Title

Student1, major; Student2, major; Mentor, department

Background: About 11% of women of reproductive age in the United States have experienced infertility, with the defective ovarian function as the leading cause (1). The ovary is the primary female reproductive organ and contains different developmental stages of follicles as the basic functional unit. Each follicle is made up of a central germ cell oocyte and the surrounding somatic cells. There is a finite number of primordial follicles set just after birth, and these follicles remain in a quiescent state to represent ovarian reserve, a marker of fertility potential. The dormant primordial follicles are activated in regular waves and sequentially develop to primary, secondary, and antral stages from birth until menopause (**Fig. 1**). As the follicle grows, it synthesizes and secretes sex steroid hormones (e.g. estrogen and progesterone) to maintain the function of the reproductive tract as well as contribute to the general health of the endocrine, cardiovascular, and immune systems (2). Meanwhile, the follicle matures and ovulates its enclosed oocyte for fertilization and pregnancy. Increasing evidence indicates that both industrial and environmental chemicals such as endocrine disrupting chemicals (EDCs) and anti-cancer drugs can result in female ovarian toxicity (ovotoxicity) and increase women's risk of hormonal imbalance, ovarian failure, anovulation, and infertility (3-5).

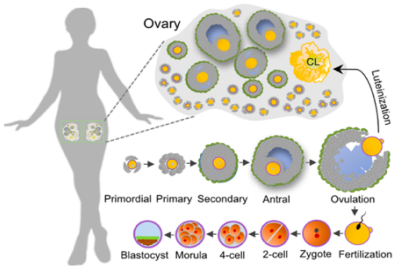

Figure 1. Ovary structure and dynamics of follicle development, oocyte maturation, ovulation and fertilization, and early embryo development.

The ovotoxicity of specific chemicals can be assessed based on two broad outcomes: (1) the health of the developing follicle and oocyte and subsequent ovulation and fertilization, and (2) the ability of the follicles to produce sex steroid hormones. Unfortunately, the lack of optimal *in vitro* models makes the current gold standard for testing the ovotoxicity of chemicals rely on the use of laboratory animals (OECD, 1995). However, *in vivo* models are time-consuming, laborious, costly, and harmful to animals, which does not comply with the Principal of 3Rs (Refine, Replace, and Reduce) to ensure animal welfare. As ovarian follicles represent a robust target for evaluating chemical's ovotoxicity, in the laboratory of [mentor], he and his team have used an alginate hydrogel-based encapsulation method to grow both mouse and human preantral follicles outside of the context of the ovary, which is termed encapsulated *in vitro* follicle growth (eIVFG) (6-8). The eIVFG maintains the 3D architecture of the follicles and supports follicle growth, hormone secretion, as well as oocyte maturation and ovulation, suggesting a promising model for *in vitro* ovotoxicity testing. However, this method is relatively slow, and multiple rounds of follicle isolation, culture, and chemical exposure are necessary to complete dose-response and time-course experiments, making the current eIVFG scenario not optimal, particularly when a rapid screening is required in the case of unpredictable and emerging environmental threats. Moreover, multiple rounds of follicle isolation require large numbers of animals.

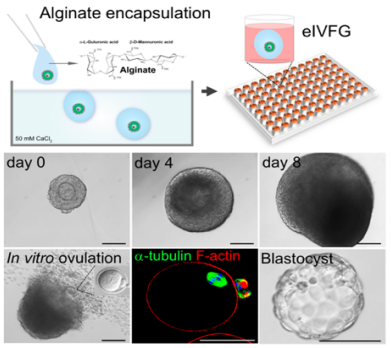

Figure 2. The eIVFG supports follicle development, oocyte maturation, and ovulation. The ovulated oocytes are capable of being fertilized through *in vitro* fertilization (IVF) and give rise to embryos that develop to the blastocyst stage.

Research Question: In order to establish an efficient and reliable female reproductive toxicity screening platform, can *in vitro* global primordial follicle activation and follicle cryopreservation be used to transform the current mouse follicle preparation strategy into a high-throughput and standardized follicle isolation, encapsulation, storage, and culture platform?

Project Goals and Objectives: The goal of this project is to develop a high-throughput *in vitro* ovotoxicity testing platform to screen chemicals' female reproductive toxicity. The objectives include: (1) to globally activate mouse primordial follicles for collecting a substantial and a homogeneous population of preantral follicles for eIVFG; and (2) to bank the isolated ovarian follicles through cryopreservation for establishing a long-term and ready-to-use follicle storage platform.

Project Significance: This project is significant to public health as the proposed platform will translate a bench assay for a single compound into a robust high-throughput screening assay, providing rapid and reliable evidence to understand the impacts of pharmaceutical drugs and environmental contaminants on the female reproductive system, as well as provide guidance for preventative and protective measures to be conducted. Equally important, the banking of ovarian follicles through global follicle activation and cryopreservation allows us to significantly minimize the cost, dependence, and use of animals.

Project Design:
Two students are needed to work on this project because of the time commitment of the procedures listed below. It is also essential the students are experienced in their chosen methods to reduce the amount of mistakes and the data obtained is efficient and accurate.

Aim 1: To globally activate primordial follicles for collecting a substantial and a homogeneous population of mouse preantral follicles. There are approximately 1-2 million and 10,000 primordial follicles present at birth in human and mouse ovaries, respectively, but only 0.1% of them in humans and 5% of them in mice will be activated and ultimately mature to antral stage for ovulation (9). Previous studies demonstrated that mice with deletion of genes of PTEN and FOXO3 or with over expression of PI3K in oocytes, the key components of PTEN-PI3K-AKT-FOXO3 signaling that regulates follicle activation, exhibited global activation of primordial follicles as well as normal maturation of the follicles (10-13). These results suggest an ideal regimen to obtain a substantial and homogenous population of preantral follicles using a small number of animals. Here, we will use the pharmacologic method to globally activate mouse primordial follicles, which has shown success in other studies (10). Specifically, we will collect and treat 5-day old CD-1 mouse ovaries with bpV(pic) at 100 μM, a PTEN inhibitor, and 740Y-P at 500 μg/mL, a PI3K activating peptide, for 48 hours (hr), to activate the PTEN-PI3K-AKT-FOXO3 signaling. We will then culture the treated ovarian pieces for an additional 8 days to allow activated primordial follicles to further develop to the secondary stage. We will then use the enzymatic method to retrieve a homogeneous population of secondary follicles with diameter at 100-130 μm as [mentor] described in previous studies (6, 14, 15). We will also test other pharmacologic conditions, including the bpV(pic) and 740Y-P concentration and animal age for ovary collection and treatment to further optimize the follicle activation efficiency. We expect that the global primordial follicle activation will allow us to collect 800-1,000 follicles per ovary, which is >100-fold higher than the method using non-treated ovaries.

Aim 2: To bank the isolated preantral follicles through cryopreservation for establishing a long-term and ready-to-use follicle storage platform. Vitrification is a process that allows the solidification of the cells and extracellular milieus into a glass-like state (16). This method is simple, cost-effective, and requires less procedural time and vitrification of gametes, embryos, or ovarian tissues is increasingly used as assisted reproductive technologies (ART) and for fertility preservation (17), suggesting that this technique can maintain reproductive cell viability and functionality. Therefore, we will use the vitrification method to cryopreserve isolated mouse preantral follicles from **Aim 1**. Since vitrification has been used in fertility treatments over long-term periods, it is expected this study will show similar results (18). We will make minor modifications to previous vitrification protocols based on follicles' specific characteristics. Briefly, follicles will be equilibrated in the equilibration solution (ES), vitrification solution (VS), and VS supplemented with synthetic polymers (VS+PXZ). The synthetic polymers will be used because they can further prevent intracellular ice formation (IIF) and devitrification (17,19, 20). Follicles will be vitrified and stored using a CBS High Security Tissue Straw in a liquid nitrogen vapor system with up to 200 follicles being vitrified in one straw. We will next evaluate the effect of vitrification on follicle quality, including both short-term and long-term after cryopreservation. Specifically, we will first warm and recover vitrified follicles after 8-weeks and 4 months post vitrification. The follicles will be encapsulated with 0.5% alginate first and cultured *in vitro* using eIVFG (**Fig. 2**). We will compare follicle reproductive outcomes between vitrified and thawed follicles and freshly isolated follicles. The parameters to be compared include follicle growth and survival, hormone secretion, *in vitro* ovulation, and oocyte fertilization and embryo development outcomes.

Project Timeline, Anticipated Results and Dissemination: The project timeline is shown in the table on the right. We anticipate that the global follicle activation will allow us to obtain 1600-2000 preantral follicles per mouse, which is >100-fold increase compared to manual follicle preparation, significantly reducing the use of animals. Moreover, the follicle cryopreservation will enable us to obtain a large amount of ready-to-use encapsulated follicles for *in vitro* ovotoxicity testing. We will further use a representative set of chemicals with known ovotoxicity to validate this platform. The research data will be presented at Discover USC . We will use the results to write and publish the manuscript in a peer-reviewed scientific journal.

Task Description	2019					2020			
	Aug	Sep	Oct	Nov	Dec	Jan	Feb	March	April
Perform experiments of global follicle activation, isolation, vitrification, follicle growth and ovulation	X	X	X						
Perform experiments of follicle hormone secretion and gene expression analyses				X	X	X			
Perform experiment of evaluating follicle quality long-term after cryopreservation. Analyze data and prepare for presentations/publications							X	X	X

Personal Statements:

Student1: I will be responsible for the proposed experiments in **Aim 1**. I have been in [mentor's] lab since the beginning of the Fall semester in 2018. I am a Junior of the class of 2020, majoring in Biology. I plan to go on to medical school to become an OB/GYN. I am passionate about women's health and my interest in women's health extends to my other extracurriculars on and off campus. On campus I am involved with Changing Carolina Peer Leaders, where I do health promotion and education on sexual health and sexual assault intervention and prevention. And off campus I volunteer with STSM as an advocate for sexual assault survivors and I also volunteer at Palmetto Baptist in the NICU and Women's Surgery departments. I was looking for a research opportunity that aligned with my interest in women's health. Since working with [mentor], I have learned so much about the reproductive system and have been able to apply things that I have learned in class to a practical lab setting while also learning many different lab procedures and methods. The experience that I will gain from [mentor]'s lab is unique and invaluable. I would love the opportunity to continue my research with the help of the Magellan Scholar grant, as it would improve my application to medical school as well as further my knowledge in women's health, which will be beneficial as I move further in my career plans. I believe this experience will be something I will be able to carry with me throughout my entire career.

Student2: I will be responsible for the proposed experiments in **Aim 2**. As a Biology major and student on the Pre-Medical track, working with [mentor] has been an essential part in furthering my passion for women's healthcare. I have learned invaluable information and laboratory protocols from working in the lab since February 2018. Since beginning my work in the lab, I have applied for and received both the Magellan Mini-Grant to help cover costs of materials for the beginning work on this project, as well as the Science Undergraduate Research Fellowship (SURF) Grant to help fund my time committed to this project. [Mentor] also gave me the opportunity to co-author another study, which is now in the process of being published in the *Future Oncology* Journal. I have completed the CITI ethics training, which has increased my understanding and appreciation of working in a lab. Receiving the Magellan Scholar grant will help me to continue working on this project to help grow my knowledge of women's reproductive health. Furthermore, working on this project will give me an edge over other applicants to medical school, since I plan to pursue a career in obstetrics and gynecology as a physician. My hope is to use the knowledge gained here to continue research throughout medical school and into my career as a physician to make advancements in women's healthcare.

References:

1. Chandra A, Copen CE, Stephen EH. Infertility and impaired fecundity in the United States, 1982-2010: data from the National Survey of Family Growth. Natl Health Stat Report. 2013(67):1-18, 1 p following 9. Epub 2014/07/06. PubMed PMID: 24988820.
2. Buyuk E, Nejat E, Neal-Perry G. Determinants of female reproductive senescence: differential roles for the ovary and the neuroendocrine axis. Semin Reprod Med. 2010;28(5):370-9. doi: 10.1055/s-0030-1262896. PubMed PMID: 20845237.
3. EPA US. Endocrine Disruption 2017. Available from: www.epa.gov/endocrine-disruption.
4. Patel S, Zhou C, Rattan S, Flaws JA. Effects of Endocrine-Disrupting Chemicals on the Ovary. Biol Reprod. 2015;93(1):20. doi: 10.1095/biolreprod.115.130336. PubMed PMID: 26063868.
5. Craig ZR, Wang W, Flaws JA. Endocrine-disrupting chemicals in ovarian function: effects on steroidogenesis, metabolism and nuclear receptor signaling. Reproduction. 2011;142(5):633-46. doi: 10.1530/REP-11-0136. PubMed PMID: 21862696.
6. Xiao S, Duncan FE, Bai L, Nguyen CT, Shea LD, Woodruff TK. Size-specific follicle selection improves mouse oocyte reproductive outcomes. Reproduction. 2015;150(3):183-92. Epub 2015/06/28. doi: 10.1530/REP-15-0175. PubMed PMID: 26116002; PMCID: PMC4527888.
7. Xiao S, Zhang J, Romero MM, Smith KN, Shea LD, Woodruff TK. In vitro follicle growth supports human oocyte meiotic maturation. Sci Rep. 2015;5:17323. Epub 2015/11/28. doi: 10.1038/srep17323. PubMed PMID: 26612176; PMCID: PMC4661442.
8. Xiao S, Zhang JY, Liu MJ, Iwahata H, Rogers HB, Woodruff TK. Doxorubicin Has Dose-Dependent Toxicity on Mouse Ovarian Follicle Development, Hormone Secretion, and Oocyte Maturation. Toxicological Sciences. 2017;157(2):320-9. doi: 10.1093/toxsci/kfx047. PubMed PMID: WOS:000405698400006.
9. Findlay JK, Hutt KJ, Hickey M, Anderson RA. How Is the Number of Primordial Follicles in the Ovarian Reserve Established? Biol Reprod. 2015;93(5):111. Epub 2015/10/02. doi: 10.1095/biolreprod.115.133652. PubMed PMID: 26423124.
10. Kim SY, Ebbert K, Cordeiro MH, Romero M, Zhu J, Serna VA, Whelan KA, Woodruff TK, Kurita T. Cell autonomous phosphoinositide 3-kinase activation in oocytes disrupts normal ovarian function through promoting survival and overgrowth of ovarian follicles. Endocrinology. 2015;156(4):1464-76. Epub 2015/01/17. doi: 10.1210/en.2014-1926. PubMed PMID: 25594701; PMCID: PMC4399322.
11. Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, Du C, Tang W, Hamalainen T, Peng SL, Lan ZJ, Cooney AJ, Huhtaniemi I, Liu K. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. Science. 2008;319(5863):611-3. Epub 2008/02/02. doi: 10.1126/science.1152257. PubMed PMID: 18239123.
12. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science. 2003;301(5630):215-8. Epub 2003/07/12. doi: 10.1126/science.1086336. PubMed PMID: 12855809.
13. Li J, Kawamura K, Cheng Y, Liu S, Klein C, Liu S, Duan EK, Hsueh AJ. Activation of dormant ovarian follicles to generate mature eggs. Proc Natl Acad Sci U S A. 2010;107(22):10280-4. Epub 2010/05/19. doi: 10.1073/pnas.1001198107. PubMed PMID: 20479243; PMCID: PMC2890455.
14. Xiao S, Zhang J, Liu M, Iwahata H, Rogers HB, Woodruff TK. Doxorubicin Has Dose-Dependent Toxicity on Mouse Ovarian Follicle Development, Hormone Secretion, and Oocyte Maturation. Toxicol Sci. 2017;157(2):320-9. Epub 2017/03/23. doi: 10.1093/toxsci/kfx047. PubMed PMID: 28329872; PMCID: PMC6074798.
15. Xiao S, Coppeta JR, Rogers HB, Isenberg BC, Zhu J, Olalekan SA, McKinnon KE, Dokic D, Rashedi AS, Haisenleder DJ, Malpani SS, Arnold-Murray CA, Chen K, Jiang M, Bai L, Nguyen CT, Zhang J, Laronda MM, Hope TJ, Maniar KP, Pavone ME, Avram MJ, Sefton EC, Getsios S, Burdette JE, Kim JJ, Borenstein JT, Woodruff TK. A microfluidic culture model of the human reproductive tract and 28-day menstrual cycle. Nat Commun. 2017;8:14584. Epub 2017/03/30. doi: 10.1038/ncomms14584. PubMed PMID: 28350383; PMCID: PMC5379057.
16. Rall WF, Fahy GM. Ice-Free Cryopreservation of Mouse Embryos at -196-Degrees-C by Vitrification. Nature. 1985;313(6003):573-5. doi: DOI 10.1038/313573a0. PubMed PMID: WOS:A1985ABQ6600049.
17. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, Vanderpoel S, Racowsky C. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Hum Reprod Update. 2017;23(2):139-55. Epub 2016/11/10. doi: 10.1093/humupd/dmw038. PubMed PMID: 27827818; PMCID: PMC5850862.

18. Campos Jacira Ribeiro, Rosa-e-Silva Julio Cesar, Carvalho Bruno Ramalho, Vireque Alessandra Aparecida, Silva-de-Sá Marcos Felipe, Rosa-e-Silva Ana Carolina Japur de Sá. Cryopreservation time does not decrease follicular viability in ovarian tissue frozen for fertility preservation. Clinics [Internet]. 2011 [cited 2019 Feb 18] ; 66(12): 2093-2097. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1807-59322011001200015&lng=en. http://dx.doi.org/10.1590/S1807-59322011001200015.
19. Ting AY, Yeoman RR, Campos JR, Lawson MS, Mullen SF, Fahy GM, Zelinski MB. Morphological and functional preservation of pre-antral follicles after vitrification of macaque ovarian tissue in a closed system. Hum Reprod. 2013;28(5):1267-79. doi: 10.1093/humrep/det032. PubMed PMID: 23427232; PMCID: PMC3627338.
20. Ting AY, Yeoman RR, Lawson MS, Zelinski MB. In vitro development of secondary follicles from cryopreserved rhesus macaque ovarian tissue after slow-rate freeze or vitrification. Hum Reprod. 2011;26(9):2461-72. doi: 10.1093/humrep/der196. PubMed PMID: 21705370; PMCID: PMC3157627.