Ansen Tan
Engineering Honors
Capstone Final Report

An Examination of the Ovarian Extracellular Matrix Composition in Reproductive-Age Women

Introduction

Mammalian reproduction requires the fusion of the male reproductive cell, or sperm, with the female reproductive egg, or ovum. For women, the ovarian follicle represents the functional reproductive unit and the precursor to the mature ovum. While men initiate sperm production during puberty and will continue generating sperm for the rest of their lives, women are born with all of their ovarian follicles and do not have the ability to create more. Prior to puberty, ovarian follicles are preserved in their primordial or dormant state; this state of dormancy is also known as quiescence. Afterwards, the female body selects a handful of primordial follicles to activate and undergo a process called folliculogenesis or the maturation of the ovarian follicle. Once activated, the primordial follicle will develop into a primary follicle, then into a secondary follicle, and finally into an antral follicle which will eventually release the ovum once fully mature. It is important to note that only one ovarian follicle will complete the full follicle cycle and once the egg is released, concurrently activated follicles will die. Once a woman's follicle reserve has been sufficiently depleted, they will enter menopause.

While the female reproductive system and folliculogenesis have been greatly studied, how exactly the body determines which quiescent follicle to activate is still largely enigmatic to the field of women's reproductive health. Ovarian tissue can be

rudimentarily separated into two components: 1) germ (follicles) and somatic cells (granulosa, thecal, and stromal cells) and 2) the extracellular matrix (ECM). The ECM represents a complex network of proteins and other molecules that provide structural support, organize, and connect cells within the tissue. In other words, if the ovarian tissue were a house, the follicles would be the residents and the ECM would represent the supportive beams, stairs, and hallways. Interestingly, it has been shown that the ovarian extracellular matrix plays a significant role in the signaling pathways and mechanical cues responsible for regulating follicle quiescence. While a comprehensive analysis of ovarian ECM across the reproductive lifespan has yet to be published, examining the chemical makeup of proteins and molecules within the ovarian ECM may elucidate the mechanisms responsible for modulating ovarian function. This study seeks to investigate the differences in ECM composition between different regions of the human ovary as well as how the composition varies with respect to age.

The human ovary can be compartmentalized into two distinct regions: the cortex and the medulla (Figure 1a). The cortex represents the thin outer layer of the ovary which contains the ovarian reserve of dormant or quiescent follicles. Upon activation, follicles will migrate towards the center of the ovary, or medulla, which houses growing follicles. Differences in ECM composition between the cortex and medulla may suggest that certain proteins or molecules within the ECM play a key role in determining the state of follicle quiescence and activation.

Achieving a greater understanding of the process of follicle development will directly contribute to the field of women's health as a whole. This is because the female reproductive system plays a profoundly important role in regulating bodily functions and overall well-being, probably to a far greater extent than the male reproductive system. This is evident in the fact that once a woman enters menopause, they will experience a plethora of symptoms ranging from fatigue and drastic changes in mood to osteoporosis and hot flashes. Hormones such as estrogen and progesterone not only contribute to folliculogenesis but also play a significant role in maintaining bone and skin health, mood and temperature regulation, and other bodily functions. Young women who undergo cancer treatments can suffer from premature ovarian insufficiency (POI) which represents one of many conditions that impair the ovary's ability to function regularly and activate quiescent follicles. The significance of this project will hopefully establish the groundwork for future investigations into the ovarian ECM composition and its role in the activation and selection of quiescent follicles. If the mechanisms behind follicle activation can be concretely understood and controlled, damaged or abnormal ovaries prior to menopause can be treated and normal hormone production can be restored in order to maintain healthy bodily functions.

Methods

This investigation has two main components: immunofluorescent staining and fluorescence quantification. I will be gathering my data from five donor tissue samples and will be focusing on six prominent proteins which are known to exist ubiquitously

throughout the ovarian ECM: collagen I, collagen IV, collagen VI, fibronectin, laminin, and perlecan.

Immunofluorescent Staining

Ovarian tissue from five donors was harvested and subsequently fixed in paraformaldehyde (PFA) in order to covalently crosslink molecules and prevent the loss of any molecular or proteal fragments. Embedded tissues were then sectioned using a microtome at a width of 5 microns. Sections from the five donors contained both ovarian cortex and medulla tissues. Cortex and medulla regions of each section will be stained against six antibodies: anti-Perlecan, rat (1:100); anti-Collagen IV, rabbit (1:500); anti-Collagen VI, rabbit (1:250); anti-Fibronectin, mouse (1:200); anti-Laminin, rabbit (1:100); and anti-Collagen I, rabbit (1:250). Additionally, all tissue samples will be stained against DAPI (4',6-diamidino-2-phenylindole) in order to visualize the nucleic DNA in fixed cells; this will allow us to determine the number of nuclei within a defined region.

Given the current limitations of existing fluorescence microscopy technologies, all six antibodies cannot be applied in a single round of staining since increasing the number of antibodies will result in exponentially increasing crosstalk or interference. Therefore, each round will stain against two antibodies alongside DAPI totalling four rounds of staining per section. With five donors (two regions of interest per donor) and four rounds of immunofluorescent staining, twenty rounds of immunofluorescent staining is required. A brief overview of this process is detailed below in Figure 1.

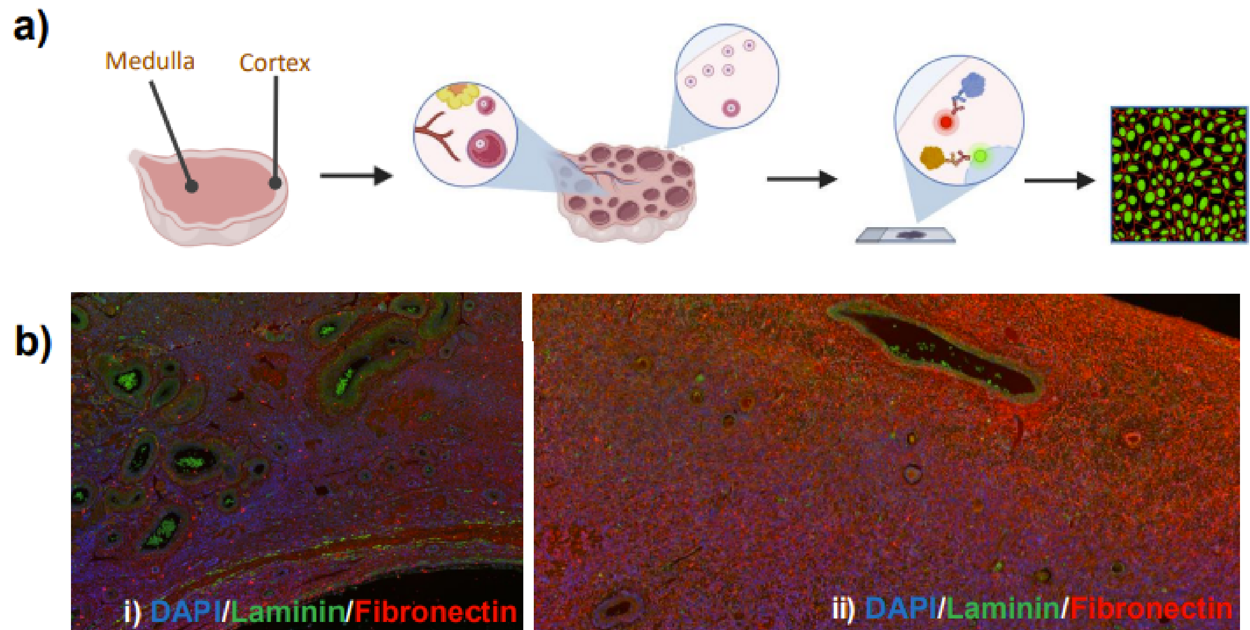

Figure 1. a) An overview of the extraction, staining, and imaging process. Donor tissue containing both cortex and medulla is fixed, embedded, section, stained against six antibodies, then imaged. b) Confocal microscopy is then used to photo-excite two antibodies per round (including DAPI) in both the medulla (i) and cortex (ii). Each antibody will correspond to their respective excitation wavelength.

The following section details the two-day immunofluorescent staining approach for **one** round of staining for **one** tissue slide. Sectioned tissue samples are first imaged under light microscopy in order to determine the presence of relevant ovarian components (follicles, vasculature, etc.) and eliminate contaminated sections. Sections are then deparaffinized with two rounds of ten minute xylene soaking and treated with five concentrations(100%, 90%, 70%, 50%, 30%) of ethanol for five minutes each. Hydrogen peroxide is then added at 3% in water for thirty minutes. Slides are then washed with deionized water and treated with a heated mediated antigen retrieval in 0.1M sodium citrate buffer for thirty minutes. Afterwards, samples are incubated in a vegetable steamer for twenty minutes in order to activate the buffered antigen retrieval.

Finally, each tissue section is permeabilized with 5mL of 0.1% triton/DPBS for fifteen minutes, then treated with a block solution (500mM Glycine, 10% Normal Goat Serum 8mL) for one hour, and incubated overnight with the targeted primary antibodies at 4° C.

The following day, incubated slides are washed three times with 5mL of PBST (twenty minutes each) and then incubated with secondary antibodies (1:1000) and DAPI (1:1000) for two hours. It is important to note that samples incubated with secondary antibodies must be handled in near darkness as any exposure to natural light may oversaturate and degrade the fluorescent markers; the following steps (including imaging) must be performed in a dimly lit environment. Three final DPBS washes are applied for twenty minutes each. Each slide is then mounted and cured overnight.

Slides are then subsequently imaged using confocal microscopy by exciting samples with precise wavelengths to capture pictures such as the ones featured in Figure 1b. Tile scans were acquired using the following settings: 2048 x 2048 pixel resolution, 600 imaging speed, phase of 20.58, and a line average of 32. With five donors, two regions of interest per donor, four rounds of staining, and three image acquisitions per region of interest, a total of 120 images were captured. Confocal images were captured and processed using the LAS X Life Science Microscope Software.

Fluorescence Quantification

Each image acquisition was exported from LAS X to ImageJ for quantification. Given that each round of staining contained three fluorescent markers (two antibodies

alongside DAPI), a total of 360 channels were analyzed. To start, fluorescence was quantified by converting each channel into its own unique grayscale image in order to record both the mean intensity of fluorescence and the total area of intensity across each replicate (Figure 2i). The number of nuclei was then acquired by converting the DAPI channel into a binary image with a >200 pixel size discrimination (Figure 2ii).

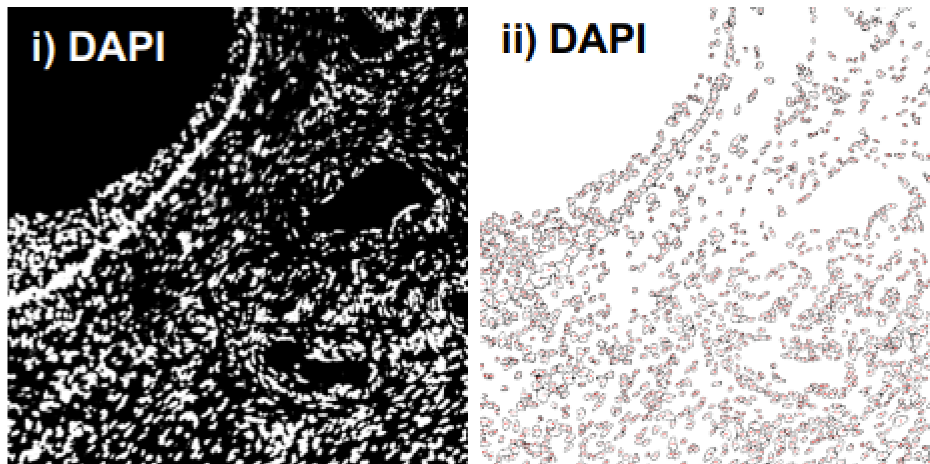

Figure 2. i) Fluorescent intensity and mean area of intensity was obtained by converting channels into grayscale images and ii) the total number of nuclei per replica was gathered by converting the DAPI channel into a binary image so that ImageJ could automatically detect particles above 200 pixels.

The intensity of fluorescence of a certain protein will indicate its concentration and presence within each replica. The total area of intensity is then divided by the number of nuclei (DAPI) in order to normalize our data with respect to the number of nuclei and calculate the area of intensity per nuclei.

Results

The calculated average mean intensity was then plotted against each donor and each region of interest for every protein using the Prism graphing software (Figure 3). These results seem to suggest that the most abundant ECM proteins from our analysis, collagens (Figure 3ii, iii, vi) and fibronectin (Figure 3iv) are similarly present in the ovarian medulla and cortex. Additionally, perlecan (Figure 3i) and laminin (Figure 3vi) were more prevalent in the medulla in nearly all cases. Theses proteins are involved in angiogenesis, mechanical stability, and cell proliferation or differentiation, and may be found in greater abundance in the center of the ovary, which is highly vascularized and responsible for supporting the development of activated follicles. Proteins such as fibronectin drastically varied in intensity while others such as collagen I, IV, and VI were observed consistently throughout the cortex.

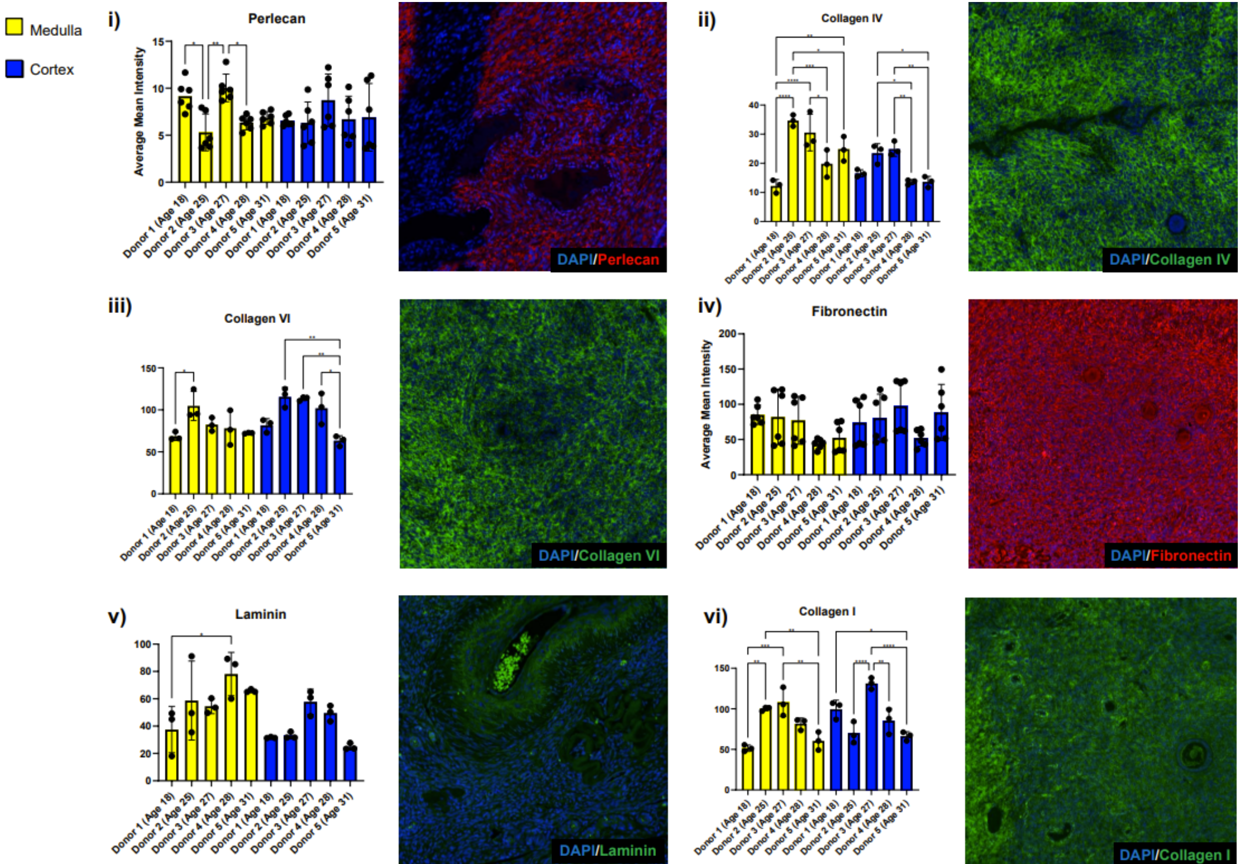

Figure 3. The average mean intensity of 6 ECM proteins was plotted for each donor, with the medulla in yellow and cortex in blue. Supporting images are presented to the right of each plot as a visualization of each antibody alongside DAPI (blue).

Next, the area of intensity and normalized area of intensity per number of nuclei was plotted against all six proteins of interest in both the medulla and cortex (Figure 4). Interestingly, while Figure 3 may not have elucidated confident significant results in terms of ECM composition, Figure 4 paints a different picture. When comparing the protein composition against antibodies, we see that the ECM composition is relatively similar between the medulla and cortex regions of the ovary. This implication is further bolstered by the fact that normalizing the data against the number of nuclei produces extremely similar results.

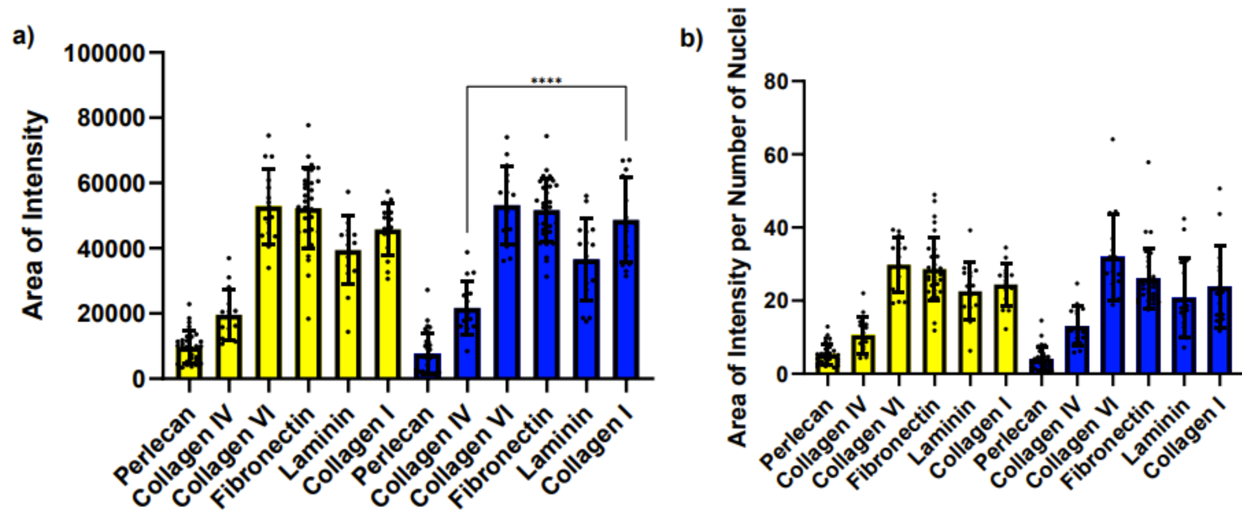

Figure 4. a) The total area of intensity for each ECM component was compared between the medulla (yellow) and cortex (blue) showing a similarity in ECM composition and b) normalizing the total area of intensity against the number of nuclei present further demonstrated the analogous makeup of the ECM throughout the ovary.

Next, we compared the ECM composition of each protein in each region of interest with respect to age in reproductive-age women (Figure 5). While these results seemingly support the implications of Figure 4, these differences cannot be confidently stated since only one donor from each age was used in this experiment. Additional donors from each range would be required to make a proper assessment. However, from our five donors, these results seem to suggest that there are no significant changes in protein composition of the ovarian ECM as reproductive-age women grow older.

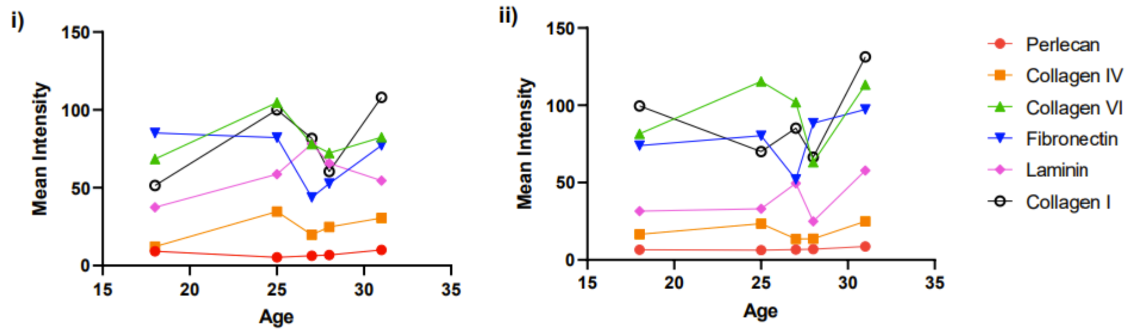

Figure 5. An examination of average mean intensity of each protein with respect to age revealed no significant changes among our donors in ECM composition in both the medulla (i) and cortex (ii).

Discussion

While a comprehensive analysis of ovarian ECM composition across the reproductive lifespan has yet to be published, this investigation provides a brief insight into the differences in ECM composition between the medulla and the cortex as well as discrepancies with respect to age. Our findings showed that the medulla and cortex share relatively similar amounts of essential ECM proteins. These results indicate several potential hypotheses. For one, given that this experiment only looked at the essential proteins necessary for structural support, vascularization, and cell proliferation and death, this may suggest that other niche and less ubiquitous components of the ECM may play a more crucial role in follicle activation. Additionally, this study examined the ECM composition of healthy, reproductive-age women due to limited resources. Perhaps an analysis of changes in ECM composition across a greater age range would elucidate more meaningful data. For instance, future studies could explore the ECM composition during drastic changes in ovarian function such as pre-pubescent tissue, women undergoing puberty, and women undergoing menopause. Furthermore, an

interesting direction that subsequent research could take includes looking at the ECM discrepancies with respect to race. In our study, four of the five donors were caucasian meaning that any comparison to the single non-caucasian donor would not result in sound conclusions.

The application of tissue engineering within the field of women's reproductive health represents a unique approach to explaining how the female body regulates its ovarian reserve. The dawn of the 21st century experienced an influx of research examining the intersection between cells and their surrounding ECM. Rather than exploring these components separately, tissue engineering aims to connect these two functional components to explain enigmatic phenomena. While this study may not have produced any definitive answers to the questions posed, it serves as a proof-of-concept experiment that future researchers can build upon.