Do IRX3 and IRX5 Direct Cytoplasm Traffic Between Oocytes During Germline Cyst Breakdown?

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I have supervised this work, read this thesis, and certify that it has my approval.
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Abstract

Poor oocyte quality and a diminished ovarian reserve contribute significantly to female infertility. The ovarian reserve is determined by the finite supply of primordial follicles a woman has at birth. Evidence suggests primordial follicles form as a result of germline cyst breakdown wherein many oocytes surrender their cytoplasmic contents to selected oocytes that are ultimately surrounded by pre-granulosa cells to form the intimate unit. We previously discovered that Iroquois homeobox transcription factors, IRX3 and IRX5 (IRX3/5), support follicle survival via intercellular communication between oocytes and their supporting granulosa cells. We observed that IRX3 and IRX5 colocalize to pre-granulosa cells and oocytes during the time of germline cyst breakdown; however, the means by which they promote follicle integrity are unknown. We hypothesized that IRX3/5 direct cytoplasmic content transfer between oocytes during germline cyst breakdown to ensure healthy oocytes within new primordial follicles. We tested this hypothesis using embryonic ovaries from wild type and *Irx3/5* double knockout (*Irx3*$^{-}$*Irx5*^{EGFP}/*Irx3*$^{-}$*Irx5*^{EGFP}, *Irx3/5* DKO) mice collected at E14.5 and cultured in vitro for 6 days. Ovaries were harvested, processed, and every 5th section was collected for H&E staining. Ovary sections were imaged and analyzed using ImageJ to trace oocytes in germline cysts, primordial and primary follicles to determine their areas, perimeter, and quantity. If the hypothesis was true, we would expect that *Irx3/5* DKO ovaries would harbor more, smaller oocytes; however, results showed no significant difference in oocyte structural measurements or quantities at any follicle stage when wild type and *Irx3/5* DKO ovaries were compared using a t-test. Based on these findings, we turn our attention to determine the molecular mechanisms that facilitate intercellular communication during and after primordial follicle formation and examine when significant structural differences arise at later stages of development.

Introduction

Infertility affects 11% of women in the United States and can affect a woman's physical and mental health, relationships, and opportunities (Chandra et al., 2013). Thirty percent of infertility in women is due to ovarian or ovulation related issues. A woman is born with all the oocytes she will have throughout her lifetime (her ovarian reserve), and this supply determines a woman's reproductive lifespan (Svetlana et al., 2019). If the quality or quantity of the ovarian reserve is diminished, premature infertility is possible. This diminishing of the reserve can occur prior to birth due to genetic and developmental factors.

One possible contributing factor to ovarian reserve suppression are genetic defects concerning IRX3 and IRX5 (IRX3/5) (Fu et al., 2020). Iroquois homeobox transcription factors *Irx3*, *Irx5*, and *Irx6* make up the IrxB cluster which is located on chromosome 16 in humans and chromosome 8 in mice (Houweling, 2001). These transcription factors support development in several tissues including the limbs, heart, and central nervous system of mice. The Jorgensen Lab discovered that *Irx3* and *Irx5*, but not *Irx6*, are expressed in the developing ovary where they collaborate to promote follicle formation (Fu et al., 2020).

Our lab discovered that a double knockout of IRX3 and IRX5 (*Irx5*^{EGFP}/*Irx3*$^{-}$*Irx5*^{EGFP} or *Irx3/5 DKO*), led to deficiencies in oocytes and their supporting granulosa cells, causing cell death. IRX3/5 facilitate intercellular communication between these cells, which is critical during

primordial follicle formation and later oocyte development. IRX3/5 are expressed in the developing ovary from embryonic day 12.5 (E12.5) to the day of birth (P0). This period correlates to the establishment of the ovarian reserve and oogenesis (Fu et al., 2018). Intercellular communication during oogenesis is critical for growth and health of follicles.

The ovarian reserve and a woman's fertility lifespan are determined by the finite supply of primordial follicles a woman has at birth. Healthy oocytes tend to be larger and have more connections with the surrounding supporting cell types. The distribution of follicle sizes and cell nuclei sizes within the oocyte can also indicate follicle health (Nasiri & Eftekhari-Yazdi, 2015). Evidence suggests primordial follicles form as a result of germline cyst breakdown wherein many oocytes surrender their cytoplasmic contents to selected oocytes that are ultimately surrounded by pre-granulosa cells to form the intimate unit. This unidirectional transfer includes cytoplasmic organelles, ribonucleic acid, and proteins sourced from the donor oocytes. The donor oocytes die after surrendering their cytoplasmic contents while the receiving oocytes survive to become primary follicles (Lei & Spradling 2016). Cytoplasmic resources could be used to determine the health of oocytes and, ultimately, the health of embryos. If these surviving follicles are of a high enough quality, they will eventually become viable ova, enabling reproduction. We hypothesized that IRX3/5 may direct cytoplasmic cargo transfer between oocytes during germline cyst breakdown and that this direction may be measured in structural changes in oocyte areas during this developmental window.

The effects of *Irx3/5 DKO* are deleterious, and they cause embryonic lethality around embryonic day 15 (E15). Previous experiments involving these mutant ovaries required expensive, difficult kidney capsule organ transplantation culture methods. These experiments, studying outcomes 10 days after E12.5, reported significantly decreased area and perimeter of primordial follicles in *Irx3/5* DKO mice; however, how IRX3/5 promote follicle integrity and survival is unknown (Fu et al., 2018). This experiment sought to culture ovaries within organ culture media prior to E15 using *in vitro* techniques, allowing the ovaries to develop through primordial follicle formation prior to the developmental stage in the kidney capsule experiments to determine when structural differences arise and how IRX3/5 may contribute to this process.

Methods

Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Animal housing and all described procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin–Madison and were performed in accordance with National Institute of Health Guiding Principles for the Care and Use of Laboratory Animals.

Mouse strains included CD1 outbred mice (Crl:CD1(ICR), Charles River, MA) and $Irx3^{-}Irx5^{EGFP}/Irx3^{-}Irx5^{EGFP}$ (*Irx3/5* DKO), all of which were maintained on a CD1 genetic background. Genotyping was carried out as previously reported (Fu et al., 2020). Timed mating was identified as the presence of a vaginal plug after mating which was designated as day 1 of pregnancy. Uteri were collected from heterozygous $Irx3^{+-}Irx5^{EGFP}/Irx3^{-}Irx5^{EGFP}$ females to harvest E14.5 embryos. Ovarian tissues from the female embryos were genotyped and *Irx3/5* DKO (n=5) and wildtype (n=7) ovaries were used for in vitro culture.

Harvested embryonic ovaries were cultured in organ culture media for 6 days. The culture media is used for standard organ cultures and contains antibiotics. Five of the ovaries used DMEM without phenol red to avoid its estrogenic effects. However, later analysis confirmed there was no significant difference between oocytes cultured in DMEM with or without phenol red. The culture was done on micellular well inserts using daily total media changes. On the 6th day of culture, the embryonic ovaries were processed and embedded in paraffin using formalin. Samples were labelled with a code to blind us to their genotypes to avoid bias. We sectioned the paraffin blocks at 5µm thickness and mounted them on microscopic slides. Every 5th section was subjected to H&E staining to provide an accurate oocyte count.

Ovary sections were imaged at a specified magnification and pixel area. Images were analyzed using ImageJ to trace oocytes in germline cysts, primordial follicles, and primary follicles. Primordial follicles, primary follicles, and germline cysts were identified using previously described methods (Myers et al., 2004). Traces were used to compare wild type and *Irx3/5* DKO areas, perimeters, and counts for each type of oocyte. Oocyte areas, perimeters, and counts were standardized to the area of the ovary they were measured in and the counts per measured area were reported as follicle count densities. Wild type and *Irx3/5* DKO oocytes were averaged by ovary then by genotype and were compared using a t-test.

Results

We found the area, perimeter, and count densities of wild type and *Irx3/5* DKO oocytes did not differ significantly. None of the primordial, primary, nor germline cyst oocytes showed significant differences between wild type and *Irx3/5* DKO when analyzed using a t-test. Quantities of primary, primordial, and germline cyst oocytes varied between different ovaries within both wild type and *Irx3/5* DKO groups. Qualitatively, some ovaries appeared to have more robust primary follicles than others but did not show a statistically significant difference (Figure 1).

Some significant differences confirmed expected developmental changes during this period of follicle formation. Across wild type and *Irx3/5* DKO areas of the primary oocytes were significantly greater than those of the germline cyst oocytes and primordial oocytes (Figure 2). This is consistent with expectations that cytoplasmic transfer increases the size of the surviving primary oocytes. Likewise, the count densities of germline cyst oocytes were much greater than those of primordial and primary oocytes. Since germline cysts were forming during this period, this is consistent with expected trends of a growing number of germline cyst oocytes (Myers et al., 2004).

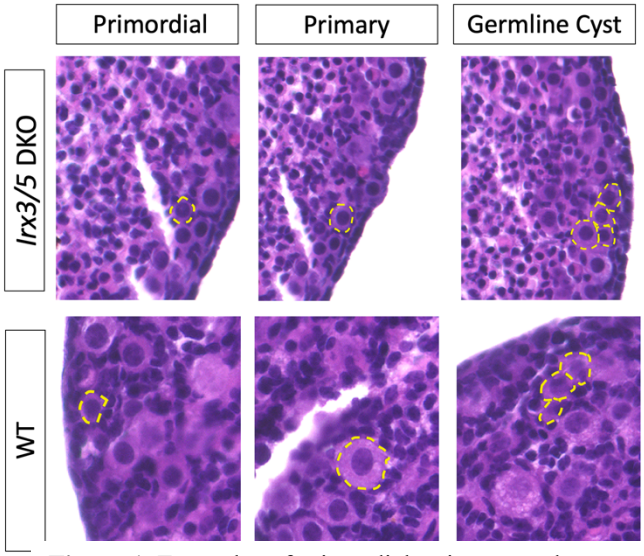

Figure 1. Examples of primordial, primary, and germline cyst oocytes in wild type (WT) and Irx3/5

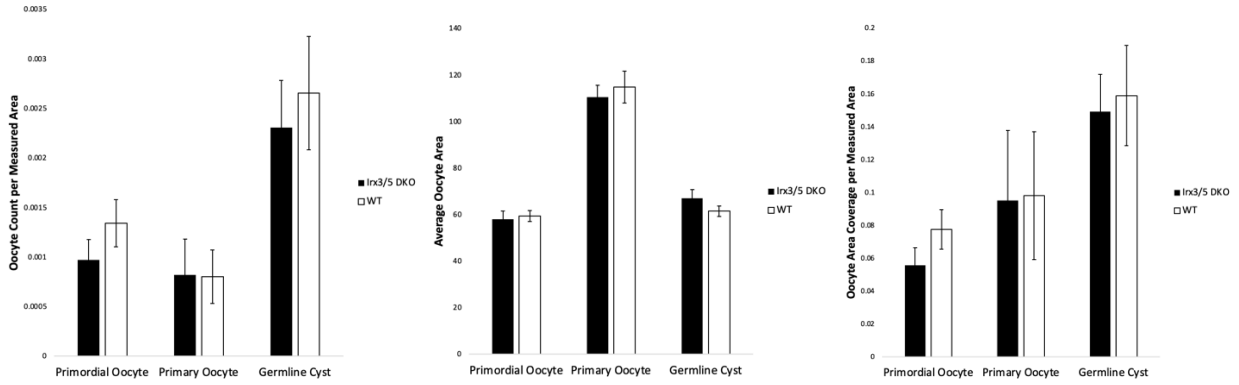

Figure 2. Oocyte count per measured area (density), oocyte area, and the proportion of the measured area covered by oocytes did not differ significantly between wild type and *Irx3/5* DKO ovaries.

Discussion

Our results showed no significant difference in oocyte structural measurements nor quantities at any follicle stage when wild type and *Irx3/5* DKO ovaries were compared. If our hypothesis were true, we would expect that *Irx3/5* DKO ovaries would harbor more, smaller oocytes. Thus, we conclude that IRX3/5 may not direct cytoplasmic content transfer during germline cyst breakdown in a way that forms structural differences.

While this hypothesis has been rejected, it will inform future studies to determine the role of IRX3/5 in supporting oocyte development. Since significant structural oocyte differences have been measured following germline cyst breakdown in *Irx3/5* DKO mice, we know IRX3/5 impact development before and during germline cyst breakdown (Fu et al., 2018). The lack of structural differences following 6 days of culture suggest that structural differences will arise in developmental stages during the germline cyst breakdown process, between the stage of this study and previous studies. Thus, future research should determine when significant structural differences arise during these later stages of development during and following germline cyst breakdown.

As an alternative to our initial hypothesis, IRX3/5 may direct events within pre-granulosa cells and selected oocytes during primordial follicle formation to establish intercellular interactions required for long-lasting follicle integrity without creating structural differences at this stage. IRX3/5 may facilitate follicular survival through cytoplasmic transfer without increasing the amount of cargo transferred and not creating larger follicles through amount of cargo transferred. They may facilitate quality rather than quantity of transfer.

IRX3/5 are expressed in germ cells and oocytes during the window of this study and previous research links their presence to intercellular communication (Fu et al., 2018). They may use alternative processes such as working with molecular agents to prepare communicative actions prior to breakdown that don't demonstrate effects until germline cyst breakdown is complete. They could potentially facilitate bidirectional communication between germ cells and oocytes rather than assisting with unidirectional cytoplasmic transfer (Matzuk et al., 2002). Both potential roles could impact future oocyte health. IRX3/5 may facilitate molecular

communication between cells during the developmental window of this study to effect structural differences later in development. Thus, future research should examine molecular mechanisms during and following primordial follicle formation to determine the role of IRX3/5 in facilitating intercellular communication.

Conclusion

This thesis is limited to the examination of IRX3/5 during the 6 days of development following E14.5. Due to complexities associated with culturing organs for greater than 7 days and the embryonic lethality of the *Irx3/5* DKO, further investigation into the stages of oocyte development may require novel methods. Identification and traced measurements of primary, primordial, and germline cyst oocytes were done by hand without machine learning or programs that more complex quantifications may rely on. This mechanical difference also limited the study to partial sampling of each imaged section.

While the results of this study were insignificant, they will direct further inquiry into the role of IRX3/5 in oocyte development. Future research may determine when structural differences between wild type and *Irx3/5* DKO arise during later stages of development. It may also examine potential molecular actors and functions related to IRX3/5 to determine their role during and following primordial follicle formation and germline cyst breakdown. This experiment will inform our group's future efforts to uncover the mechanisms of IRX3/5 and how their absence contributes to deleterious effects on oocyte health and female infertility.

This thesis sought to determine if IRX3/5 created significant structural differences during primordial follicle and germline cyst formation. We hypothesized that IRX3/5 direct cytoplasmic content transfer between oocytes during germline cyst breakdown to ensure healthy oocytes within new primordial follicles. The insignificant differences found in this study suggest that IRX3/5 create structural differences that arise during later stages of primordial follicle formation or later in development. It also raises an alternative hypothesis that IRX3/5 direct events in pre-granulosa cells and selected oocytes during primary follicle formation to establish intercellular interactions that could result in future follicle integrity.

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