Project Title: Identifying the mechanisms regulating oocyte maturation in the ovary: A multidisciplinary study

Student: Prodromos Chatzikyriakou
Supervisor: Dr Mark Fenwick

Introduction: During foetal development and early childhood the vast majority of ovarian follicles degenerate becoming atretic and only very few will proceed to maturation and ovulation[1]. The reasons and mechanism behind this phenomenon still remain unknown despite the fact that it can profoundly affect female fertility.
In this project, we attempted to examine whether the proximity of the follicle to the ovarian vasculature influences its chances to develop into a mature oocyte. It is well-known that many developmental signals act in a different manner depending on their concentrations, forming gradients emanating from the morphogen's source[2]. Blood is the primary form of transport for nutrients, waste by-products, oxygen and CO_2 to- and from all tissues. This makes it an ideal candidate to differentially influence and determine/control the fate of follicles in the developing ovary.

Methods: IHC staining of 5μm ovary sections from 8 day-old mice (n=3) fixed in 10% formalin. We used VASA/DDX4 as a follicular marker and CD31 to determine the presence of endothelial cells as previously described[3]. The sections were imaged at very high resolution (Fig. 1) to allow precise measurements of the distances between the relevant structures in question.
The follicles were subsequently manually staged depending on the criteria listed in Table 1 (Fig. 2). The ImageJ plug-in Point Picker (version March 6, 2013) (Philippe Thévenaz, Biomedical Imaging Group, Swiss Federal Institute of Technology, Lausanne, Switzerland) was employed to extract the coordinates of the each follicle categorised and their size and centroid were calculated. The endothelial cells of the ovary were also delineated and their coordinates derived in a similar manner.

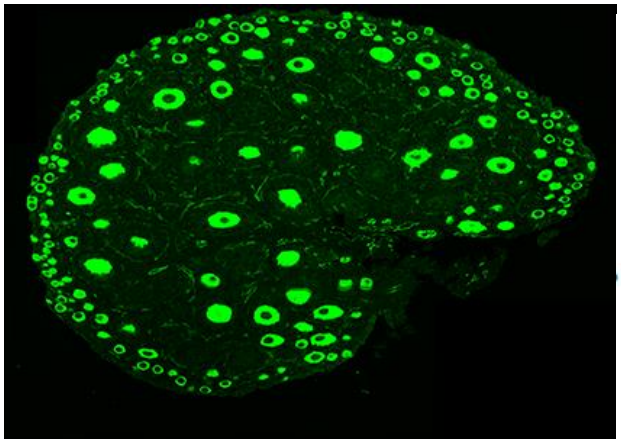

Figure 1 Immunofluorescence staining showing follicles as round green objects and endothelial cells.

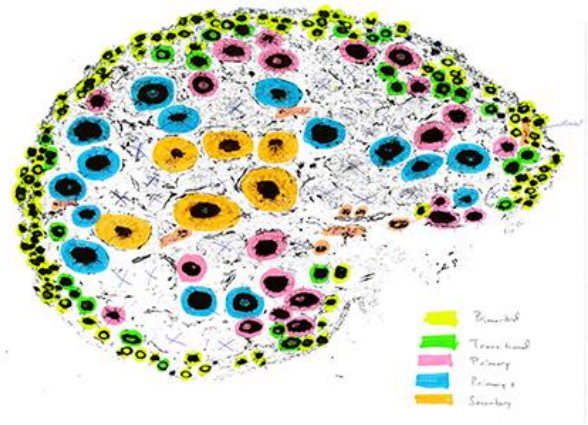

Figure 2: Follicle stagging.

Initial Inclusion Criterion	Appearance of nuclear staining (DAPI) indicating that the oocyte was sectioned near the core
Primordial	Presence of flat, squamous granulosa cells only
Transitional	Presence of one cuboidal granulosa cell
Primary	Completely encircled by a single layer of cuboidal granulosa cells
Primary+	Presence of an additional granulosa cell not part of the initial layer
Secondary+/-	More than two layers of granulosa cells

Table 1: Staging scheme used to categorise follicles according to their developmental phase.

A variety of statistical tests (linear regression models, Ripley's *K*-function with and without Complete Spatial Randomness assumptions, Besag's transformation of Ripley's *K*-function for inhomogeneous samples) to query the data for spatial interactions between blood vessels and the follicles depending on their developmental stage. All spatial point statistical analyses were done in R (version 3.1.1) using in-house code and the package `spatstat`[4].

Results: All the statistical tests we performed failed to identify a significant (p<0.5) relationship between the smaller distances from vasculature and follicles of later developmental stages. Model-fitting and point analysis did not show any statistically significant correlation either. It may initially appear as a negative result but this could be potentially explained by the small number of sections used (n=3), the choice of the age we examined or other parameters not accounted for.

Discussion: We used a novel methodology adapted from geospatial statistics to examine if the oocyte maturation in the female ovary is influenced by the proximity to the vasculature and the subsequent differential access to a variety of factors that could control their fate. This proof-of-principle methodology described here showed that applying topological information in the study of biological processes is possible and could shed light to interactions between structures depending on their relative positions.
The intricacies of ovarian development might have played a role in the results we got and further research would be needed to investigate them further. For example, it is well described that the more developed follicles tend to segregate in the middle of the ovary. Also, it was evident that the bigger, more mature follicles were surrounded by a dense capillary network which could not be excluded by our analyses. It is not clear if this is initiated by the developing follicle or the presence of a preexisting blood vessel promotes follicle development in the vicinity. Markers for neovascularisation and angiogenesis could provide valuable information on this aspect.

Acknowledgements: The author would like to deeply thank the Group Leader Dr Mark Fenwick for his supervision, challenging discussions, upbeat approach and for giving me the opportunity to undertake this project in the first place. Postgraduate students Sofia Granados Aparici and Isam Sharum have been instrumental for this project. They taught me, guided me and without them this project wouldn't have taken this form; I am very grateful to both of them. I would like to extend my thanks to Dr Gianluca Marcelli who provided very valuable ideas, statistical assistance and dedicated his time to this project. Finally, I would like to thank my funder, the Society for Reproduction and Fertility, for their generous support.

References

1 Matsuda, F., Inoue, N., Manabe, N. & Ohkura, S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *Journal of Reproduction and Development* **58**, 44-50 (2012).
2 Gurdon, J. & Bourillot, P.-Y. Morphogen gradient interpretation. *Nature* **413**, 797-803 (2001).
3 Fenwick, M. A. *et al.* Investigations of TGF-β signaling in preantral follicles of female mice reveal differential roles for bone morphogenetic protein 15. *Endocrinology* **154**, 3423-3436 (2013).
4 Baddeley, A. & Turner, R. Spatstat: an R package for analyzing spatial point patterns. *Journal of statistical software* **12**, 1-42 (2005).