Encapsulation of bovine primordial follicles in rigid alginate does not affect growth dynamics

Kathryn L. McElhinney[1-3], Erin E. Rowell[1-2], and Monica M Laronda[1, 3-4]

[1]Stanley Manne Children's Research Institute, Ann & Robert H. Lurie Children's Hospital of Chicago; [2]Department of Surgery, Feinberg School of Medicine, Northwestern University; [3]Department of Pediatrics, Division of Endocrinology, Feinberg School of Medicine, Northwestern University; [4]Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

Background

Premature ovarian insufficiency (POI) results from the depletion of the ovarian reserve to less than 1,000 primordial follicles before the age of 40. There are many causes of POI, including idiopathic, genetic, autoimmune, and iatrogenic. **Ovarian tissue cryopreservation (OTC) is the only pre-treatment fertility preservation option available to patients who cannot ovulate or for those whom ovarian stimulation and egg retrieval are not advisable.** The OTC process includes the surgical removal of an ovary and cryopreservation of the ovarian cortex for future autotransplantation. The success rate and functional lifespan of the trans-planted tissue varies significantly with only 20 - 40% of patients having a successful pregnancy after transplant and the functional duration of the transplanted tissue ranging from 2 months - 12 years. There is a mass activation event following transplantation that results in an 80% reduction in primordial follicles which make up the ovarian reserve. To quell this mass activation event and optimize current autotransplantation techniques, a better under-standing of the factors that influence primordial follicle activation and quiescence is needed. New ovarian fertility restoration techniques are desperately needed that support long-term restoration in a safe way, especially for those that have metastatic disease. Additionally, transgender individuals and those with differences of sexual development may undergo elective or medically necessary gonadectomy and decide to cryopreserve and save tissue that contains germ cells. This population would also benefit from alternative restoration technologies. A bioprosthetic ovary made of isolated follicles in a three-dimensional printed gelatin scaffold restored hormone production and fertility in ovariectomized mice. This approach could potentially allow ovarian follicles to be isolated from any metastatic cells prior to transplantation, enhancing the safety of fertility restoration. An engineered bioprosthetic scaffold that incorporates controls for primordial follicle activation would also improve graft longevity. Primordial follicles, which reside in the stiffer and denser ovarian cortex, grow better when encapsulated in stiffer environments (Figure 1). Disruption of the extracellular matrix (ECM) in murine ovaries increased primordial follicle activation, while exogenous physical pressure added to these ovaries restores primordial follicle quiescence. In our study, we sought to investigate **how different encapsulating rigidities would affect the quiescence and growth of isolated or in situ bovine primordial follicles**. We further explored the impact of isolating primordial follicles from bovine ovarian cortical tissue to **determine if the isolation process is an irreversible activation event within follicles**. Our findings contribute to the understanding of primordial follicle activation and will inform the development of future fertility restoration techniques.

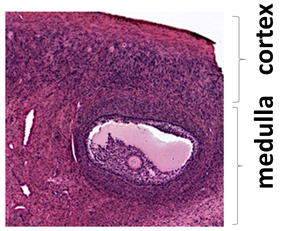

Figure 1. Bovine cortical extracellular matrix is 1.1 times the density of medullary extracellular matrix. Laronda, M. M. *et al. Biomaterials* **50,** 20–29 (2015).

Results

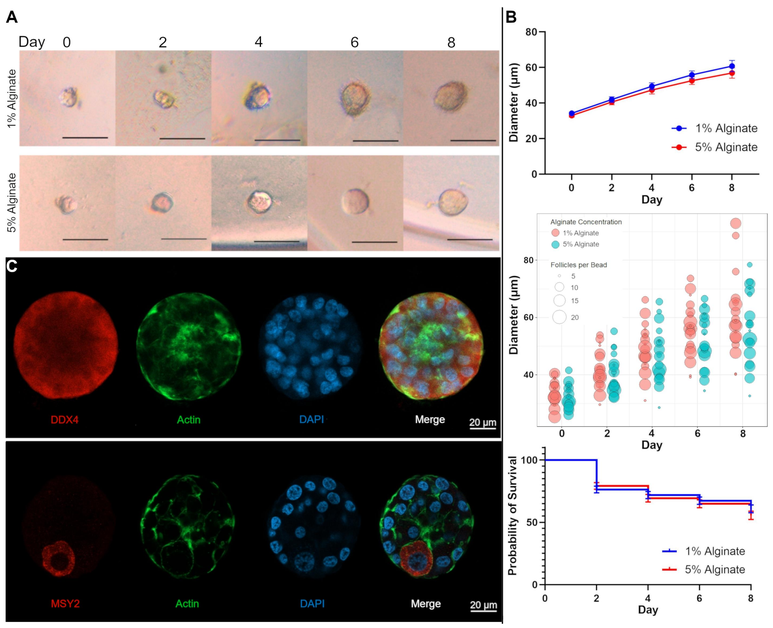

Figure 3. Growth and survival of primordial follicles encapsulated in differently rigid alginate gels. (A) Primordial follicles encapsulated in 1% and 5% alginate were examined under light microscopy every other day for all 8 days of culture. The same representative follicle is shown over time from each culture condition. Scale bars are 100µm. (B) Growth curves were plotted using mean ± SEM of primordial follicle diameter. A multivariable regression utilizing day, number of follicles encapsulated per bead, and alginate concentration was constructed to analyze the effects of these covariates on follicle diameter. Survival curves between the two concentrations of alginate were also constructed. (C) After 8 days in culture, the presence of bovine follicles was stained for DDX4 and MSY2 using whole mount immunofluorescence techniques.

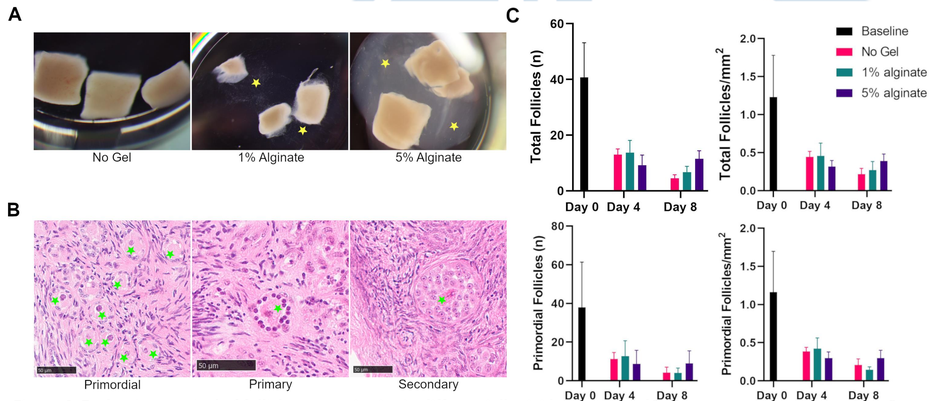

Figure 4. Evaluating survival of follicles grown in situ in differentially rigid conditions. (A) Tissue was cultured for up to 8 days in either no gel, 1% alginate, or 5% alginate. Alginate is marked with yellow stars. (B) Representative images of follicles found in in situ tissue that was cultured for up to 8 days. Primordial, primary and secondary follicles are noted by green stars. Scale bars are 50µm. (C) Total and primordial follicles were counted in tissues fixed on days 0, 4 and 8 to assess for follicle numbers in cultures without gel, in 1% alginate, and in 5% alginate. The mean± SEM are graphed for each condition and timepoint.

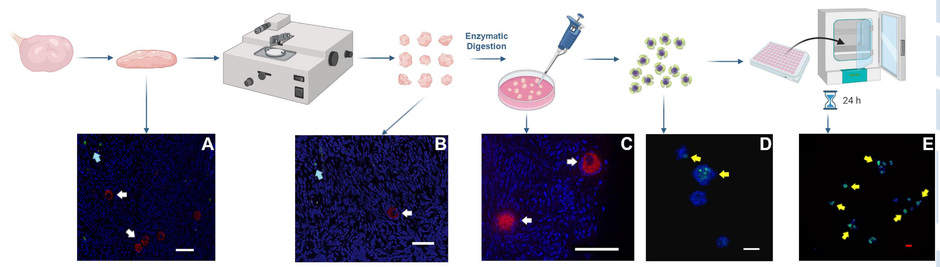

Figure 5. EdU uptake during tissue processing for primordial follicle isolation. Bovine cortex tissue samples were reserved at specific time points during the ovarian tissue processing and primordial follicle isolation process for EdU incorporation. Tissue in panel A was incubated with EdU immediately after the ovarian cortex was removed using the tissue slicer. Tissue in panel B was incubated immediately after processing by the tissue chopper. Tissue in panel C was incubated after enzymatic digestion. Tissue in A-C was co-stained for MSY2 using immunofluorescence techniques. Isolated follicles in panel D were incubated immediately after mechanical isolation and follicles in panel E were incubated after 1 day in culture. White arrows are pointing to follicles stained red with MSY2 in the tissue. Blue arrows are pointing to stromal cells stained green in the tissue indicating EdU incorporation in cells away from follicles. Yellow arrows are pointing to isolated follicles with green stained granulosa cells indicating EdU incorporation. White scale bars are 50µm and the red scale bars is 100µm.

Methods

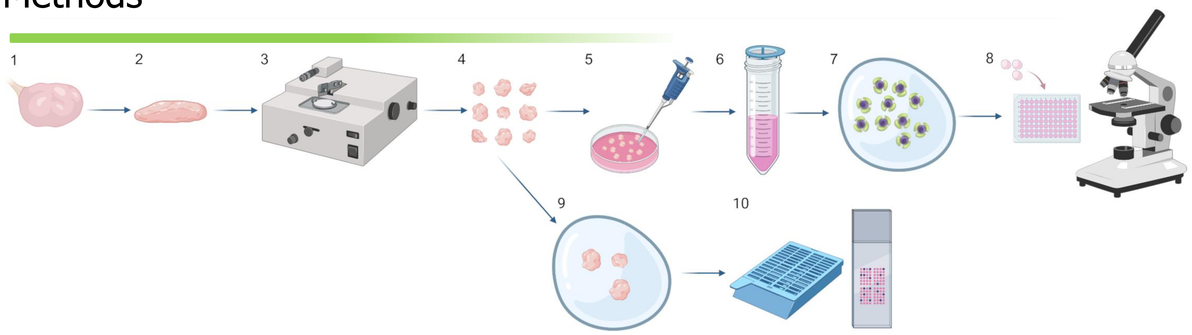

Figure 2. The above schematic outlines the process of isolating and culturing both bovine cortical tissue pieces and follicles in 1% and 5% alginate gel beads. Bovine primordial follicles were isolated from ovarian cortex. A median of 10 follicles (range 5-24) were encapsulated per bead in either 1% or 5 % alginate across 5 experiments. Bovine ovaries were obtained within 24 hours of animal sacrifice and then bisected (1). Ovaries were then sliced into 0.5mm thick slices using a Stadie-Riggs Tissue Slicer (2). Tissue slices were then further processed into 1 by 1 by 0.5mm pieces using a tissue chopper (3-4). Tissue pieces were then either encapsulated in alginate gel for *in situ* experiments (9) or underwent enzymatic and mechanical digestion (4-5). The sample was then passed through a cell strainer and follicles were eluted using a holding media (6). Isolated follicles were then crosslinked in either 1% or 5% alginate (7). The encapsulated follicles and tissue were subsequently crosslinked in a calcium sulfate solution (7-8). Follicles were then cultured for 8 days with light microscopy imaging taken every other day along with media exchanges (8). Growth and survival curves were constructed. I*n situ* experiments were cultured for 0, 4 and 8 days. Cortex tissue was then submitted for histologic processing and H&E staining (10). Follicles were then counted and staged. A total of 12 pieces of ovarian cortex were analyzed for each condition on each day. All statistical analyses were performed using Graph Pad Prism 9.

Conclusions

- Isolating primordial follicles is an activating event that is not affected by rigid environments.
- Primordial follicle quiescence likely relies on multiple cues, both physical and biochemical.
- More work is needed to better understand each of these cues and how they relate to each other to understand primordial follicle activation and its role in premature ovarian insufficiency and to develop future fertility preservation technologies.

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