

AWARD NUMBER: W81XWH-15-1-0082

TITLE: A New Paradigm for Ovarian Sex Cord-Stromal Tumor Development

PRINCIPAL INVESTIGATOR: Qinglei Li

CONTRACTING ORGANIZATION: Texas A&M Agrilife Research
College Station, TX 77843

REPORT DATE: May 2016

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE May 2016		2. REPORT TYPE Annual		3. DATES COVERED 15 Apr 2015 - 14 Apr 2016	
4. TITLE AND SUBTITLE A New Paradigm for Ovarian Sex Cord-Stromal Tumor Development				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-15-1-0082	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Qinglei Li, Yang Gao E-Mail: gli@cvm.tamu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) TEXAS A&M AGRILIFE RESEARCH COLLEGE STATION TX 77843-0001				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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: Transforming growth factor beta (TGFB) signaling regulates key reproductive events via TGFB1/TGFB2. To determine potential effect of overactivation of TGFB signaling in the oocyte, we generated a mouse model containing a constitutively active TGFB1 using growth differentiation factor 9 (<i>Gdf9</i>)-Cre (i.e., TGFB1-gCA). Follicle counting demonstrated that the number of primordial, primary, and secondary follicles was reduced in TGFB1-gCA ovaries compared with controls at P7. Concomitantly, abnormal follicle structures were detected in TGFB1-gCA ovaries, evidenced by INHA staining. These results suggest that sustained activation of TGFB1 using <i>Gdf9</i> -Cre disrupts folliculogenesis via affecting ovarian reserve and follicle growth/development. Apoptosis analysis using ovaries at critical timepoints during follicular development did not reveal alteration of oocyte apoptosis in TGFB1-gCA ovaries. Immunostaining was performed to determine the molecular identify of the tumors. The results showed that ovarian tumor tissues from TGFB1-gCA mice were positive for granulosa cell markers FOXL2, INHA, and FOXO1, supporting the formation of granulosa cell tumors in these mice. In the ovary culture system, SB-505124 seemed to improve follicle development in TGFB1-gCA ovaries. Therefore, sustained activation of TGFB1 using <i>Gdf9</i> -Cre leads to the development of ovarian neoplasms reminiscent of granulosa cell tumors.					
15. SUBJECT TERMS Ovarian tumor, TGF-beta signaling, Follicular development, Proliferation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 12	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	9
5. Changes/Problems.....	10
6. Products.....	10
7. Participants & Other Collaborating Organizations.....	10
8. Special Reporting Requirements.....	11
9. Appendices.....	11

1. Introduction

Transforming growth factor beta (TGFB) signaling regulates multiple cellular functions and key reproductive events via transmembrane receptors (TGFB β 1/TGFB β 2) and intracellular mediators of SMAD proteins. Until now, the function of TGFB signaling in mouse oocytes remains elusive. The aim of this project during the reporting period is to define the oncogenic role of constitutively active TGFB β 1 in the oocyte using *Gdf9*-Cre and the role of sustained activation of oocyte TGFB β 1 in ovarian tumor development using an *in vitro* approach.

2. Keywords

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

3. Accomplishments

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

The major goal during this reporting period is to define the oncogenic role of constitutively active TGFB β 1 in the oocyte using *Gdf9*-Cre and the role of sustained activation of oocyte TGFB β 1 in ovarian tumor development using an *in vitro* approach. The milestones are: 1.) The effect of sustained activation of TGFB β 1 in the oocyte identified (8 months), and 2.) The role of sustained activation of TGFB β 1 in the oocyte *in vitro* identified (4 months). These milestones have been achieved during this period.

- *What was accomplished under these goals?*

1.) Major activities and specific objectives

A. We collected postnatal ovaries from control and TGFB β 1-gCA females at postnatal days and performed histological assays and follicle number analysis. The specific objective of these studies is to determine the effect of sustained activation of TGFB β 1 using *Gdf9*-Cre on primordial follicle activation.

B. We performed apoptosis analysis using ovaries from both control and TGFB β 1-gCA mice at P3, P7, P12, and P21. The specific objective is to determine whether oocyte death and apoptosis is a potential contributing factor to granulosa cell tumor development.

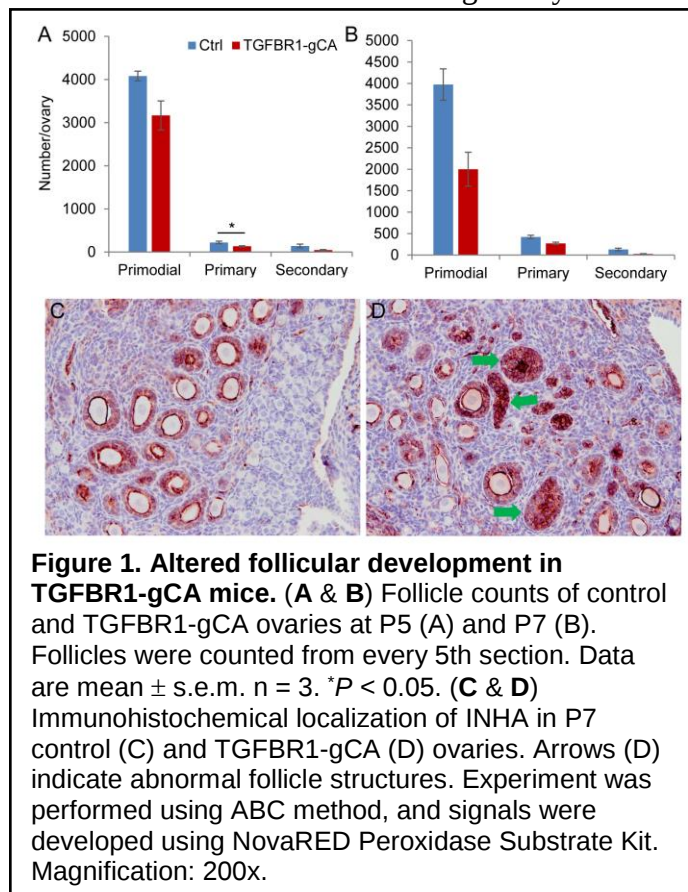
C. We performed immunohistochemistry analysis of ovaries from control and TGFB β 1-gCA mice at the age of 8 weeks. We used multiple granulosa cell markers including alpha inhibin (INHA), forkhead box protein O1 (FOXO1), forkhead box L2 (FOXL2), and anti-Mullerian hormone (AMH) and germ cell marker DEAD-box helicase 4 (DDX4). SRY-box 9 (SOX9), a Sertoli cell marker was also analyzed. The specific objective of these studies is to determine the molecular type of ovarian tumors in our model.

D. We collected ovaries and isolated oocyte cumulus complexes from control and TGFB1-gCA mice. We performed ovary culture in the absence or presence of TGFB1 inhibitor, SB 505124. The effect of TGFB1 inhibition on follicular development was analyzed.

2.) Significant results or key outcomes

A. Overactivation of TGFB1 using *Gdf9*-Cre perturbed follicular development at an early stage. Because *Gdf9*-Cre is expressed in primordial follicles, we performed histological analysis of follicular development in control and TGFB1-gCA mice at early postnatal stages. To perform follicle counting, ovaries were first serially sectioned (5 μ m) and stained with periodic acid Schiff's (PAS) and hematoxylin. Follicles were counted from every 5th section. Follicle classification was based on morphological criteria (Myers *et al.* 2004, Bristol-Gould *et al.* 2006). Morphologically, a primordial follicle contains an oocyte that is partially or completely encapsulated by one layer of squamous somatic cells, whereas a primary follicle has an oocyte surrounded by one layer of cuboidal granulosa cells. Follicles at transitional stages may contain both squamous and cuboidal granulosa cells, and were defined as primary follicles if surrounding somatic cells were mainly cuboidal. A secondary follicle consists of 2 or more layers of granulosa cells but has no antrum. Primordial and primary follicles were counted, regardless of the status of the oocyte nucleus. Since the oocyte diameter for primordial and primary follicles averages $\sim 14 \mu$ m, the same follicle could be counted every two sections. Hence, the estimated primordial and primary follicle numbers per ovary were calculated by multiplying the cumulative number of counts of each ovary by 5 and dividing by 2. For secondary follicles, only oocytes with nucleus were counted and a correction factor of 5 was applied to estimate the total number of secondary follicles per ovary (Bristol-Gould *et al.* 2006, Ren *et al.* 2012).

Follicle quantification did not show a significant difference in primordial follicles between control and TGFB1-gCA mice at postnatal day 5 (P5), although there was a reduction of primary follicle numbers in the TGFB1-gCA mice versus controls (Figure 1A). At P7, the number of primordial follicles, primary follicles, and secondary follicles was reduced in TGFB1-gCA ovaries compared with controls (Figure 1B). At this stage, abnormal follicle structures were detected in the ovary of TGFB1-gCA mice, evidenced by INHA staining (Figure 1C and D). These results suggest that



sustained activation of TGFBR1 in the oocytes using *Gdf9*-Cre disrupts ovarian folliculogenesis by affecting ovarian reserve and subsequent follicle development.

B. Activation of TGFBR1 using *Gdf9*-Cre did not promote apoptosis in the oocyte. It has been suggested that loss of oocytes during follicular development may alter the differentiation and cell fate of ovarian granulosa cells (Pitman *et al.* 2012). We therefore performed apoptosis analysis to determine whether there is a link between oocyte apoptosis and abnormal granulosa cell differentiation and tumor formation. To achieve this goal, we utilized a commercially available *In situ* Apoptosis Detection Kit to stain ovarian samples at P3, P7, P12, and P21 (n = 5). Briefly, paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol before treated 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 diaminobenzidine (DAB) and slides were counterstained with Methyl Green. To validate this kit, we showed that samples treated with DNase I contained abundant apoptotic cells (i.e., positive control), whereas negative controls where TdT was substituted with water showed only background staining (Figure 2A and B). Our apoptosis analysis using ovarian samples from several critical time points during early follicular development did not reveal alteration of oocyte apoptosis in TGFBR1-gCA ovaries (Figure 2C and D). Extensive apoptosis was detected in ovarian somatic cells at P21 in TGFBR1-gCA mice compared with controls, where apoptosis was confined to certain follicles that were atretic or would potentially be atretic (Figure 2E and F). Thus, these results suggest that oocyte apoptosis may not be a main contributing factor to ovarian tumor development in our mouse model.

C. Molecular analysis of ovarian tumor type. To define the molecular identity of ovarian tumors in TGFBR1-gCA mice, we performed immunostaining to determine expression of a granulosa cell lineage maker FOXL2 (Schmidt *et al.* 2004) and 3 other granulosa cell-expressed proteins, FOXO1, INHA, and AMH. DDX4, a germ cell marker, was also included. Briefly, Tissue processing and embedding were carried out using the histology core facility of the Department of Veterinary Integrative Biosciences at

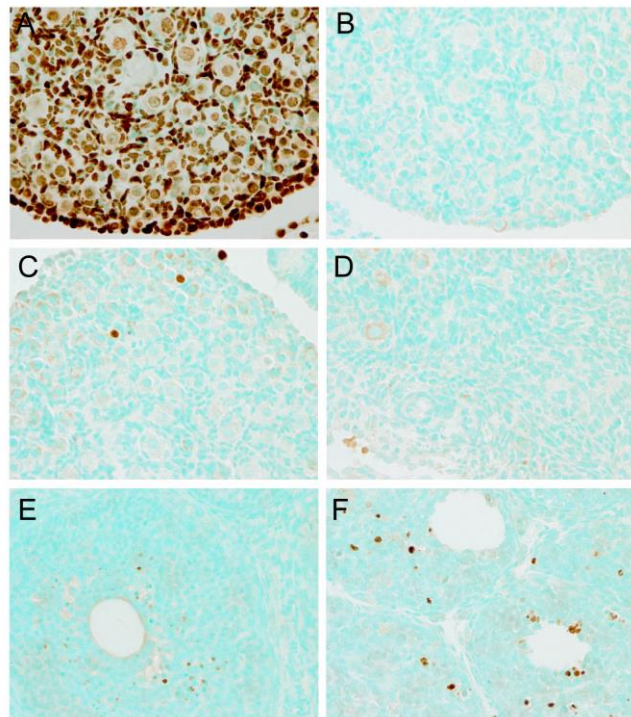


Figure 2. Apoptosis analysis of postnatal ovaries from control and TGFBR1-gCA mice. (A) P3 ovarian sections treated with DNase I as positive control. (B) Negative control where TdT was replaced with water. (C-F) Representative images of apoptosis analysis of P3 and P21 control (C and E) and TGFBR1-gCA (D and F) ovarian sections. Apoptotic cells were labeled with terminal deoxynucleotidyl transferase (TdT) and signals were detected using DAB. Sections were counterstained with Methyl Green. At least 4 independent samples per group were used in this analysis. Magnification: 400x.

Texas A&M University. Paraffin sections (5 μ m) were used for both immunofluorescence and immunohistochemistry as described (Li *et al.* 2011, Gao *et al.* 2014). Briefly, sections were deparaffinized in xylene and rehydrated in graded alcohol. Then the slides were subject to antigen retrieval using citrate buffer. For immunohistochemical analysis, sections following antigen retrieval were treated with H₂O₂ and blocked with 5% non-immune serum, and incubated with primary and secondary antibodies and Avidin/Biotin Complex (ABC; Vector Laboratories). The signals were developed using NovaRED Peroxidase Substrate Kit (Vector Laboratories). The sections were counterstained with hematoxylin and mounted with Permount. For immunofluorescence microscopy, the sections were blocked with 5% bovine serum albumin (BSA) and sequentially incubated with primary antibodies and secondary antibodies conjugated with Alexa Fluor 594 (1:400; Invitrogen). The slides were mounted using ProLong Gold Slowfade media with DAPI. The immunofluorescence signals were examined using an IX73 fluorescence microscope. Images were captured using an XM10 CCD camera and cellSens® Digital Imaging Software (Olympus). Isotype-matched IgGs were included as negative controls for these assays.

The localization of FOXL2 (Figure 3A), INHA (Figure 3C), FOXO1 (Figure 3E), AMH (Figure 3G), and DDX4 (Figure 3I) was detected in the granulosa cell or oocyte compartment of the control mice, while ovarian tumor tissues from TGFBR1-gCA mice were immunoreactive with FOXL2 (Figure 3B), INHA (Figure 3D), and FOXO1 (Figure 3F), supporting the development of granulosa cell tumors in these mice. However, expression of AMH was close to background level in the tumor tissues (Figure 3H). These tumors did not express DDX4 (Figure 3J). Representative negative controls using rabbit and goat IgGs were respectively shown in

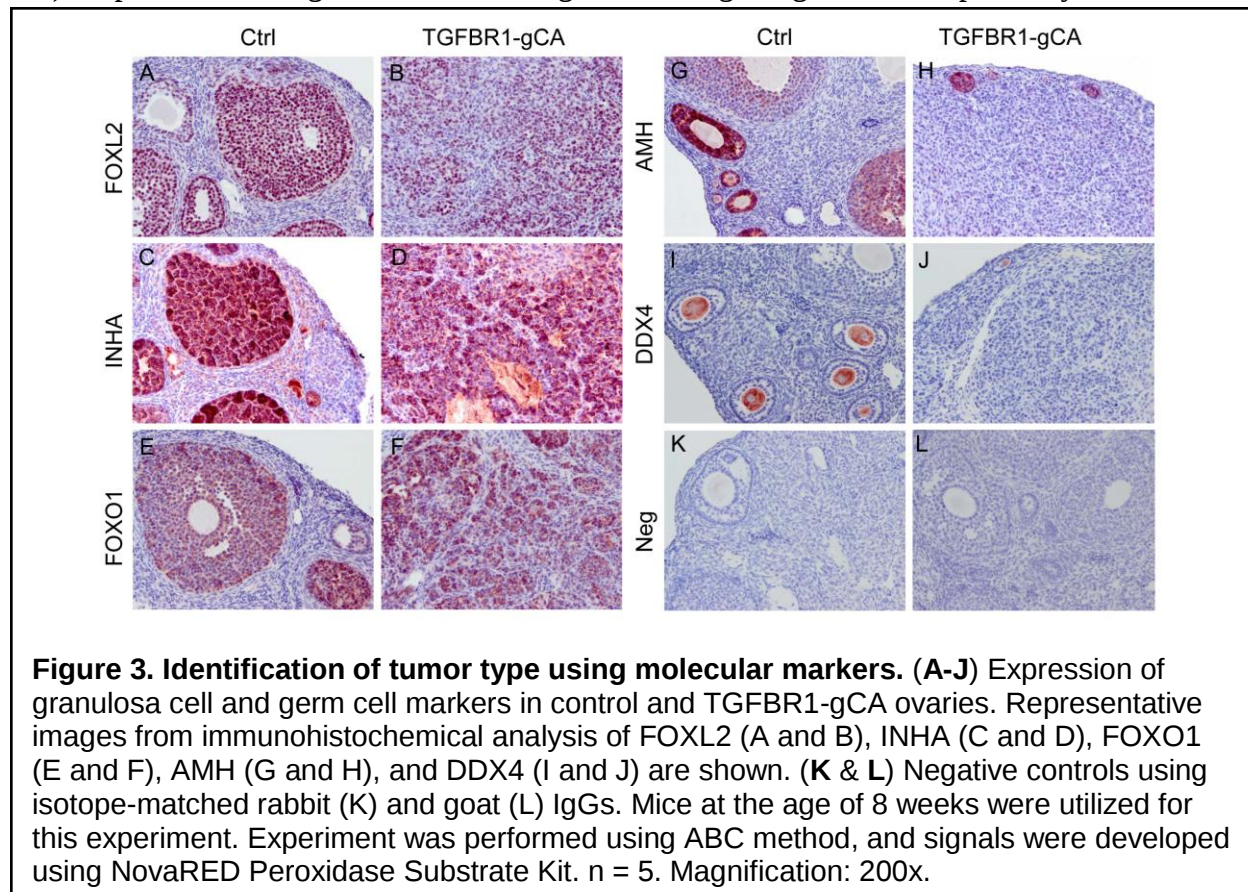
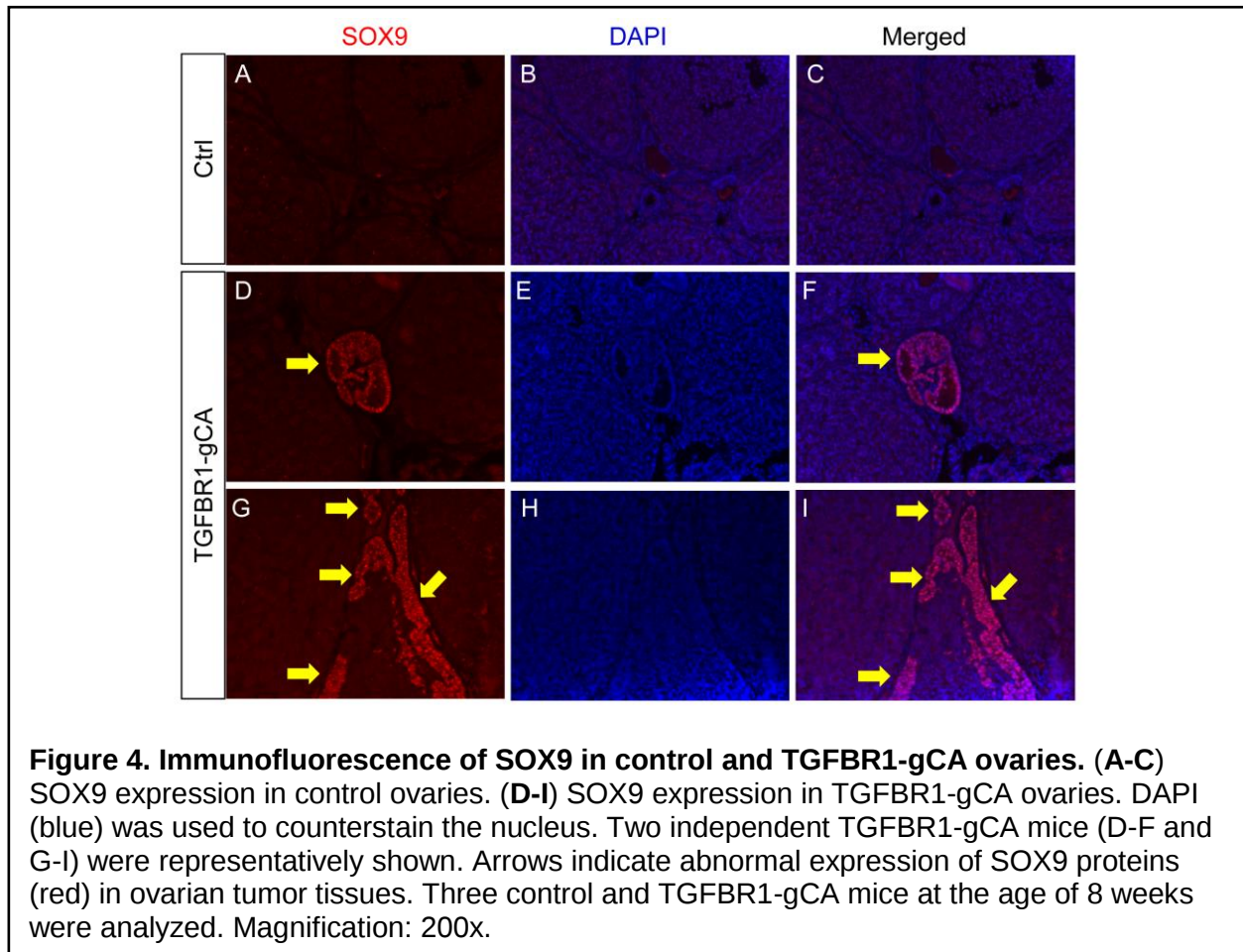


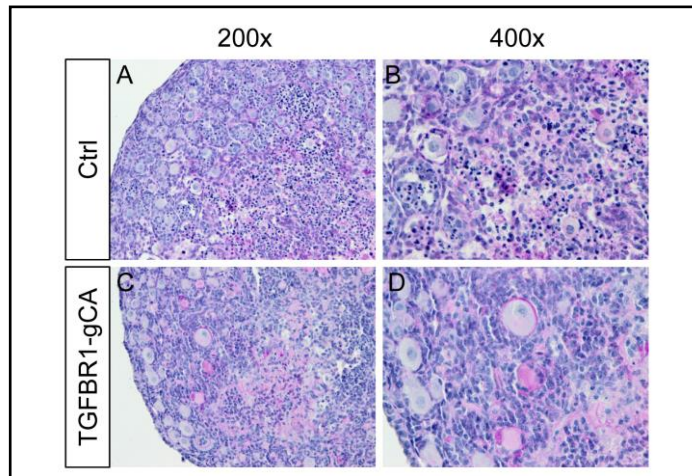
Figure 3K and L. Because granulosa cell tumors can express Sertoli cell marker (Liu *et al.* 2015), we examined whether Sertoli cell-like components were present in the TGFBR1-gCA tumors. Our results showed expression of SOX9 in ovarian tumors resulting from TGFBR1 overactivation (Figure 4D-I; arrows). Thus, sustained activation of TGFBR1 using *Gdf9-Cre* causes the formation of ovarian neoplasms reminiscent of granulosa cell tumors.



D. Identify the role of sustained activation of oocyte TGFBR1 in ovarian tumor development using *in vitro* approach. We took advantage of a small molecule inhibitor of TGFBR1 to further elucidate the oncogenic property of the constitutively active TGFBR1 using *in vitro* culture approach. In brief, we collected ovaries from control and TGFBR1-gCA mice and cultured them using Transwell membrane insert (Dutta *et al.* 2014). Ovaries were cultured in the presence or absence of SB-505124 for 10 days at 37°C supplemented with 5% CO₂. Culture was fed every 2 days. After that, the ovaries were fixed and processed for histological analysis. We also isolated oocyte-granulosa cell complex (OGC) by using 0.1% collagenase digestion of P12 ovaries (Eppig & O'Brien 1996). While OGCs retrieved from the control mice were morphologically normal and similar in size, OGCs obtained from the TGFBR1-gCA mice varied in size and contained large abnormal structures, suggesting that overactivation of TGFBR1 using *Gdf9-Cre* disrupts folliculogenesis and promotes the formation of abnormal OGCs. Interestingly, our culture experiment revealed disorganized and abnormal follicle structures within vehicle-treated TGFBR1-gCA ovaries (Figure 5A and B). Incubation of TGFBR1-gCA ovaries with SB-

505124 (10 μ M) seemed to improve follicle development in the TGFBR1-gCA ovaries (Figure 5C and D). Therefore, these studies reinforce that ovarian tumor development in TGFBR1-gCA mice is due to enhanced TGFBR1 activity.

Figure 5. Histological analysis of cultured TGFBR1-gCA ovaries treated with vehicle and TGFBR1 inhibitor. (A & B) TGFBR1-gCA ovaries treated with vehicle control. (C & D) TGFBR1-gCA ovaries treated with SB-505124. Ovary culture was performed using Transwell membrane insert in the presence or absence of SB-505124 (10 μ M) for 10 days before histological analysis using periodic acid Schiff's (PAS) and hematoxylin staining. Magnification: 200x (A & C) and 400x (B & D).



- 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, we plan to continue the research activities described in the original proposal. Two major goals are: 1.) Explore the stage-specific function of constitutively active TGFBR1 in the oocyte; and 2.) Discover novel regulatory mechanisms of granulosa cell proliferation resulting from constitutive activation of TGFBR1 in the oocyte. We will create a new mouse model using *Zp3-Cre* to determine whether the effect of TGFBR1 activation is follicle stage dependent. We will also perform RNA sequencing analysis to understand the mechanism of the ovarian tumor phenotype in our model system.

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

Nothing to Report.

- 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

Yang Gao and Qinglei Li. Sustained activation of transforming growth factor beta signaling using growth differentiation factor 9-driven iCre disrupts postnatal folliculogenesis and promotes malignant transformation of ovarian somatic cells (**Poster presentation**). *22nd Annual Meeting, Texas Forum for Reproductive Sciences*, Houston, Texas, USA, April 21 & 22, 2016.

- 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

This experiment created a mouse model that develops ovarian tumors (i.e., TGFBR1-gCA), 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	Contributed to sample collection, histological analysis, oocyte granulosa cell complex isolation, immunostaining, apoptosis detection, and data analysis
Qinglei Li	PI	3 Calendar	Contributed to sample collection, data analysis, and project supervision
David Threadgill	Co-investigator	0.6 Calendar	Contributed to experimental design
Robert Burghardt	Co-investigator	0.6 Calendar	Contributed to ovarian tissue culture

- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.
- 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*

- Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Cook RW, Kipp JL, Shea LD, Mayo KE & Woodruff TK (2006) Postnatal regulation of germ cells by activin: the establishment of the initial follicle pool. *Dev Biol* 298, 132-148.
- Dutta S, Mark-Kappeler CJ, Hoyer PB & Pepling ME (2014) The steroid hormone environment during primordial follicle formation in perinatal mouse ovaries. *Biology of Reproduction* 91.
- Eppig JJ & O'Brien MJ (1996) Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* 54, 197-207.
- Gao Y, Bayless KJ & Li Q (2014) TGFBR1 is required for mouse myometrial development. *Mol Endocrinol* 28, 380-394.
- Li Q, Agno JE, Edson MA, Nagaraja AK, Nagashima T & Matzuk MM (2011) Transforming growth factor beta receptor type 1 is essential for female reproductive tract integrity and function. *PLoS Genet* 7, e1002320.
- Liu Z, Ren YA, Pangas SA, Adams J, Zhou W, Castrillon DH, Wilhelm D & Richards JS (2015) FOXO1/3 and PTEN Depletion in Granulosa Cells Promotes Ovarian Granulosa Cell Tumor Development. *Mol Endocrinol* 29, 1006-1024.
- Myers M, Britt KL, Wreford NG, Ebling FJ & Kerr JB (2004) Methods for quantifying follicular numbers within the mouse ovary. *Reproduction* 127, 569-580.

- Pitman JL, Mcneilly AS, Mcneilly JR, Hays LE, Bagby GC, Sawyer HR & McNatty KP (2012) The fate of granulosa cells following premature oocyte loss and the development of ovarian cancers. *Int J Dev Biol* 56, 949-958.
- Ren Y, Cowan RG, Migone FF & Quirk SM (2012) Overactivation of hedgehog signaling alters development of the ovarian vasculature in mice. *Biology of reproduction* 86, 174.
- Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC & Treier M (2004) The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development* 131, 933-942.