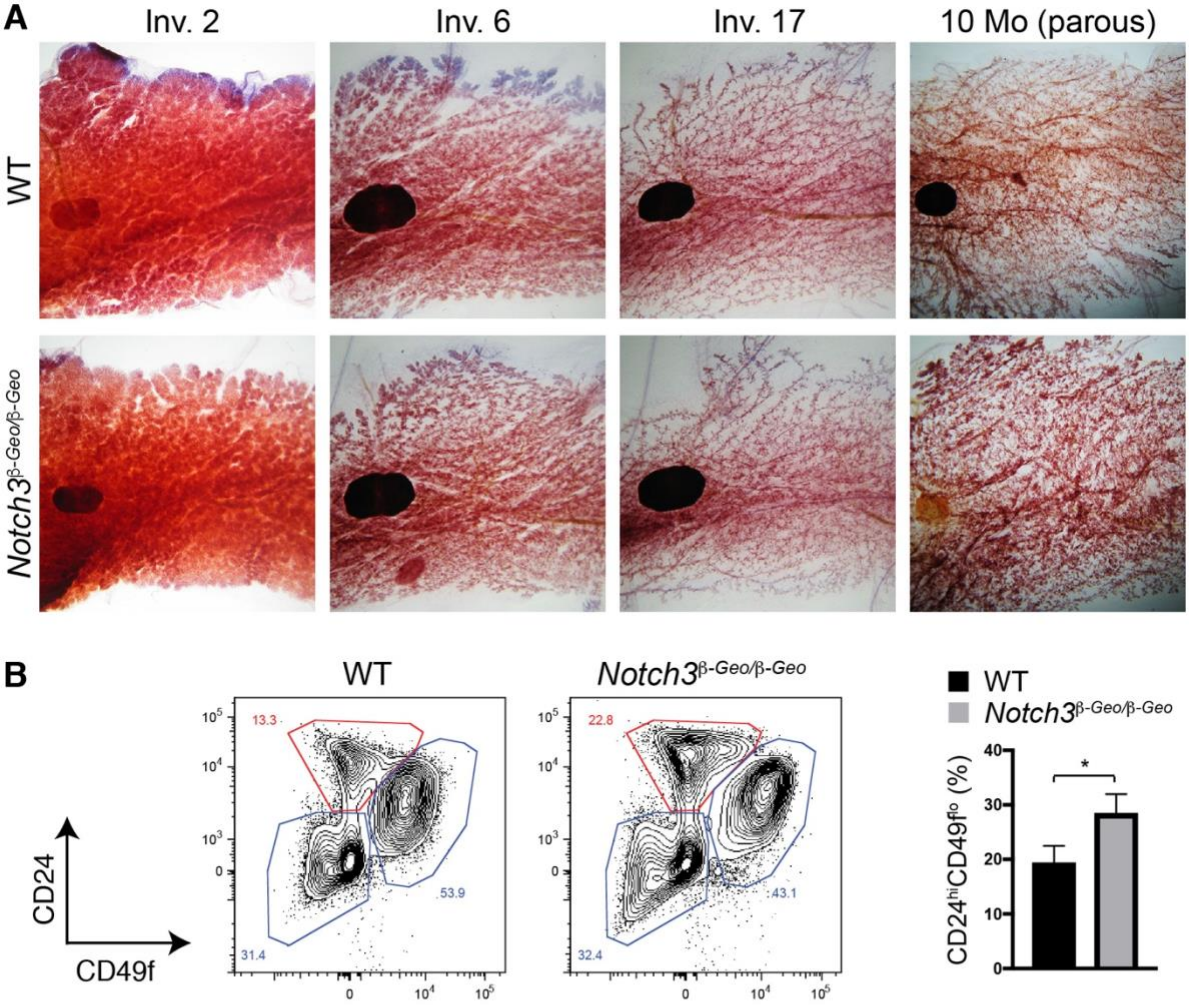

Fig. 5. Postpartum *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice showed normal post-lactational involution but developed mammary hyperplasia associated with expansion of CD24^{Hi}CD49f^{Lo} subpopulation. (A) Whole-mount mammary glands of parous wild-type and *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice at involution day 2, 6 & 17 and 10 months of age. (B) Representative flow cytometry analysis of mammary epithelial cells in parous wild-type and *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice at 6-8 months of age, and quantification of the CD24^{Hi}CD49f^{Lo} subpopulation. * $p < 0.05$.

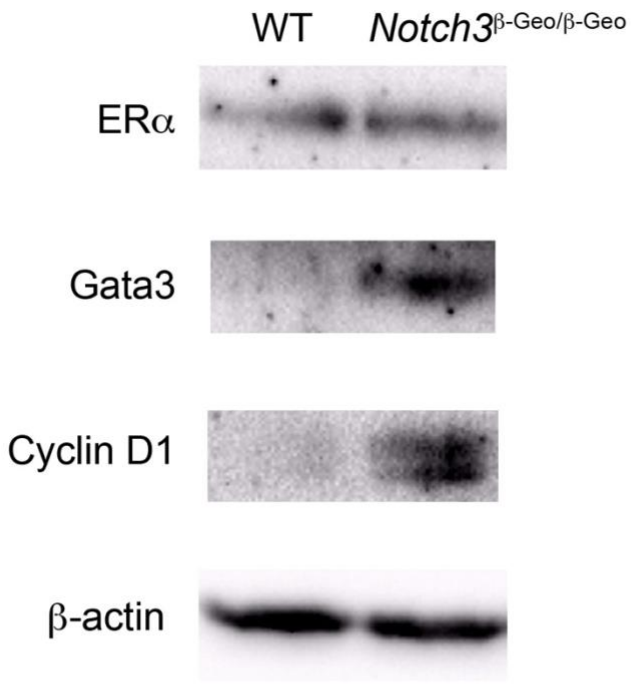

Fig. 6. Western blot analysis in the mammary tissues of multiparous wild-type and *Notch3*$^{\beta\text{-Geo}/\beta\text{-Geo}}$ mice. Animals were sacrificed at 5 months of age (5 weeks after forced-weaning of pups from the third pregnancy).

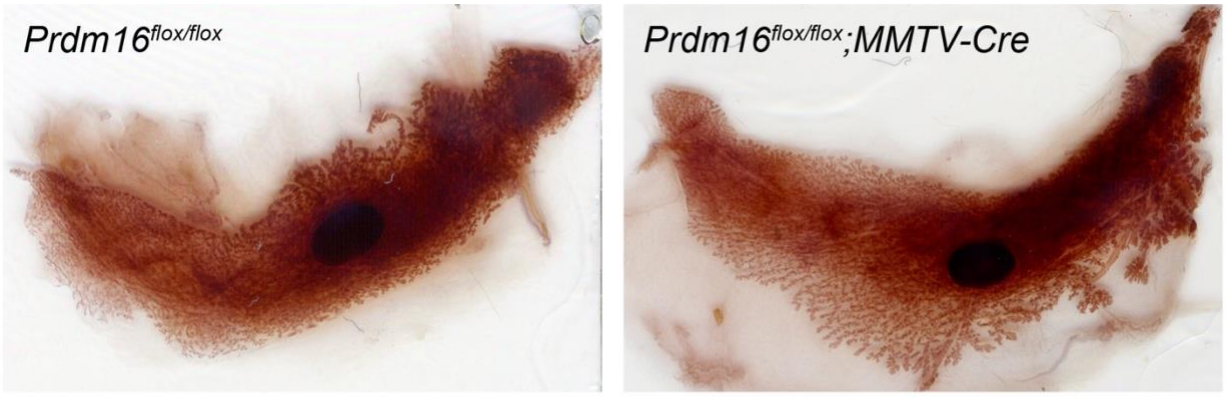

Fig. 7. Representative images of whole-mount mammary glands from *Prdm16*$^{flox/flox}$ and *Prdm16*$^{flox/flox}$*;MMTV-Cre* mice at day 6 of post-lactational involution.

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.

The results were reported in an abstract submitted to the AACR Annual Meeting 2021 (Title: Notch3 functions as a tumor suppressor in ER-positive breast cancer. Abstract Number: 3111)
This project was also described in the Annual Report of the Cancer Center and Research Institute at the University of Mississippi Medical Center that reaches to local communities for the purpose of enhancing public understanding and increasing interest in cancer research.

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.

It remains to be determined whether Notch3 exerts a tumor-suppressive function in part through the promotion of brown adipocyte differentiation thereby ameliorating the inflammatory microenvironment in the postpartum mammary gland. Therefore, we will perform experiments outlined in SOW Major Task 4 to determine the impact of brown adipocytes on postpartum mammary gland. To this end, we have generated *Prdm16$^{flox/flox}$;MMTV-Cre* mice, which harbor mammary-specific deletion of *Prdm16* and show defective brown adipocyte differentiation during involution. We will examine the inflammatory mediators, macrophages, myeloid-derived suppressor cells, regulatory T cells, as well as lymphatic vessels in the postpartum mammary gland of these mice (in comparison with the wild-type mice). If altered inflammatory milieu is observed, we will test whether treatment with resveratrol (or roscovitine) ameliorates pro-inflammatory microenvironment and decelerates tumor cell proliferation/dissemination.

To further understand tumor-suppressive function of Notch3 in the mammary epithelium, we will perform experiments outlined in SOW Major Task 5 to determine alterations in self-renewal and differentiation of PI-MECs or other stem/progenitor populations in parous Notch3 knockout mice. We have already showed altered expressions of Cyclin D1 and Gata3 in the *Notch3$^{\beta\text{-}Geo/\beta\text{-}Geo}$* postpartum mammary gland. We will perform lineage tracing experiment using *Rosa$^{LSL\text{-}YFP}$;WAP-Cre* and *Notch3$^{\beta\text{-}Geo/\beta\text{-}Geo}$;Rosa$^{LSL\text{-}YFP}$;WAP-Cre* mice to determine whether Notch3 regulates parity-induced stem-like cells and whether Gata3 plays an important role in this context.

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).

The observation that mammary epithelial cells from the postpartum *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice contributed specifically to the ductal end buds in the host mice suggests that Notch3 regulates mammary stem cells in the postpartum mammary gland. This may represent a new avenue for the investigation of cellular origin and initiation of postpartum breast cancer.

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 found that Notch3 knockout mice suppressed (rather than promoted) mammary tumor xenografts. We have identified candidates of Notch3 target genes that potentially function in the immune system. Thus, in addition to the proposed tumor-suppressive functions in the mammary epithelium and brown adipocytes, our results suggest an oncogenic role for Notch3 in immune cells.

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Our original approach was to transplant *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mammary epithelial cells into epithelium-divested wildtype mammary fat pad, and wildtype epithelial cells into epithelium-divested *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ fat pad using 4-week-old donor and host mice. These mice would then undergo pregnancy, lactation and post-lactational involution. However, lactation in transplants was not possible because the transplanted epithelium was not attached to the nipple. It has been reported that apoptosis and remodeling of the transplanted mammary tissues commenced immediately after birth of pups (Jackson-Fisher et al., 2004). As an alternative, we performed transplantation of mammary epithelial cells from postpartum *Notch3*$^{\beta\text{-}Geo/\beta\text{-}Geo}$ mice into postpartum wild-type mammary glands. By X-gal staining of the mammary glands, we were able to distinguish transplants outgrowth from the host mammary epithelium.

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.

COVID-19 pandemic had a negative impact on the progression of this project, including temporary shutdown of the lab and delays in routine orders due to supply chain issues.

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.*

Chung W-C, Egan SE, Xu K. Notch3 functions as a tumor suppressor in ER-positive breast cancer. AACR 2021 Annual Meeting, April 10-15, 2021. Abstract #3111

- **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 (PI)	No change
Name: Wen-Cheng Chung (Scientist I)	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?
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.

Nothing to report

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.*

AACR 2021 Annual Meeting, April 10-15, 2021. Abstract #3111

Title: Notch3 functions as a tumor suppressor in ER-positive breast cancer
Short Title: Notch3 in ER-positive breast cancer

Wen-Cheng Chung[1], Sean E. Egan[3], Keli Xu[1,2]
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The Notch signaling pathway has been implicated in the development as well as tumorigenesis of the mammary gland. Notch3 promotes luminal cell specification and marks a highly clonogenic luminal progenitor population. Aberrant Notch3 activation has been shown to promote the growth of basal breast cancer as well as resistance to hormonal therapy in metastatic luminal breast cancer. Interestingly, recent studies also suggest a tumor-suppressive function of Notch3 in mammary epithelium through regulation of estrogen receptor α (ER). Here we report the expression and functional analysis of Notch3 in the mouse mammary gland. X-gal staining of the *Notch3*$^{\beta\text{-}Geo/+}$ mammary gland showed that Notch3 was highly expressed in luminal cells throughout mammary development with exception at the initiation of post-lactational involution, when Notch3 expression was restricted to the basal cells. Deletion of Notch3 caused decreased Notch activation in the $CD24^{Hi}CD49f^{Lo}$ subpopulation of the pubescent mammary gland, accompanied by a significant decrease in the number of $CD24^{Hi}CD49f^{Lo}CD61^{+}$ luminal progenitor cells. Whole-mount preparation of the *Notch3* knockout mammary glands showed normal development during puberty, pregnancy, lactation and involution. Surprisingly, parous *Notch3* knockout mice developed mammary ductal hyperplasia by 10 months of age, some of which progressed to ductal carcinoma in situ, and ultimately to invasive and metastatic cancer. Parous *Notch3* knockout mice exhibited an expansion of the $CD24^{Hi}CD49f^{Lo}$ subpopulation, and mammary tumors from these mice were composed predominantly of $CD24^{Hi}CD49f^{Lo}$ cells. The vast majority of these tumors were ER-positive and relatively well differentiated, with a papillary and/or glandular pattern. They expressed the luminal marker cytokeratin 8, and some of them co-expressed cytokeratin 14, a basal marker. All *Notch3* knockout mammary tumors showed high level expression of Cyclin D1. These results suggest that Notch3 may prevent ER-positive breast cancer through suppression of $CD24^{Hi}CD49f^{Lo}$ progenitor cell self-renewal in the postpartum mammary gland. In addition to its impact on mammary epithelium, deletion of Notch3 altered the mammary microenvironment, in particular, brown adipocyte differentiation in the mammary fat pad. Further experiments will be performed to define a potential role for Notch3-regulated brown adipose tissue in breast cancer initiation and

progression. Finally, analysis of TCGA and METABRIC human breast cancer data sets revealed a negative correlation between expressions of *NOTCH3* and *ESR1* (encoding estrogen receptor α), and association of high *NOTCH3* expression with enhanced survival of patients with luminal A and luminal B subtype breast tumors. Taken together, Notch3 can act as a tumor suppressor in the mammary epithelium, and possibly in the mammary microenvironment as well, to prevent development and metastasis of ER-positive breast cancer.