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13. SUPPLEMENTARY NOTES**14. ABSTRACT:**

Transforming growth factor beta (TGFB) family members regulate multiple cellular functions and key reproductive processes in a contextually dependent manner via the interaction with membrane associated serine/threonine kinase receptor complexes (TGFB1/TGFB2) and downstream SMAD proteins. To complement our mouse model containing a constitutively active TGFB1 using growth differentiation factor 9 (*Gdf9*)-Cre (i.e., TGFB1-gCA), we herein generated a mouse model using *Zp3*-Cre line (termed TGFB1-zCA). We performed a number of experiments including H & E staining, immunohistochemistry, and apoptosis assay to analyze potential ovarian phenotype of these mice. In contrast to TGFB1-gCA mice, the TGFB1-zCA mice did not develop ovarian tumors and demonstrated essentially normal ovarian histology and expression of granulosa cell and germ cell proteins. Using ovarian RNA from TGFB1-gCA mice and controls, we performed RNA-seq experiment. Initial analysis identified 1301 genes that were differentially regulated in the TGFB1-zCA ovaries versus controls. Interestingly, a number of genes are associated with folliculogenesis and oogenesis. Further studies will be focused on exploiting the RNA-seq data and defining key regulators and/or pathways for ovarian tumor development.

15. SUBJECT TERMS

Ovarian tumor, TGF-beta signaling, Follicular development, Proliferation, RNA-seq

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1. Introduction

Transforming growth factor beta (TGF β) family members regulate a myriad of cellular functions and key reproductive processes in a contextually dependent manner via the interaction with membrane associated serine/threonine kinase receptor complexes and downstream SMAD proteins (Massague 2012). TGFBR1/TGFBR2, the type 1 and 2 receptors for TGF β s, mediate essential signaling of TGF β ligands. Increasing lines of evidence have documented the critical involvement of TGF β signaling in reproductive development, function, and dysfunction (Knight & Glister 2006, Li 2014). The function of TGF β signaling in mouse oocytes is not clear. The aim of this project during the reporting period is to identify the oncogenic insult of sustained activation of TGF β signaling in the oocyte. To complete aim, we have proposed to explore the stage-specific function of constitutively active TGFBR1 in the oocyte, and discover novel regulatory mechanisms of granulosa cell proliferation.

2. Keywords

Ovarian tumor, Sex cord-stromal tumor, TGF-beta signaling, Overactivation, *Gdf9*-Cre, *Zp3*-Cre, Folliculogenesis, Proliferation, Apoptosis, Malignant transformation

3. Accomplishments

- *What were the major goals of the project?*

The major goal during this reporting period is to identify the oncogenic insult of sustained activation of TGF β signaling in the oocyte. The proposed milestones are: 1.) To create TGFBR1 *Zp3*-Cre constitutively active mice, and 2.) To define key regulators/pathways for sex cord-stromal tumor development. We have achieved the first milestone, and made significant progress toward the second milestone.

- *What was accomplished under these goals?*

1.) Major activities and specific objectives

A. Major Task 3: Explore the stage-specific function of constitutively active TGFBR1 in the oocyte

Subtask 1: Generating *TGFBR1 Zp3*-Cre constitutively active mice

Subtask 2: Validation of the model

Under the proposed Major Task 3, we have generated *TGFBR1 Zp3*-Cre constitutively active mice (TGFBR1-zCA) and characterized these mice using histological, immunohistochemical, and apoptotic analyses, which will be detailed in the following “*Significant results or key outcomes*” section. The proposed milestone for Major Task3 has been completed.

B. Major Task 4: Discover novel regulatory mechanisms of granulosa cell proliferation resulting from constitutive activation of *TGFBR1* in the oocyte

Subtask 1: Sample preparation

Subtask 2: Next Generation Sequencing

Subtask 3: Data mining and validation

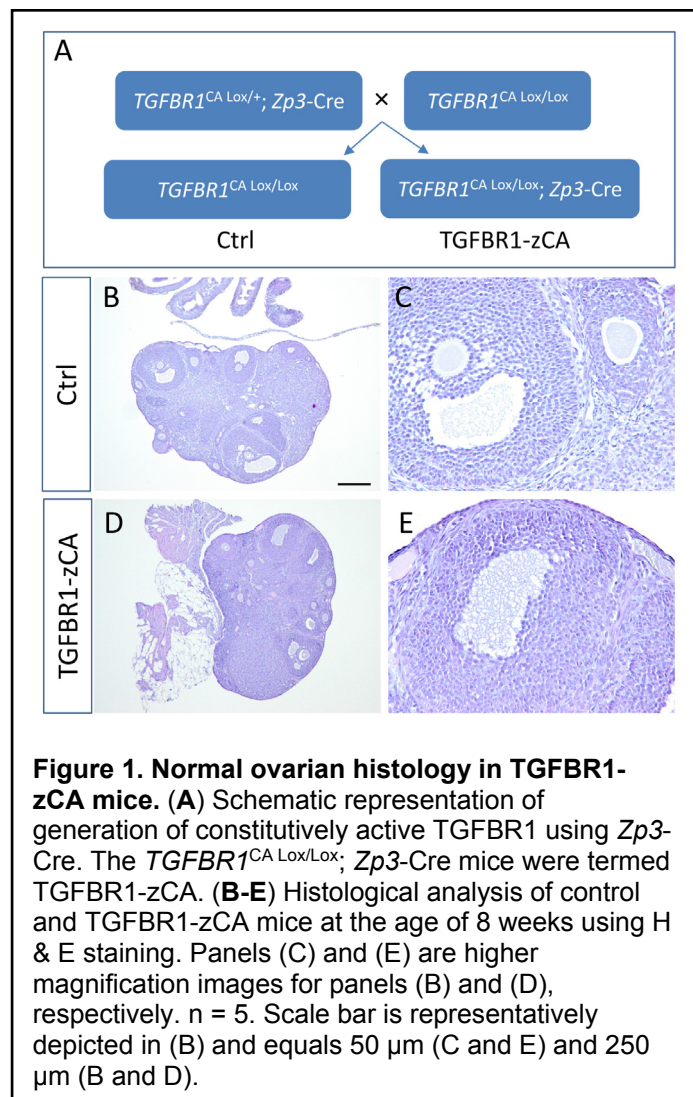
Under the proposed Major Task 4, we have prepared ovarian RNA samples, performed RNA-seq, and conducted preliminary analysis of RNA-seq results. The findings will be detailed in the following “*Significant results or key outcomes*” section. We will focus on Subtask 3 in the no-cost extension period to complete the proposed milestone of defining key regulators/pathways for sex cord-stromal tumor development.

2.) Significant results or key outcomes

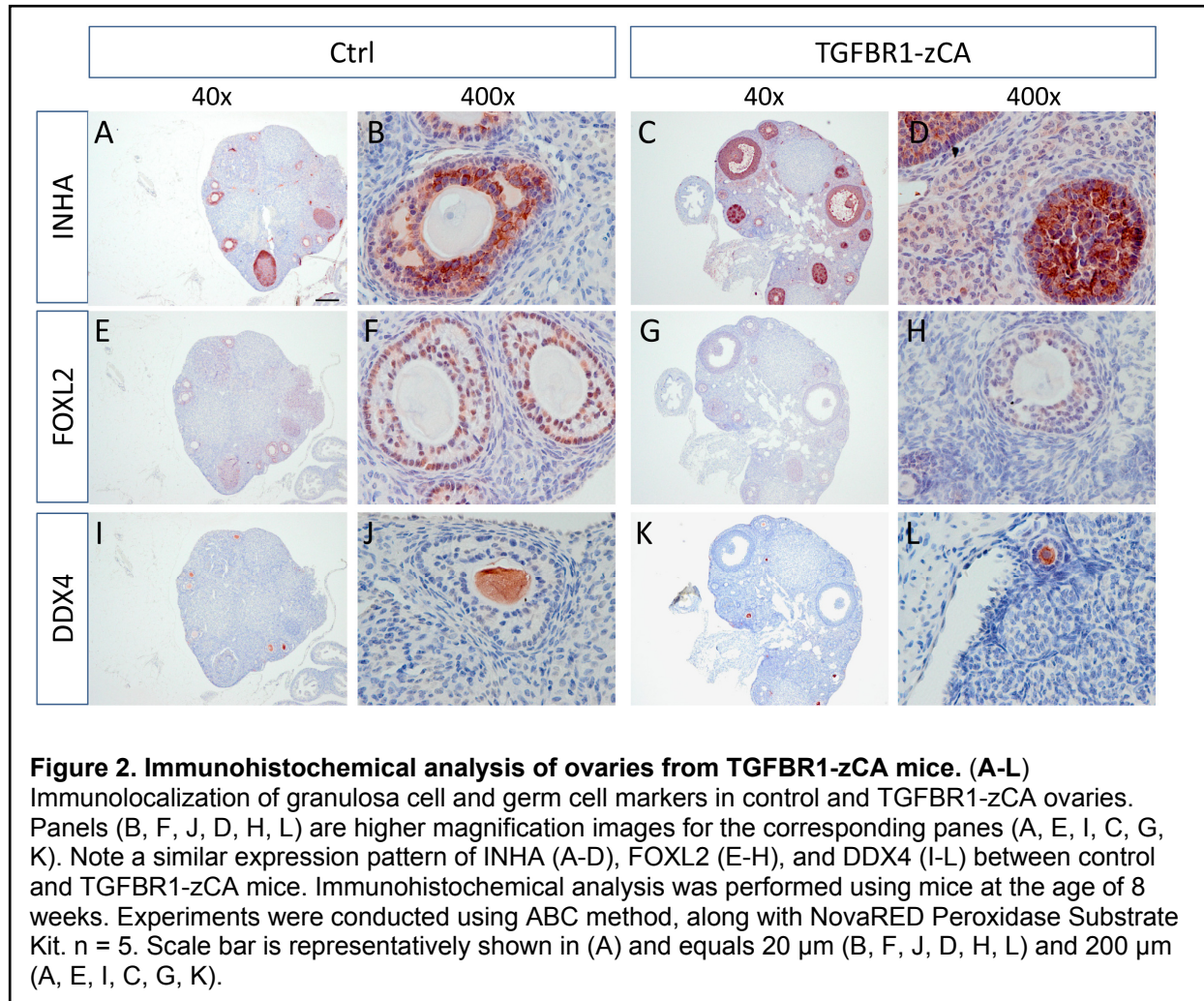
A. Major Task 3: Explore the stage-specific function of constitutively active *TGFBR1* in the oocyte

Gdf9-iCre is expressed in the germ cell from the primordial follicle stage (Lan *et al.* 2004). *Zp3*-Cre, which is expressed in growing oocytes, but not non-growing oocytes of primordial follicles (de Vries *et al.* 2000). To complement our *TGFBR1*-gCA mice, we generated a mouse model using *Zp3*-Cre line, which was purchased from The Jackson Laboratory (Stock no. 003651) (de Vries *et al.* 2000). First, we crossed the *Zp3*-Cre mice with *TGFBR1*^{CA} *Lox/Lox* mice to obtain *TGFBR1*^{CA} *Lox/+*; *Zp3*-Cre male mice. Then these mice were bred with *TGFBR1*^{CA} *Lox/Lox* females to generate the *TGFBR1*^{CA} *Lox/Lox*; *Zp3*-Cre female mice (termed *TGFBR1*-zCA; Figure 1A).

Next we analyzed potential ovarian phenotype of the *TGFBR1*-zCA mice at the age of 8 weeks (n = 5). In contrast to the *TGFBR1*-gCA mice, no ovarian abnormality was found macroscopically (not shown). To determine potential microscopic abnormalities, we performed H & E staining of the ovaries from both control and *TGFBR1*-zCA mice. The ovaries from both control and *TGFBR1*-zCA mice contained follicles at different developmental stages that appeared to be histologically normal (Figure 1B-E).



To examine whether molecular changes occur in the TGFB β 1-zCA mice, we performed immunohistochemical analysis using control and TGFB β 1-zCA ovaries and antibodies directed to inhibin alpha (INHA), forkhead box L2 (FOXL2), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4). Immunohistochemistry was performed using avidin-biotin complex (ABC) kit from Vector Laboratories as described (Li *et al.* 2011, Gao *et al.* 2016). Briefly, sections were deparaffinized and rehydrated before antigen retrieval, which was performed by boiling the sections in acidic sodium citrate buffer (pH 6.0) using a microwave. Following H₂O₂ treatment and blocking, the sections were first incubated with primary antibodies directed to INHA (AbD Serotec), FOXL2 (Abcam), and DDX4 (Cell Signaling). Subsequently, the sections were incubated with secondary antibodies and ABC reagents. Signals were developed using NovaRED Peroxidase Substrate Kit (Vector Laboratories). As expected, granulosa cells from control mice were positively stained for INHA and FOXL2 (Figure 2A, B, E, and F), while oocytes were immunoreactive with anti-DDX4 antibody (I and J). In line with the normal histological observations in the TGFB β 1-zCA ovaries (Figure 1D and E), no differences in the expression pattern of INHA, FOXL2, and DDX4 were found in the ovaries between TGFB β 1-zCA mice (Figure 2C, D, G, H, K, and L) and control mice (Figure 2A, B, E, F, I, and J).



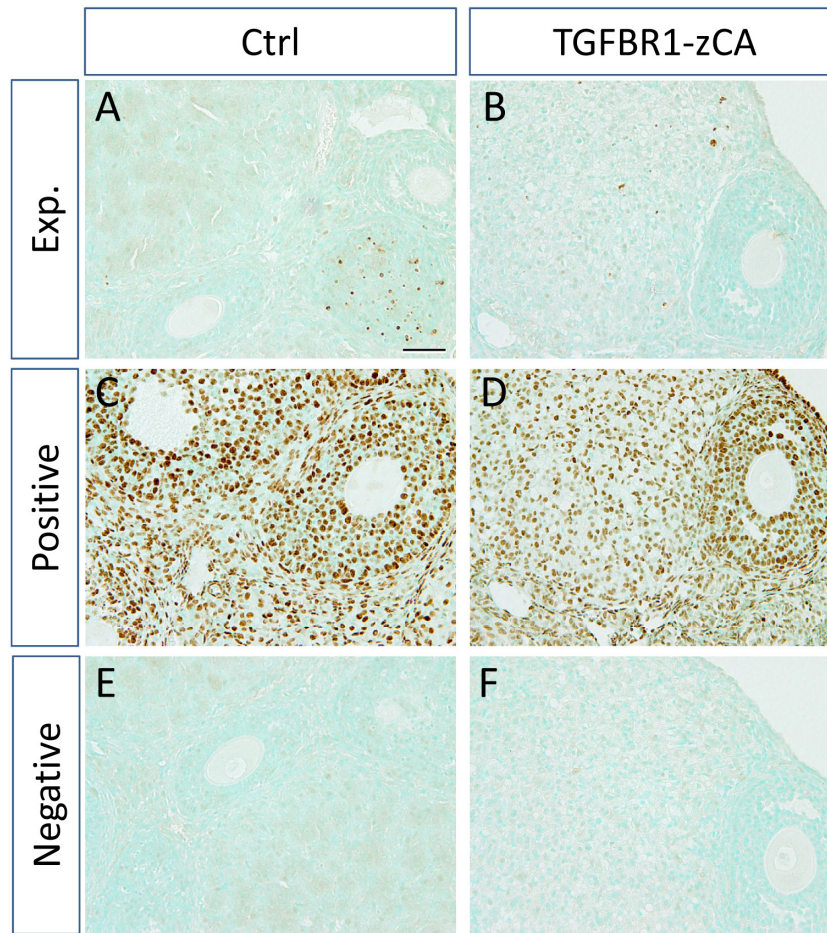


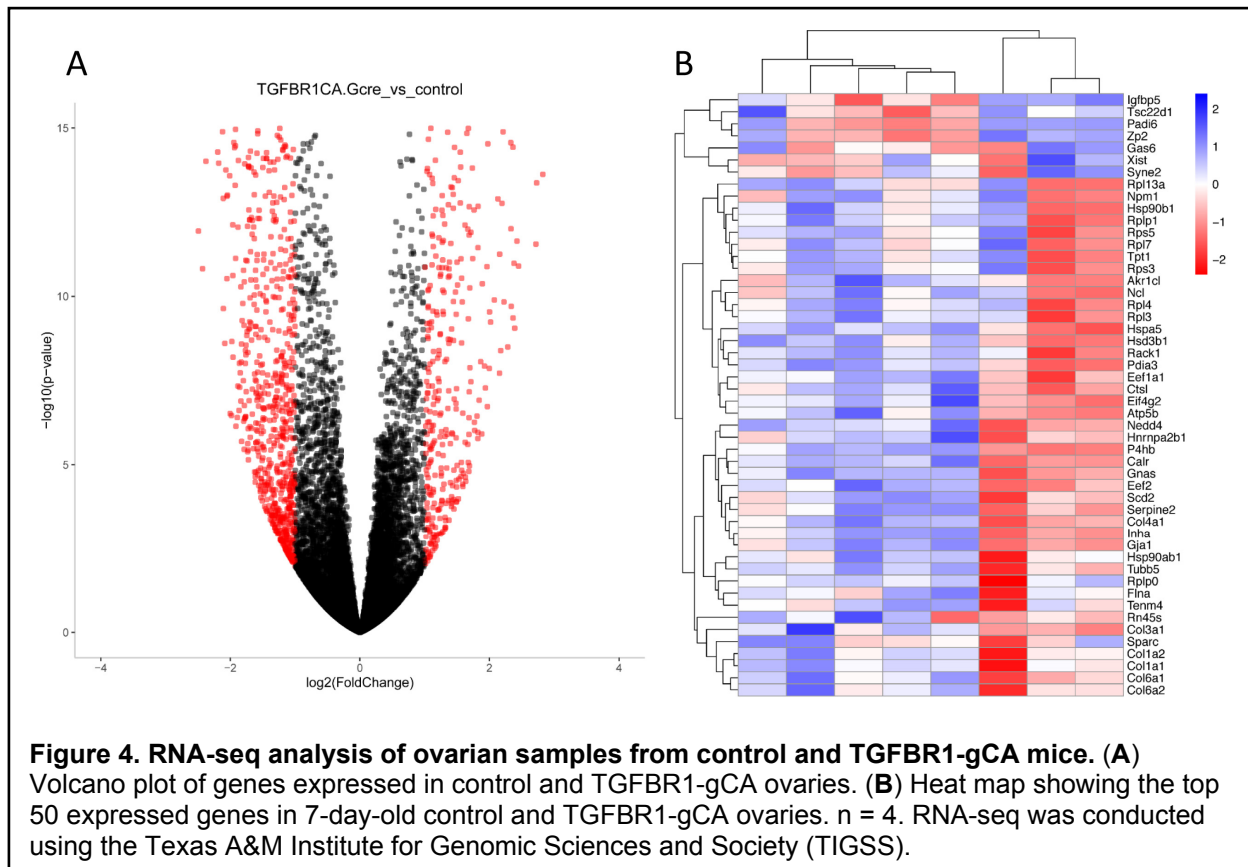
Figure 3. Analysis of apoptotic cells in ovaries from control and TGFBR1-zCA mice. (A and B) Analysis of apoptotic cells using 8-week-old control and TGFBR1-zCA ovaries. (C and D) Positive controls where ovarian sections were treated with DNase I. (E and F) Negative control where terminal deoxynucleotidyl transferase (TdT) was replaced by water. TdT and DAB were used to label apoptotic cells within the ovary. Sections were counterstained with Methyl Green. n = 5. Scale bar is representatively shown in (A) and equals 50 μ m (A-F).

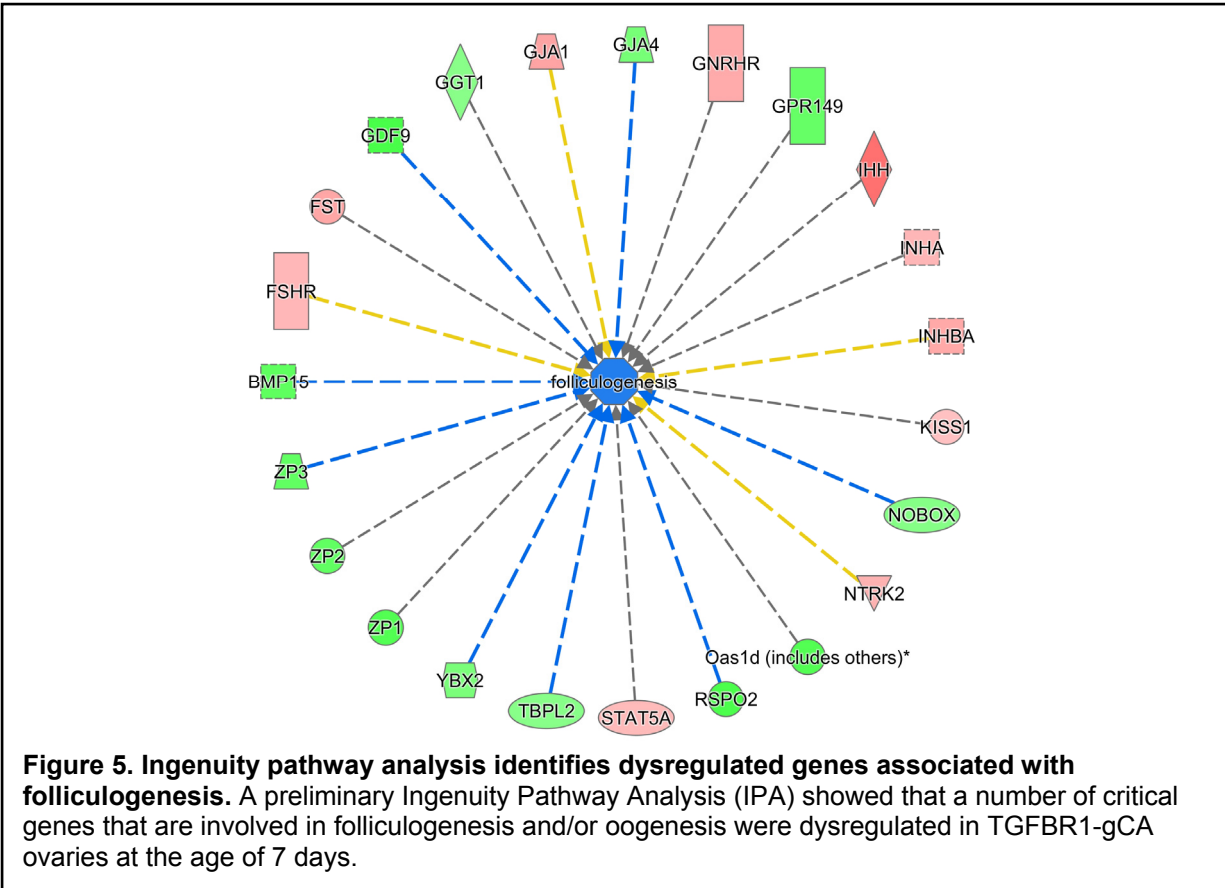
Since cell proliferation and death are altered in tumors, we used a commercially available ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore) to analyze apoptosis in ovarian samples from control and TGFBR1-zCA mice. Briefly, paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol before the treatment with Proteinase K. Then 3% H₂O₂ was applied to inactivate endogenous peroxidases. Apoptotic cells were labeled with terminal deoxynucleotidyl transferase (TdT) and incubated with streptavidin-horseradish peroxidase (HRP) conjugate. The signals were detected using DAB substrate and slides were counterstained with Methyl Green. Compared to controls, apoptosis did not appear to be altered in TGFBR1-zCA ovaries (Figure 3A and B). Corresponding positive (Figure 3C and D) and negative (Figure 3E and F) controls were included.

The finding that TGFBR1-zCA did not develop ovarian tumor phenotype posed several possibilities including potential requirement of activation of TGFBR1 during primordial stage of follicle development for ovarian tumorigenesis, direct or indirect activation of TGFBR1 within somatic cell compartment in TGFBR1-gCA ovaries, and/or low efficiency of TGFBR1 activation in TGFBR1-zCA ovaries. Our qPCR analysis using total RNAs prepared from TGFBR1-zCA and controls did not show increased expression of *TGFBR1^{CA}* transcripts in TGFBR1-zCA ovaries (not shown), which is in line with the incapability of detecting recombined conditional beta-catenin in the ovary by the original research using *Zp3-Cre* line potentially due to the low ratio of the oocytes to somatic tissue in the ovary (de Vries *et al.* 2000).

B. Major Task 4: Discover novel regulatory mechanisms of granulosa cell proliferation resulting from constitutive activation of TGFBR1 in the oocyte.

RNA-seq is a powerful tool with high sensitivity and resolution to profile gene expression. To identify novel regulators/pathways of ovarian granulosa cell tumors, we isolated total RNA from TGFBR1-gCA and control mice at the age of 7 days and performed RNA-seq analysis. The assay and data processing were performed using Texas A&M Institute for Genomic Sciences and Society (TIGSS). The volcano plot and heat map of the top 50 expressed genes were shown (Figure 4). Initial analysis has identified 1301 genes that were differentially regulated. An Ingenuity Pathway Analysis identified a number of genes that are associated with folliculogenesis and oogenesis (Figure 5; top of next page). The RNA-seq data will be further exploited. Spatial expression of genes of interest will be examined when possible.





- What opportunities for training and professional development has the project provided?
Nothing to Report.
- How were the results disseminated to communities of interest?
Nothing to Report.
- What do you plan to do during the next reporting period to accomplish the goals?
In the next reporting period (i.e., no-cost extension), we will focus on Subtask 3 (i.e., data mining and validation) to complete the proposed goal and milestone of identifying key regulators and/or pathways for sex cord-stromal tumor development.

4. Impact

- What was the impact on the development of the principal discipline(s) of the project?
The potential impact of this project is to identify new diagnostic and therapeutic targets for ovarian granulosa cell tumors.

- What was the impact on other disciplines?
Nothing to Report.
- What was the impact on technology transfer?
Nothing to Report.
- What was the impact on society beyond science and technology?
Nothing to Report.

5. Changes/Problems

- Changes in approach and reasons for change

We initially planned to use TGFBR1 *Zp3*-Cre constitutively active (CA) mice for RNA-seq experiment. However, these mice did not develop ovarian tumors, making them unsuitable for the proposed experiment. Thus, we made a minor modification of the experimental approach to use TGFBR1 *Gdf9*-Cre CA ovaries (day 7) for the proposed experiment. Approval of the amendment of animal use protocol was obtained from both institutional IACUC and DOD's ACURO.

- Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.
- Changes that had a significant impact on expenditures
Nothing to Report.
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

6. Products

- Publications, conference papers, and presentations

Xin Fang, Yang Gao, Qinglei Li. SMAD3 Activation: A Converging Point of Dysregulated TGF-Beta Superfamily Signaling and Genetic Aberrations in Granulosa Cell Tumor Development? *Biology of reproduction*. 2016 95(5):105. [Review paper]

- Website(s) or other Internet site(s)
Nothing to Report.
- Technologies or techniques
Nothing to Report.
- Inventions, patent applications, and/or licenses
Nothing to Report.

- Other Products

The mouse model (i.e., TGFBR1-gCA) created by this project develops ovarian tumors, which could be potentially used for preclinical testing of therapeutic agents.

7. Participants & Other Collaborating Organizations

- What individuals have worked on the project?

Name	Project Role	Nearest person month worked	Contribution to the project
Yang Gao	Graduate Student	6 Calendar	Mouse breeding, immunostaining, apoptosis detection, and data analysis
Xin Fang	Graduate student	3 calendar	Genotyping and histological analysis
Qinglei Li	PI	3 Calendar	Mouse breeding, data analysis, and project supervision
David Threadgill	Co-investigator	0.6 Calendar	Data analysis
Robert Burghardt	Co-investigator	0.6 Calendar	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?

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TGF-beta signaling in endometrial cell function and dysfunction

National Institutes of Health, NICHD

The major goal of this project is to identify the mechanisms whereby TGF β signaling regulates endometrial cell function and determine how abnormal TGF β signaling causes endometrial dysfunction and fertility problems.

Role: PI

- What other organizations were involved as partners?

Nothing to report.

8. Special Reporting Requirements

None.

9. Appendices

References Cited under *Significant results or key outcomes*

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