The potential molecular mechanisms regulating early ovarian folliculogenesis in the domestic cat (*Felis catus*).

A Cumulative Dissertation

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me. This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

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List of Abbreviations

µl	microlitre
µm	micrometre
ARMCX4	Armadillo repeat containing 4
AR	Androgen receptor
ART	Assisted reproductive techniques
ATP	Adenosine triphosphate
BAMBI	Bone morphogenetic protein and activin membrane bound inhibitor
BHLHE40	Basic helix-loop-helix family member E40
BMP	Bone morphogenetic protein
BMP4	Bone morphogenetic protein 4
BMP15	Bone morphogenetic protein 15
BMPER	Bone morphogenetic protein binding endothelial regulator
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCDC63	Coiled-coil domain containing 63
cDNA	Complementary deoxyribonucleic acid
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CYP17A1	Cytochrome P450 family 17 subfamily A member 1
CYP19A1	Cytochrome P450 family 19 subfamily A member 1
DHEA	Dehydroepiandrosterone

DNA	Deoxyribonucleic acid
DNAH5	Dynein axonemal heavy chain 5
DNAH7	Dynein axonemal heavy chain 7
dpc	Days post conception
DRC7	Dynein regulatory complex subunit 7
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGLN2	Egl-9 family hypoxia inducible factor 2
ESR1 (ERα)	Estrogen (Oestrogen) receptor 1
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
FURIN	Furin, paired basic amino acid cleaving enzyme
GDF	Growth differentiation factor
GDF-9	Growth differentiation factor 9
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
HIF	Hypoxia-inducible factor
HIF-1	Hypoxia-inducible factor 1
HIF-1α	Hypoxia-inducible factor 1 alpha
HIF1AN	Hypoxia inducible factor 1 subunit alpha inhibitor

HIST1H1T	Histone 1, H1t
HPG	Hypothalamic-pituitary-gonadal
HSD3B	3-beta-Hydroxysteroid dehydrogenase
HSD17B	17-beta-Hydroxysteroid dehydrogenase
HSD17B1	17-beta-Hydroxysteroid dehydrogenase 1
HSD17B2	17-beta-Hydroxysteroid dehydrogenase 2
HSD17B7	17-beta-Hydroxysteroid dehydrogenase 7
IGF1	Insulin-like growth factor 1
IVA	*In vitro* activation
IVF	*In vitro* fertilisation
IVG	*In vitro* growth
IVM	*In vitro* maturation
LB1	Lysis buffer 1
LCM	Laser capture microdissection
LH	Luteinising hormone
LHCGR	Luteinising hormone/choriogonadotropin receptor
LN^2	Liquid nitrogen
MMP	Matrix metalloproteinase
MMP1	Matrix metalloproteinase 1
MMP2	Matrix metalloproteinase 2
MMP3	Matrix metalloproteinase 3

MMP7	Matrix metalloproteinase 7
MMP9	Matrix metalloproteinase 9
MMP12	Matrix metalloproteinase 12
MMP13	Matrix metalloproteinase 13
MMP21	Matrix metalloproteinase 21
PBS	Phosphate buffered solution
PFKL	Phosphofructokinase liver type
PGC	Primordial germ cell
PGR	Progesterone receptor
PGRMC1	Progesterone receptor membrane component 1
PGRMC2	Progesterone receptor membrane component 2
PI3K/Akt	Phosphatidylinositol-3-kinase/protein kinase B
PTEN/PI3K/Akt	Phosphatase and tensin homolog deleted on chromosome 10/ phosphatidylinositol-3-kinase/protein kinase B
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RIN	Ribonucleic acid (RNA)-integrity number
RNA	Ribonucleic acid
SMURF2	E3 ubiquitin protein ligase 2
SPAG1	Sperm associated antigen 1
StAR	Steroidogenic acute regulatory protein
STS	Steroid sulfatase

TGF-β	Transforming growth factor beta
TWSG1	Twisted gastrulation bone morphogenetic protein signalling modulator 1

Zusammenfassung

Ovarialfollikel von Säugetieren werden im weiblichen Eierstock bei verschiedenen Arten entweder vor oder nach der Geburt gebildet. Beim ersten Stadium von Ovarialfollikeln handelt es sich um ruhende Primordialfollikel. Diese bestehen auseiner primäre Eizelle, die in der Prophase I der ersten meiotischen Teilung verharrt und von Granulosazellen umgeben ist. Die Anzahl der Primordialfollikel, die im Eierstock gebildet werden, kann zwischen Hunderten, Tausenden oder sogar Millionen liegen, wobei es bei den verschiedenen Arten Unterschiede gibt. Es wird angenommen, dass die Höchstzahl feststeht und diese Population ruhender Primordialfollikel als Eizellreservoir für die künftige Fruchtbarkeit dient. Die Follikulogenese in den Eierstöcken umfasst die Aktivierung ruhender Primordialfollikel und die Entwicklung zu Primär-, Sekundär- und Antralfollikeln bis hin zur Ovulation der Eizelle. Die Regulierung der ovariellen Follikulogenese ist für die weibliche Fruchtbarkeit von entscheidender Bedeutung. Viele Studien, die überwiegend an Nagetieren durchgeführt wurden, konzentrierten sich auf die Aufklärung der molekularen Mechanismen, die an der Entwicklung von Ovarialfollikeln und Eizellen beteiligt sind. Bei größeren Tiermodellen gibt es daher noch große Wissenslücken. Dies gilt insbesondere für die frühe Follikelentwicklung, vom primordialen bis zum sekundären Stadium, für die die molekularen Mechanismen bei Säugetieren noch nicht vollständig geklärt sind.

Anhand der Hauskatze (*Felis catus*) als Modellorganismus stellten wir die Hypothese auf, dass die Entwicklung vom primordialen zum primären und vom primären zum sekundären Follikel durch spezifische Signalwege reguliert wird und daher auch durch spezifische Genexpressionsmuster charakterisiert ist. Durch eine Reihe von Experimenten wollten wir die Transkription und Proteinexpression während der frühen Follikelentwicklung beschreiben. Die Eierstöcke von Hauskatzen stammten aus Tierheimen, in denen routinemäßige Ovarektomien durchgeführt wurden. Mit Hilfe mechanischer Sektion wurden Follikel früher Entwicklungsstadien aus den Eierstöcken von Hauskatzen isoliert, d.h. Eizellen mit den sie umgebenden somatischen Zellen. Wir sammelten primordiale, primäre und sekundäre Follikel. Vor der Isolierung der Ribonukleinsäure (RNA) wurden die Follikelproben für jeden Typ gepoolt, um die Anzahl der Follikel pro Probe zu erhöhen. Für die RNA-Sequenzierung wurden Bibliotheken vorbereitet und kundenspezifische Index-Primer ermöglichten es uns, die Bibliotheken für die Sequenzierung zu poolen.

In unserer ersten Veröffentlichung stellten wir die Analyse der RNA-Sequenzierungsdaten vor, die Gene aufzeigte, die während der frühen Follikelentwicklung bei dieser Spezies signifikant

unterschiedlich exprimiert wurden. Wir haben spezifische molekulare Mechanismen identifiziert, die an der Regulierung der frühen Follikelbildung beteiligt sein könnten. So fanden wir zum Beispiel heraus, dass die Phosphatidylinositol-3-Kinase und die Proteinkinase B (PI3K/AKT) sowie die Signalwege des transformierenden Wachstumsfaktors β (TGF-β) an der frühen Follikelentwicklung bei der Hauskatze beteiligt sein könnten. Darüber hinaus haben wir festgestellt, dass die extrazelluläre Matrix (ECM) des Eierstocks auch bei dieser Tierart an beiden Entwicklungsübergängen beteiligt zu sein scheint. Zur Validierung verglichen wir die Genexpressionswerte der RNA-Sequenzierungsergebnisse mit den Daten der quantitativen Reverse-Transkriptions-Polymerase-Kettenreaktion (qRT-PCR) für zwei Gene: Knochenmorphogenetisches Protein 15 (BMP15) und Histon 1, H1t (HISTH1T). In den RNA-Sequenzierungsdaten fanden wir heraus, dass die Gene von BMP15 und HISTH1T zwischen den frühenEntwicklungsphasen signifikant unterschiedlich exprimiert wurden. Die qRT-PCR-Daten stimmten mit diesen Ergebnissen überwiegend überein, In der Analyse für die erste Veröffentlichung identifizierten wir auch Gene, die an der ovariellen Steroidbiogenese während der frühen Follikelentwicklung beteiligt sind. In unserer zweiten Veröffentlichung stellten wir die Hypothese auf, dass Signale von Gonadotropinen und Sexualsteroiden, vermittelt über Rezeptoren, an der frühen Follikelbildung beteiligt sind und dass frühe Follikel bei der Hauskatze eine Quelle für Sexualsteroide sein könnten, so dass weitere Untersuchungen zu diesem Thema durchgeführt wurden. Wir führten immunhistologische Untersuchungen an Ovargewebeschnitten durch und untersuchten die Lokalisierung von Gonadotropinrezeptoren, Sexualsteroidrezeptoren und steroidogener Enzyme. Wir detektierten Signale für Gonadotropin- und Sexualsteroidrezeptorproteine in den frühen Follikeln an unterschiedlichen Stellen und in unterschiedlicher Intensität. Im Vergleich zum Primordial- und Primärstadium, in denen keine Proteinsignale für die analysierten steroidogenen Enzyme nachweisbar waren, stellten wir fest, dass im Sekundärstadium Proteinsignale für einige von ihnen nachweisbar waren. Obwohl alle frühe Follikelstadien auf der Ebene der Genexpression voll ausgestattet sind, Sexualsteroide zu produzieren, ist aufgrund der Proteindaten anzunehmen, dass nur die sekundären Follikel eine potentielle Quelle für Sexualsteroide sind, nicht aber die früheren Stadien. Wir haben ebenfalls die Genexpression von drei steroidogenen Enzymen, dem Androgenrezeptor, Progesteronrezeptoren und einem Cholesterintransporter mittels qRT-PCR gemessen. Die Expressionswerte waren zu niedrig, um einen aussagekräftigen Vergleich mit den Ergebnissen der RNA-Sequenzierung anzustellen, und für keines der mittels qRT-PCR untersuchten Gene wurde eine statistische Signifikanz für unterschiedliche Genexpressionen

zwischen den Stadien ermittelt. In Zukunft könnte eine größere Anzahl von Follikeln pro Probe dieses Problem beheben.

Zusammenfassend lässt sich sagen, dass wir zwei umfassende Studien vorgestellt haben, in denen zum ersten Mal bei der Hauskatze potenzielle Schlüsselgene, Signalwege und molekulare Mechanismen untersucht wurden, die die frühe Follikelentwicklung regulieren könnten. Wir tragen damit zu einem besseren Verständnis der Prinzipien der frühen Follikelentwicklung bei Säugetierarten bei — ein Thema von großem Interesse für die Reproduktionsbiologie. Unsere Ergebnisse könnten in Zukunft auch für die Gestaltung von *in-vitro*-Experimenten zur Kultivierung früher Follikel bei Hauskatzen nützlich sein.

Summary

Mammalian ovarian follicles are formed in the female ovary either before or after birth in different species. The first type of ovarian follicles are a population of dormant primordial follicles, which contain a primary oocyte arrested at prophase I of the first meiotic division surrounded by granulosa cells. The number of primordial follicles that are formed in the ovary can be anywhere between hundreds, thousands or even millions, which differs across species. The peak number is suggested to be fixed and this population of dormant primordial follicles serve as the oocyte reservoir for future fertility. Ovarian folliculogenesis involves the activation of dormant primordial follicles and development into primary, secondary and antral follicle stages towards the ovulation of the oocyte. The regulation of ovarian folliculogenesis is crucial to female fertility. Many studies, predominantly performed in rodents, focused on elucidating the molecular mechanisms involved in ovarian follicle and oocyte development. There are therefore still major gaps in our knowledge for larger animal models. This is particularly true for early follicle development, from the primordial primary to secondary stages, for which the molecular mechanisms remain to be fully elucidated in mammals.

Using the domestic cat (*Felis catus*) as a model organism, we hypothesised that primordial to primary and primary to secondary follicle development is regulated by specific signalling pathways and characterized by specific gene expression patterns. Through a series of experiments, we aimed to describe transcription and protein expression during early follicle development. Domestic cat ovaries were obtained from animal shelters where routine ovariectomies were performed. The mechanical dissection technique was used to isolate whole early follicles, which consist of an oocyte and the surrounding somatic cells, from domestic cat ovaries. We collected primordial, primary and secondary follicles. Prior to ribonucleic acid (RNA) isolation, follicle samples were pooled for each type to increase the number of follicles per sample. Libraries were prepared for RNA-sequencing and custom index primers allowed us to pool the libraries prior to sequencing.

In our first publication, we presented the analysis of the RNA-sequencing data, which revealed genes that were significantly differentially expressed during early follicle development in this species. We identified specific molecular mechanisms which may be involved in the regulation of early folliculogenesis. For example, we found that the phosphatidylinositol-3-kinase and protein kinase B (PI3K/Akt) and the transforming growth factor beta (TGF-β) signalling pathways were involved in early follicle development in the domestic cat. Additionally, we

identified that the extracellular matrix (ECM) of the ovary was participating during both developmental transitions in this species too. For validation purposes, we compared the gene expression levels of the RNA-sequencing results to quantitative reverse transcription polymerase chain reaction (qRT-PCR) data for two genes: bone morphogenetic protein 15 (*BMP15*) and Histone 1, H1t (*HISTH1T*). In the RNA-sequencing data, we found that *BMP15* and *HISTH1T* were significantly differentially expressed during early follicular development. The qRT-PCR data was mainly concordant with these results. During our analysis for the first publication we also identified genes involved in ovarian steroidogenesis during early follicle development. In our second publication, we hypothesised that gonadotropin and sex steroid signalling is involved in early folliculogenesis and that early follicles are a source of sex steroids in the domestic cat so further investigations were pursued. We immunostained ovarian tissue sections and investigated the localisation of gonadotropin receptors, sex steroid receptors and steroidogenic enzyme proteins. We found that gonadotropin and sex steroid receptor protein signals were detected in different follicular locations in early follicles and at different intensities during early follicle development in the domestic cat. In comparison to the primordial and primary stages, when no protein signals were detectable for the analysed steroidogenic enzymes, we found that protein signals for some of them were detectable by the secondary stage. Although early follicles are fully equipped at the level of gene expression to produce sex steroids, we conclude from the protein data that it is may be possible that only secondary follicles are a source of sex steroids but not the earlier stages. We measured gene expression levels for three steroidogenic enzymes, the androgen receptor, progesterone receptors and a cholesterol transporter using qRT-PCR. The expression levels were too low to make a conclusive comparison to the RNA-sequencing results and no statistical significance was estimated for the expression of these genes during early folliculogenesis studied using qRT-PCR. In the future, a larger number of follicles per sample may overcome this and provide more insight into a smaller subset of genes.

In conclusion, we presented two in-depth studies which investigated in the domestic cat for the first time potential key genes, signalling pathways and molecular mechanisms that may be regulating early follicle development. We are contributing toward an improved understanding of the principles of early follicle development in mammalian species – a topic of great interest in reproduction biology. Our results may also be useful for designing *in vitro* experiments for the culture of early domestic cat follicles in the future.

Chapter 1: Introduction

1.1 The mammalian ovary

The mammalian ovary has been the subject of intense investigation for many years. Andreas Vesalius in his thesis *De humani corporis fabrica libri septem* (Vesalius, 1543), may have been the first to describe the ovarian components and the ovarian follicle – a structure containing the female egg cell or oocyte (Shoham and Schachter, 1996). By the 17th century, the ovary's role as a producer of eggs had been extensively explored, with William Harvey writing in *Exercitationes de generatione*: "*Ovum esse primordium commune omnibus animalibus*" ("the egg is the common starting point for all animals", (Harvey, 1662). Shortly after, Regnier De Graaf described morphological and functional features of the female reproductive organs in *De mulierim organis generation inservientibus* (De Graaf, 1672). Since then, this field of research has immensely progressed because of conceptual advances and progress in methodologies.

Typically ovaries in female mammals are lined with a stromal epithelium layer (Hummitzsch, 2019). Directly beneath this outermost layer lies **(Figure 1)** a layer of dense connective tissue known as the *tunica albuginea* (Hummitzsch et al., 2019). Underneath this, there is a composition of surrounding fibroblasts, fibres of collagen and elastin **(Figure 1)** which make up the ovarian cortex where the ovarian follicles are located (Hummitzsch et al., 2019). The medullary region **(Figure 1)** under the cortex contains a network of blood and lymphatic vessels (Hummitzsch et al., 2019). Ovarian follicles are the basic unit of the ovary which are composed of morphologically unique and varying numbers of stage-specific somatic cells surrounding the oocyte (Prasasya and Mayo, 2019). The structure of the ovarian follicle aids in protecting the oocyte and providing the oocyte with essential molecules required to promote follicle development (Prasasya and Mayo, 2019). Ovarian follicles also play a fundamental role in steroid production necessary for the brain, skeletal and cardiovascular systems (Jamnongjit and Hammes, 2006). The development of oocytes, termed oogenesis, involves a series of processes including oocytogenesis, ootidogenesis and folliculogenesis (Lim and Choi, 2012). Folliculogenesis, also known as ovarian follicle development, involves the activation of primordial follicles and their development into primary, secondary, preantral and antral follicle stages (described in detail in section 1.4) **(Figure 1)** (Prasasya and Mayo, 2019). Successful development may lead to the release of the oocyte from the ovary during ovulation **(Figure 1)** (Prasasya and Mayo, 2019). The regulation of ovarian folliculogenesis in mammalian species is a topic of great interest in reproduction biology.

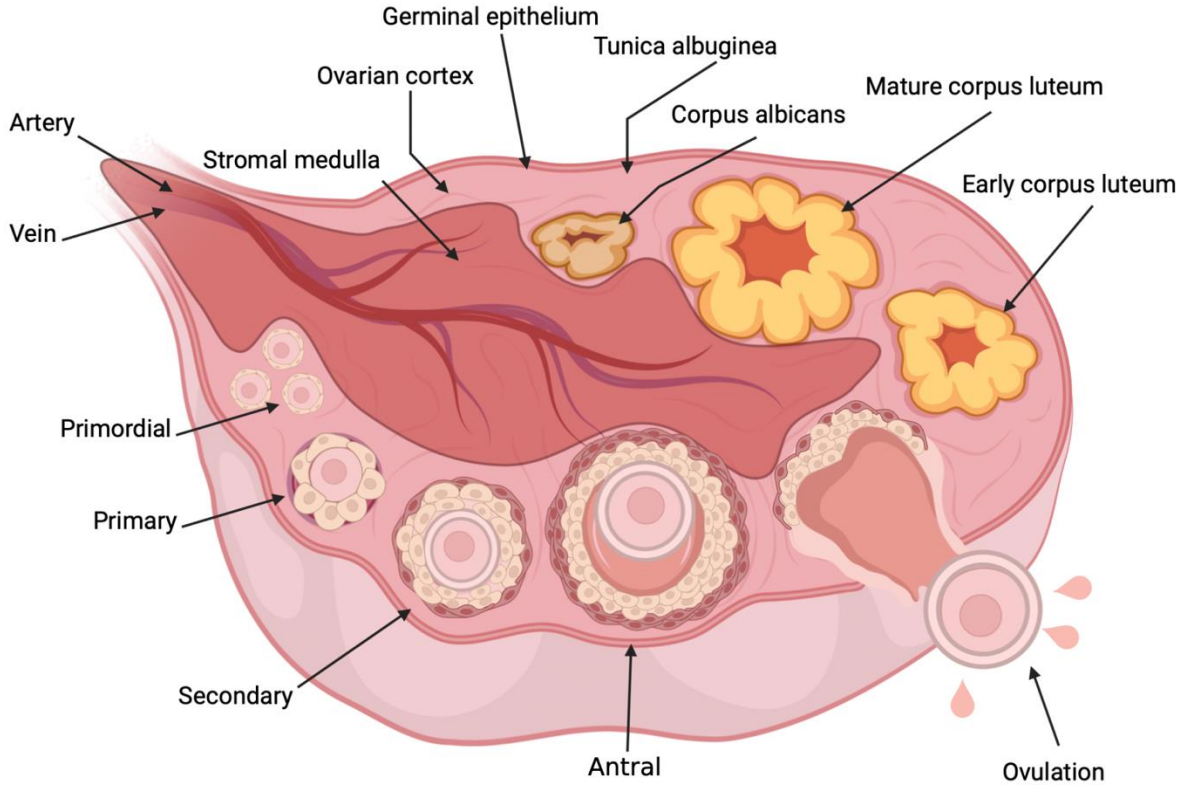

Figure 1. The typical morphological features of the mammalian ovary and a basic overview of ovarian folliculogenesis in mammals. *Ovaries are composed of an outer germinal epithelium, a tunica albuginea layer, an ovarian cortex and an inner stromal medulla. Ovarian follicles can be found throughout the ovarian cortex. The ovarian cortex is avascular, the medullary region is vascularised with branched networks of blood vessels. Ovarian follicles contain an oocyte and are surrounded by stage-specific somatic cells. Folliculogenesis includes the activation of primordial follicles and development into primary, secondary, preantral (not shown) and antral stage follicles The preovulatory follicle releases the oocyte from the ovary during ovulation. The somatic cells left behind then develop into a temporary endocrine gland known as the corpus luteum (pictured here in three stages of its development). The caption of this figure is based on information from two publications (Hummitzsch, 2019, Prasasya and Mayo, 2019). Adapted from "Ovary Anatomy", by BioRender.com (2022). Retrieved from* https://app.biorender.com/biorender-templates/t-6037f4d134362f00ac35c0e0-ovary-anatomy

1.2 The ovarian reserve in mammals

In mammals, ovarian follicle formation and the establishment of the ovarian reserve begins before or immediately after birth (Monniaux et al., 2014). Foetal life is therefore a critical period because it has direct implications for the female reproductive lifespan, which corresponds to the number of ovarian follicles within the ovarian reserve (Kaipia and Hsueh, 1997, Monniaux et al., 2014, Maidarti, 2020). To fully appreciate the process of ovarian follicle development it is worthwhile to know how female germ cells are formed. Briefly, the migration of undifferentiated primordial germ cells (PGCs), not yet a sperm or egg cell, begins from the embryonic yolk sac **(Figure 2)** through the dorsal mesentery of the hindgut toward the embryonic genital ridge (Kanamori et al., 2019). The migratory process includes several phases – separation, migration and colonisation of the genital ridge (Kanamori et al., 2019). Upon receiving signals to differentiate into female germ cells, PGCs multiply in large numbers by mitosis **(Figure 2)** and form oogonia (Kanamori et al., 2019). The colonisation of the genital ridge up to the formation of oogonia is completed at different time points in different species. For example, it occurs around 45 days post conception (dpc) in domestic cat prenatal ovaries (Monniaux et al., 2014). In humans, it is completed at the end of 35 days of embryogenesis, and in mice it is completed around ten dpc (Faddy et al., 1992, Monniaux et al., 2014). After colonisation, oogonia within germ cell nests enter meiosis and progress to the diplotene stage of the first meiotic prophase – where they become arrested in prophase I of meiosis I **(Figure 2)** and are termed primary oocytes (Wang and Pepling, 2021). Primordial follicle formation occurs over a series of events, including oocyte germ cell nest breakdown and encapsulation of the surviving oocytes **(Figure 2)** by pre-granulosa cells (Tanimoto et al., 2022). Subsequently, this results in a large population of arrested primary oocytes contained inside the primordial follicles which represents the female's ovarian reserve (Tanimoto et al., 2022). Over the female's lifetime, a selection of dormant primordial follicles may be activated and continue into ovarian follicle development (Tanimoto et al., 2022).

Ultimately, each primordial follicle has several possible fates: to remain dormant and die, to activate and develop but later be lost by atresia, or to activate and successfully develop, reaching the eventual point of ovulation **(Figure 2)** (Chen et al., 2020). How each follicle responds to developmental signals is imperative to cellular life or death.

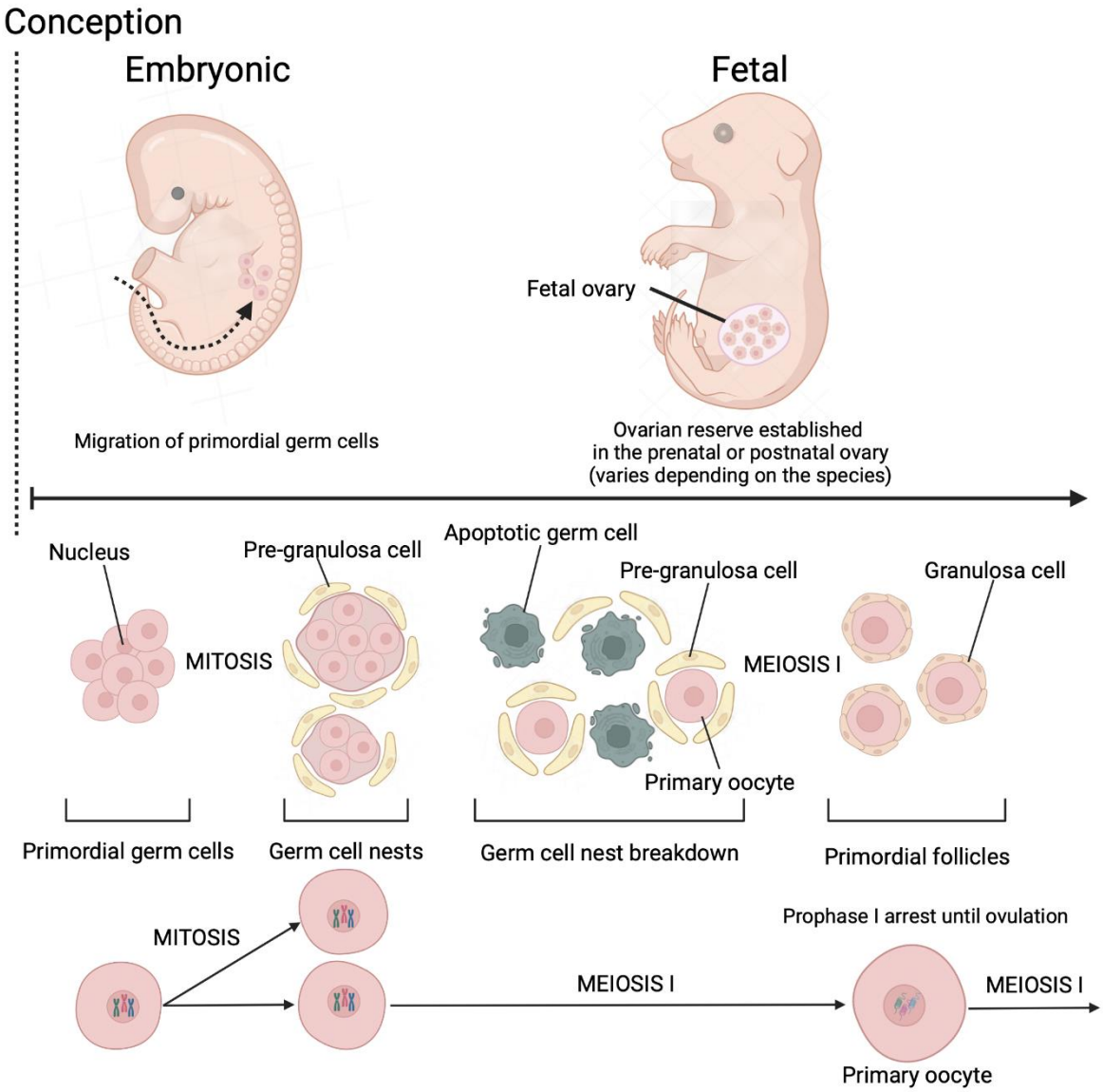

Figure 2. The origin of the ovarian reserve and primordial follicle formation in mammals. *Beginning in the embryo, primordial germ cells migrate to the genital ridge where they proliferate rapidly by mitosis, resulting in germ cell nests. The germ cell nests begin to break down, with many germ cells undergoing apoptosis as pre-granulosa cells encapsulate surviving primary oocytes. Primordial follicles are formed after germ cell nest breakdown. Primordial follicles are surrounded by a layer of squamous granulosa cells. The primary oocytes within ovarian follicles remain arrested at prophase I of the first meiotic division until ovulation. The caption of this figure is based on information from three publications (Kanamori et al., 2019, Wang and Pepling, 2021, Tanimoto et al., 2022). Originally created by me using BioRender.com (2022).*

1.3 Regulators of ovarian follicle development in mammals

Ovarian follicle development is regulated mainly by three organs, the hypothalamus, anterior pituitary and the gonads, which make up the hypothalamic-pituitary-gonadal (HPG) axis **(Figure 3a)** – a model describing the relationship between the external environment and the reproductive organs (Harris, 1955). The HPG axis involves the release of two gonadotropins, the follicle-stimulating hormone (FSH) and the luteinising hormone (LH), from the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) produced in the hypothalamus **(Figure 3a)** (Harris, 1955). The gonadotropins are essential for ovarian steroidogenesis, a molecular cascade that produces the ovarian steroids, 17-beta oestradiol and progesterone **(Figure 3b)**, critical for normal uterine function, establishment and maintenance of pregnancy and mammary gland development (Drummond, 2006). In most mammals, ovarian steroidogenesis occurs according to the two-cell, two-gonadotropin theory which posits that two ovarian follicle somatic cells **(Figure 3b)**, the granulosa and theca cells, work together to make ovarian steroids (Armstrong et al., 1979). The theca cells respond to LH signalling by increasing the expression of enzymes necessary for the conversion of cholesterol **(Figure 3b)** to progesterone and androgens such as androstenedione and testosterone (Drummond, 2006, Jamnongjit and Hammes, 2006). The granulosa cells respond to FSH signalling by increasing the expression of enzymes necessary for the conversion of theca cell-derived androgens **(Figure 3b)** into oestrogens such as, oestradiol and oestrone (Drummond, 2006, Jamnongjit and Hammes, 2006).

In turn, the process of follicle development is regulated by the pituitary gonadotropins, FSH and LH, which may be generally divided into three phases including the gonadotropin-independent phase, the gonadotropin responsive phase and the gonadotropin-dependent phase (Orisaka et al., 2021, McGee and Hsueh, 2000, Orisaka et al., 2009). Early follicle development may occur independently of FSH and LH stimulation as demonstrated in studies using FSH deficient mice (Abel et al., 2000) and women lacking gonadotropins (Goldenberg et al., 1976). Follicle development from the preantral stage to the early antral stage is controlled by intraovarian regulators (Cattanach et al., 1977) and may be stimulated by FSH (McGee and Hsueh, 2000). Studies in bovine and human have shown that supplementing *in vitro* cultures with a low dose of FSH is beneficial to preantral follicle development (McLaughlin and Telfer, 2010, McLaughlin et al., 2018). Follicle growth and maturation beyond the early antral stage including follicle recruitment, selection and ovulation, is dependent on FSH and LH (gonadotropin-dependent phase) (Kumar et al., 1997). In a study using human ovarian tissue

transplanted into hypogonadal mice, it was shown that the transplanted follicles with two layers of granulosa cells did not form antral cavities suggesting follicle dependence (Oktay et al., 1998).

For the most part however, the key genes, signalling pathways and molecular mechanisms which regulate early follicle development remain elusive in mammals. Past studies in mice and human found that the phosphatase and tensin homolog deleted on chromosome 10/phosphatidylinositol-3-kinase/protein kinase B (PTEN/PI3K/Akt) signalling pathway may play an important role in early follicle development regulation (Hsueh et al., 2015, John et al., 2008, Castrillon et al., 2003, Gallardo et al., 2008, Reddy et al., 2008, Zheng et al., 2012, Jagarlamudi et al., 2009, Li et al., 2010, Grøndahl et al., 2013, Novella-Maestre et al., 2015, Wang et al., 2016). Members from the TGF-β-family such as the anti-Müllerian hormone and bone morphogenetic proteins (BMPs) have been implicated as essential regulators of early folliculogenesis in rodent and human studies (Durlinger et al., 1999, Carlsson et al., 2006, Durlinger et al., 2001, Durlinger et al., 2002, Wang et al., 2014, Nilsson and Skinner, 2003, Nilsson et al., 2007, de Castro et al., 2016, Kedem et al., 2011). Ovarian follicles are also suggested to be under the influence of the ovarian microenvironment too, as demonstrated in a mouse study in recent years (Nagamatsu et al., 2019).

In summary, we suggest that ovarian follicle development is regulated by a complex series of extraovarian and intraovarian ligands functioning in an endocrine, autocrine and/or paracrine manner. Apart from the well-studied mouse model, the principles of mammalian ovarian folliculogenesis remain very poorly understood overall.

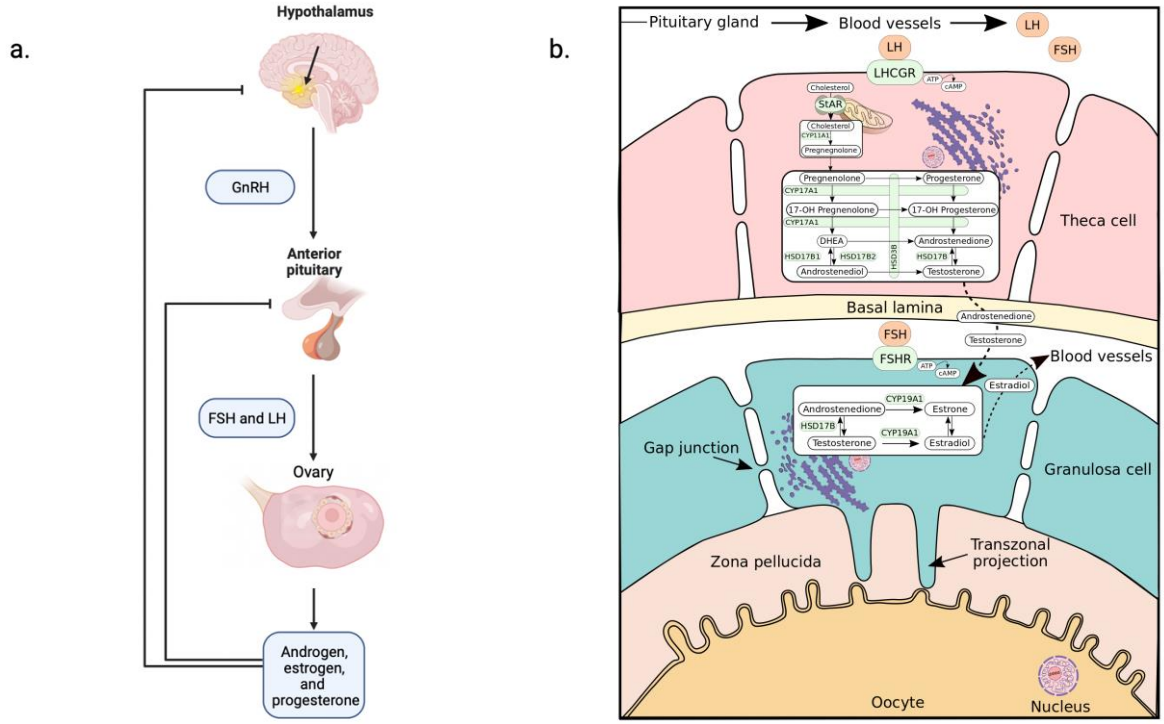

Figure 3. The HPG axis and the two-cell, two-gonadotropin theory. (a) *The HPG axis involves the GnRH produced in the hypothalamus which stimulates the release of FSH and LH from the anterior pituitary. The gonadotropins trigger the production of androgen, oestrogen, and progesterone in the ovary which can exert an inhibitory effect on the hypothalamus and pituitary by downregulating the release of GnRH.* ***(b)*** *Steroid production in ovarian follicles occurs via the two-cell, two-gonadotropin model where ovarian steroids are synthesised from cholesterol through interactions between granulosa and theca cells. LH binds to the luteinising hormone/choriogonadotropin receptor (LHCGR) and stimulates the expression of the steroidogenic enzymes necessary for androgen production in theca cells. Cholesterol is transported by steroidogenic acute regulatory protein (StAR) from the outer to the inner mitochondrial membrane where it is converted to pregnenolone by the cholesterol sidechain cleavage enzyme (CYP11A1). Pregnenolone diffuses into the smooth endoplasmic reticulum and is converted to progesterone by the 3-beta-hydroxysteroid dehydrogenase enzyme (HSD3B). Progesterone is then converted to androstenedione by 17-alpha-hydroxylase/17, 20-desmolase (CYP17A1). In granulosa cells, FSH signals via the follicle-stimulating hormone receptor (FSHR) which stimulates the expression of enzymes necessary for oestrogen synthesis. Androstenedione produced by theca cells diffuses into granulosa cells and is converted to testosterone by the enzyme 17-beta-hydroxysteroid dehydrogenase (HSD17B) or to oestrone by aromatase (CYP19A1). The CYP19A1 enzyme uses testosterone to produce 17-beta-oestradiol. However, HSD17B can also produce 17-beta-oestradiol using oestrone as a*

substrate. Abbreviations: cyclic adenosine monophosphate (cAMP), adenosine triphosphate (ATP), 17-beta-hydroxysteroid dehydrogenase 1 (HSD17B1), 17-beta-hydroxysteroid dehydrogenase 2 (HSD17B2), 17-hydroxypregnenolone (17-OH-pregnenolone) and dehydroepiandrosterone (DHEA). The caption of this figure is based on information from five publications (Harris, 1955, Drummond, 2006, Armstrong et al., 1979, Jamnongjit and Hammes, 2006, Wagner, 2020). Graphic (a) was adapted from "Hypothalamic-Pituitary-Organ Axis (Layout)", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates/t-61f81089524a7e00a0a48701-hypothalamic-pituitary-organ-axis-layout*. Graphic (b) was originally created by me using Inkscape Project. (2020). Inkscape. Retrieved from https://inkscape.org.*

1.4 Ovarian follicle development in the domestic cat

In the domestic cat, primary oocyte formation takes place during a well-defined period, with the onset occurring at approximately 40–50 days of foetal development, possibly completing 8 days after birth (Bristol-Gould and Woodruff, 2006). The activation of dormant primordial follicles **(Figure 4)** begins around 40 days after birth in the domestic cat (Monniaux et al., 2014).

In general, cat ovarian follicles resemble those described in other mammalian species in terms of cell type present during development however, the ultimate size of the follicle is proportional to the size of the animal (Griffin et al., 2006). For example, domestic cat primordial follicles contain on average a single layer of 4-10 squamous granulosa (Ariel et al., 2016) and the follicle and oocyte measure at 40 μm (Jewgenow and Göritz, 1995) and 20-30 μm (Bristol-Gould and Woodruff, 2006) in diameter, respectively **(Figure 4)**. In mice the diameter of a primordial follicle is 17 μm, in pig 34 μm and similar to the domestic cat it is 44 μm in human (mean values) (Griffin et al., 2006). Primary follicles in the domestic cat contain on average a single layer of cuboidal granulosa cells and the follicle and oocyte measure at 50 μm (Jewgenow and Göritz, 1995) and 30-40 μm (Bristol-Gould and Woodruff, 2006) in diameter, respectively **(Figure 4)**. Secondary follicles vary in size ranging between 100-400 μm (Bristol-Gould and Woodruff, 2006) with the oocytes ranging between 40-75 μm (Bristol-Gould and Woodruff, 2006) or in later stages 70-90 μm (Jewgenow and Pitra, 1993). The oocyte in early secondary follicles is surrounded by a transparent, extracellular coat called the zona pellucida **(Figure 4)** and multiple layers of granulosa cells that are surrounded by newly recruited theca cells (Bristol-Gould and Woodruff, 2006). The zona pellucida assists in species-specific sperm-egg binding, induction of the acrosome reaction and post-fertilisation secondary blockage of the polyspermy (Sinowatz et al., 2001, Wassarman et al., 1998, Wassarman, 1982). By this stage, the theca cell layer is well-established opposite the basement membrane and separates the surrounding layer of granulosa cells from the ovarian stroma (Bristol-Gould and Woodruff, 2006). Small antral follicles can be similar in diameter to late-stage preantral follicles, but they greatly increase in size as follicular fluid begins to accumulate (Bristol-Gould and Woodruff, 2006). Large antral follicles contain intact mural and cumulus granulosa layers, many theca cell layers, a large antral space and an oocyte (Bristol-Gould and Woodruff, 2006).

Development of the antral and pre-ovulatory follicle begins after puberty when the two gonadotropin hormones, FSH and LH, become involved **(Figure 4)** (Bristol-Gould and Woodruff, 2006). Puberty in the domestic cat occurs between 4-12 months of age, but this

varies depending on breed, photoperiod (light and dark exposure) and body weight (Jemmett and Evans, 1977, Lofstedt, 1982, Bristol-Gould and Woodruff, 2006). Until ovulation, the primary oocyte within developing ovarian follicles is arrested at prophase I of the first meiotic division (Laisk et al., 2019). At ovulation, the secretion of FSH and LH triggers the exit from prophase arrest, and primary oocytes complete the first meiotic division (Laisk et al., 2019). The molecular mechanisms which regulate early follicle development in the domestic cat, including primordial follicle dormancy and activation, are for the most part unknown.

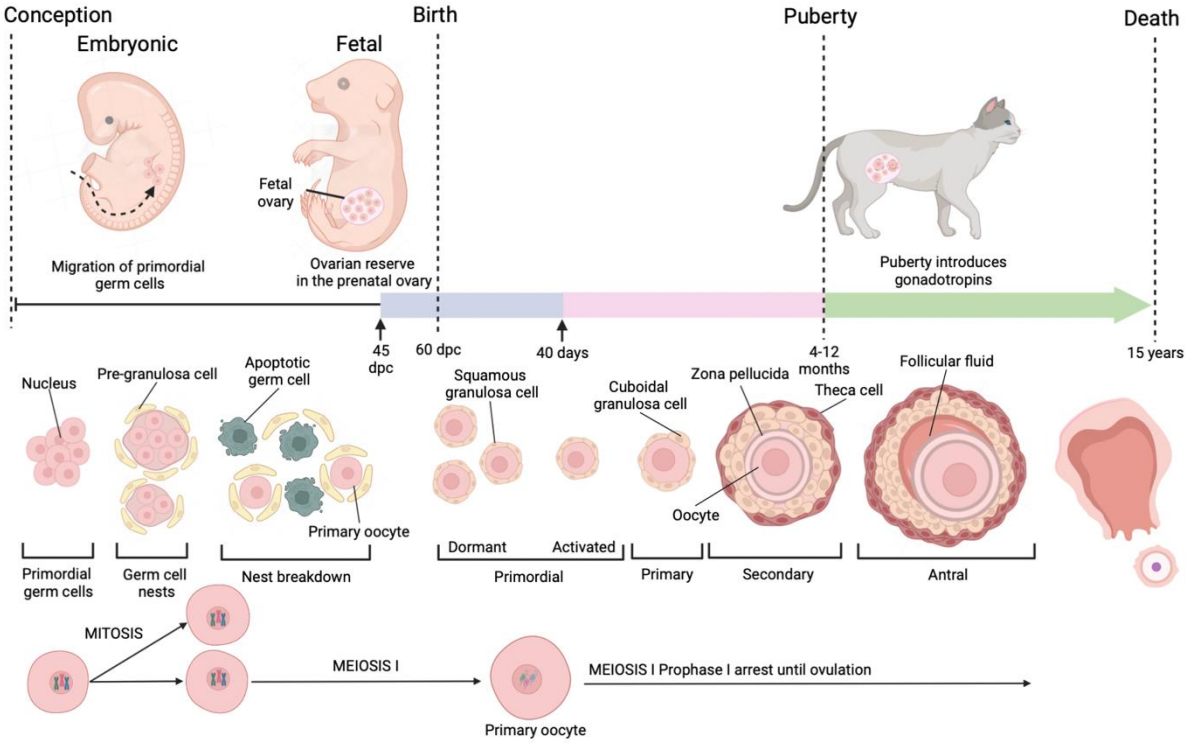

Figure 4. The ovarian reserve and folliculogenesis in the domestic cat. *The formation of primordial follicles begins 45 dpc and lasts until 40 dpc in the domestic cat. The boundary between the violet and pink bars corresponds to the stage at which most primordial follicles have been formed. At 40 dpc, dormant primordial follicles can be activated by specific signals to begin folliculogenesis. However, until puberty occurs at around 4-12 months of age, activated follicles can only grow until the early antral stage. At puberty, the input of gonadotropins allows for the progression beyond the early antral stage through to ovulation. The boundary between the pink and green bars corresponds to the first occurrence of complete follicular development culminating in ovulation. The bars indicate* ***(1)*** *meiosis initiation and formation of primordial follicles (violet bar),* ***(2)*** *folliculogenesis before puberty (pink bar), and* ***(3)*** *folliculogenesis and occurrence of ovulation after puberty (green bar). Before birth, age is indicated as dpc and after birth, age is indicated as days, months, or years. The caption of this figure is based on information from three publications (Bristol-Gould and Woodruff,*

2006, Monniaux et al., 1997, Monniaux et al., 2014). Originally created by me using BioRender.com.

1.5 Importance of studies on early follicle development in the domestic cat

Of the 37 wild cat species, many are threatened and face extinction in all or part of their native habitat such as, the cheetah (*Acinonyx jubatus*), Asian golden cat (*Catopuma temminckii*), black-footed cat (*Felis nigripes*), Iberian lynx (*Lynx pardinus*), leopard (*Panthera pardus*), Amur tiger (*Panthera tigris*), lions within the Indian population (*Panthera leo*) and snow leopard (*Panthera uncia*) to name a few (CITES-Appendix I)[1]. For critically endangered populations that are managed in captivity but whose individuals struggle to mate naturally or have infertility issues, assisted reproductive techniques (ARTs) are required as a reproductive support (Zahmel et al., 2022). This includes techniques such as *in vitro* maturation (IVM) of oocytes, *in vitro* fertilisation (IVF) and embryo transfer into a surrogate mother (Zahmel et al., 2022).

The most critical limiting factor is the lack of mature and developmentally competent oocytes suitable for current ARTs. One solution could be to use the oocytes present in the large population of dormant primordial follicles instead. Two methods essential to this outcome are *in vitro* activation (IVA) of primordial follicles and *in vitro* growth (IVG) of early follicles. In the mouse, the promise of these methods has been dramatically demonstrated through the live birth of "Eggbert" who was conceived from primordial follicles cultured within ovarian tissue pieces *in vitro* (Eppig and O'Brien, 1996). Unfortunately, Eggbert did not develop normally and died prematurely. As a consequence, the same investigators modified their procedure, adding and removing various factors at different time points of culture which remarkably resulted in the birth of 59 normal pups (O'Brien et al., 2003). Since Eggbert, the recapitulation of early follicle development *in vitro* has progressed in larger animal models such as, bovine (Gupta et al., 2008, Gupta and Nandi, 2012), porcine (Wu et al., 2001), non-human primates (Xu et al., 2009, Xu et al., 2011) and humans (Hovatta et al., 1997, Hovatta et al., 1999, Picton and Gosden, 2000, Telfer and McLaughlin, 2012, Xiao et al., 2015).

This is a promising step forward in the establishment of novel *in vitro* methods for species conservation. It also highlights that minor changes based on an insight into the molecular

[1] Accessed on the 26th of October 2022
https://checklist.cites.org/#/en/search/cites_appendices%5B%5D=I&output_layout=alphabetical&level_of_listing=0&show_synonyms=1&show_author=1&show_english=1&show_spanish=1&show_french=1&scientific_name=Felidae&page=1&per_page=20

mechanisms which regulate early follicle development can have a large impact on the success of *in vitro* experiments. For endangered felid species, the most relevant model organism to investigate early folliculogenesis is the domestic cat (Rojo et al., 2015). Several studies have already gained some insights into factors and molecular mechanisms which may regulate early folliculogenesis in the domestic cat (Fujihara et al., 2016, Fujihara et al., 2018, Jewgenow and Pitra, 1993) but this knowledge is far from complete. There are still major gaps in our fundamental knowledge on how early follicle development is regulated in this species. Furthermore, the successes observed in the rodent model are yet to be accomplished in the domestic cat.

The domestic cat is also considered a suitable model for humans as cat oocytes share similar features to human oocytes that are not present in the rodent model (Rojo et al., 2015). Studying ovarian follicle biology in model organisms may benefit human ARTs that are used a form of treatment prior to gonadotoxic chemotherapy and radiotherapy (Nicosia et al., 1985, Wallace et al., 1989, Byrne et al., 1992, McVie, 1999). Currently, the fertility preservation options for post-pubertal and adult cancer patients includes the cryopreservation of ovarian tissue, oocytes and embryos, although oocyte cryopreservation tends to be avoided in pre-pubertal cancer patients because of concerns about the immaturity of the HPG axis (Reichman et al., 2012, Hanson and Franasiak, 2020). In that case, the only option for pre-pubertal cancer patients and older patients that cannot delay chemotherapy is the cryopreservation of autologous ovarian tissue prior to treatment (Resetkova et al., 2013, Kim et al., 2018). The re-transplantation of a patient's ovarian tissue of course runs the risk of reseeding malignant cells (Dolmans et al., 2013). Therefore, there is an urgent need for alternative methods as the number of people requiring fertility preservation increases (Rodriguez-Wallberg et al., 2019). Developing alternative methods such as IVA and IVG could benefit this group of patients in the future – even though at present, the technique is still in its infancy and much more research in animal models is required first.

1.6 Focus of the study

Early ovarian folliculogenesis includes the activation of dormant primordial follicles from the ovarian reserve and their development into primary and secondary stages in mammalian species. The exact molecular mechanisms which regulate early follicle development remain to be fully elucidated. For the domestic cat model, we hypothesised that early follicle development could be characterised by specific molecular mechanisms during primordial,

primary and secondary follicle development. We expected that gene expression levels of specific proteins could serve as developmental biomarkers. The aim of this thesis was therefore to **(1)** identify potential key genes and by this the signalling pathways that characterise early follicle development in the domestic cat; **(2)** discover candidate genes for follow-up studies; and **(3)** by contrasting the signalling pathways of the domestic cat with the mouse model (and maybe humans) take a first step towards identifying general principles of follicle development in mammals.

The domestic cat is an attractive medium-sized model to study early ovarian folliculogenesis. Ovaries can be readily obtained from veterinary clinics from routine ovariectomies performed in animal shelters, and follicles can be easily accessed in ovarian tissue through cost-effective techniques such as mechanical dissection. Not only is the domestic cat an extremely popular companion animal for people, it is also the most relevant model organism to endangered wild felids and can be used as a model organism for humans too. Therefore, the results presented here may have wider practical implications for future ARTs in a variety of species.

Chapter 2: Signalling pathways and mechanistic cues highlighted by transcriptomic analysis of primordial, primary, and secondary ovarian follicles in domestic cat

2.1 Summary

In this study, published in "Scientific Reports", we hypothesised that early follicle development could be characterised by specific molecular mechanisms in the domestic cat (Kehoe et al., 2021). Our goal was to characterise gene expressions, signalling pathways and molecular mechanisms during primordial to primary and primary to secondary development in adult, non-pregnant domestic cats.

To do so, we mechanically isolated early ovarian follicles, 60 primordial (≤ 45 μm) follicles, 15 primary (55–70 μm) follicles and 3 secondary (85–110 μm) follicles. Samples were lysed, snap frozen in liquid nitrogen (LN_2), and then stored in -80°C freezer for a brief period. Next, selected samples were thawed and pooled per follicle type to provide us with 180 primordial follicles per tube, 45 primary follicles per tube and 9 secondary follicles per tube. Total RNA was extracted, complementary deoxyribonucleic acid (cDNA) generated and sequencing libraries created with custom index primers that were tagged to each library preparation to allow for demultiplexing. Libraries were quantified, normalised and then sequenced.

The data was analysed using DESeq2 through contrasts of follicle types to produce two lists of genes that were differentially expressed: primordial versus primary and primary versus secondary. This allowed us to identify significant changes in gene expression during primordial to primary and primary to secondary development, respectively. Functional annotation and enrichment analysis allowed us to identify the top gene ontology (GO) terms that were associated with biological processes implicated to play an important role during early follicle development in the domestic cat. We found the PI3K/Akt, TGF-β and hypoxia-inducible factor (HIF) 1 signalling pathways were involved during early folliculogenesis in the domestic cat. We also identified the ECM in this list too. We presented differential gene expression patterns for a subset of matrix metalloproteinase (MMP) genes which are suggested to interact with the ECM. We also compared the gene expression levels of the RNA-sequencing and qRT-PCR data for two genes: *BMP15* and *HISTH1T* whose gene expression patterns analysed by qRT-PCR were mainly concordant with the RNA-sequencing data.

The results from this publication have led to the description of early follicle developmental regulation in the domestic cat. Similarities amongst genes, signalling pathways and biological processes were discussed with reference to published work. The publication also highlighted some open research questions which could be pursued in the future.

2.2 Author contributions

Shauna Kehoe: Collection of ovarian follicle samples, characterisation and imaging of ovarian follicles, isolation of RNA, generation of RNA-sequencing libraries, quality control checks of RNA-sequencing libraries, documentation of experiments, experimental design of RNA-sequencing analysis pipeline, writing scripts in R, analysis, preparing the manuscript, qRT-PCR (unpublished data), and discussing data.

Beate C. Braun and Katarina Jewgenow: Project design, supervision, discussing data, and preparing the manuscript.

Beate C. Braun: qRT-PCR and analysis.

Susan Mbedi: Contributed reagents, materials, and advice during RNA-sequencing library preparation and quality control checks.

Paul R. Johnston: Experimental design of RNA-sequencing analysis pipeline and writing scripts in R.

All authors were involved in correcting the manuscript.

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Signalling pathways and mechanistic cues highlighted by transcriptomic analysis of primordial, primary, and secondary ovarian follicles in domestic cat

Shauna Kehoe[1]✉, Katarina Jewgenow[1], Paul R. Johnston[2,3,4], Susan Mbedi[2,5] & Beate C. Braun[1]

In vitro growth (IVG) of dormant primordial ovarian follicles aims to produce mature competent oocytes for assisted reproduction. Success is dependent on optimal in vitro conditions complemented with an understanding of oocyte and ovarian follicle development in vivo. Complete IVG has not been achieved in any other mammalian species besides mice. Furthermore, ovarian folliculogenesis remains sparsely understood overall. Here, gene expression patterns were characterised by RNA-sequencing in primordial (PrF), primary (PF), and secondary (SF) ovarian follicles from *Felis catus* (domestic cat) ovaries. Two major transitions were investigated: PrF-PF and PF-SF. Transcriptional analysis revealed a higher proportion in gene expression changes during the PrF-PF transition. Key influencing factors during this transition included the interaction between the extracellular matrix (ECM) and matrix metalloproteinase (MMPs) along with nuclear components such as, histone HIST1H1T (H1.6). Conserved signalling factors and expression patterns previously described during mammalian ovarian folliculogenesis were observed. Species-specific features during domestic cat ovarian folliculogenesis were also found. The signalling pathway terms "PI3K-Akt", "transforming growth factor-β receptor", "ErbB", and "HIF-1" from the functional annotation analysis were studied. Some results highlighted mechanistic cues potentially involved in PrF development in the domestic cat. Overall, this study provides an insight into regulatory factors and pathways during preantral ovarian folliculogenesis in domestic cat.

Artificial reproductive technology (ART) such as, germ cell cryopreservation and in vitro embryo production can potentially contribute to species conservation when populations decline to a critically small size[1]. However, in practice, the inadequate number of oocytes that are acquired from later ovarian follicle stages limits current applications within endangered species conservation breeding programs[2]. First and foremost, improving female gamete yield is essential in order to progress. Studies involved in optimising these techniques could potentially be applied to human oncofertility and fertility preservation in the future also[3].

Within the ovary, an oocyte is found inside an ovarian follicle[4]. Ovarian follicles from various developmental stages are contemporaneously present within adult animal ovaries beginning from PrF, PF, SF, to antral follicular stages. Each follicle stage is characterised by cell-specific morphological and physiological features of the oocyte and the surrounding follicular cells[4]. The activation, growth, and development of an ovarian follicle, a process termed ovarian folliculogenesis, is finely regulated by cell-to-cell interactions and coordinated gene expression

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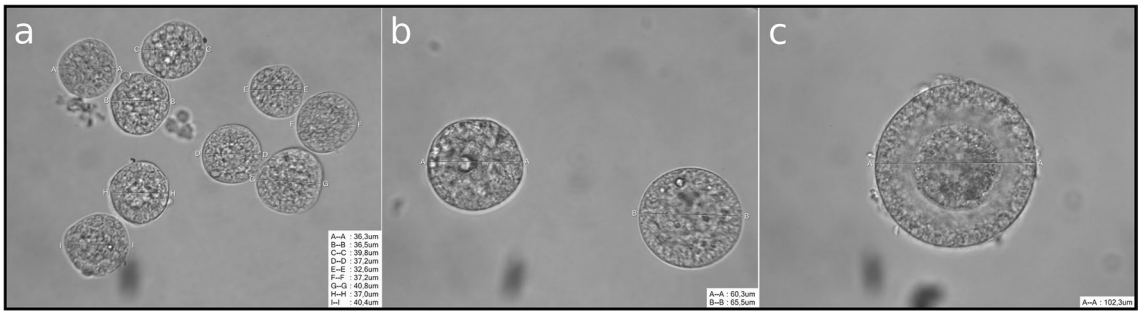


Figure 1. Microscope images of domestic cat preantral ovarian follicles. **(a)** PrF (≤ 45 µm), **(b)** PF (55–70 µm), **(c)** SF (85–110 µm with a visible zona pellucida).

in the oocyte and surrounding granulosa cells (GCs)[4]. Oocyte recovery for in vitro applications can be achieved by isolating granulosa-oocyte-complexes from later antral follicles however, the majority of oocytes (≥ 90%) are encapsulated in the earliest, dormant PrFs[5]. Therefore, harnessing oocytes from this larger resource could potentially overcome current limitations in the field[6]. Nevertheless, the recapitulation of female germ cell development from the early preantral stage is challenging and remains in its rudimentary stage for mammalian species.

At present, complete oogenesis originating from primordial germ cells has been achieved in mice[7,8]. For other species such as, ovine and feline, more advanced ovarian follicle stages have developed successfully in culture[9,10]. Embryo production from oocytes derived from advanced preantral follicles developed by IVG has been achieved in porcine, buffalo, ovine, and goat but inconsistently so[11]. Evidently, inadequate oocyte maturation rates are especially accountable for this which demands considerable research. Overall, the key to improving the success rate seems to lie within the sequential provision of essential nutrients and growth factors to the follicles based on a profound knowledge on effective biomarkers for the particular ovarian follicular stage[12]. Thus, RNA-sequencing transcriptomic analysis could address fundamental questions surrounding ovarian folliculogenesis and potentially identify genes and biological processes useful for developing IVG methods.

Mainly, elucidating molecular mechanisms during early folliculogenesis has focused on ovarian follicle gene expression in transgenic murine models[13–15]. Transcriptomic profiling from laser capture microdissection (LCM) of ovine GCs and oocytes has been performed along with gene expression profiling at a spatio-temporal level in the same species[16,17]. In bovine, transcriptome analysis of GCs from ovarian follicles has characterised differential gene expression and has investigated gene expression during development of antral follicles[18,19]. Transcriptomic profiling of human oocytes from PrFs and PFs isolated by LCM has revealed putative signalling factors associated with the maintenance and activation of PrF dormancy[20]. Many studies in domestic animals and rodents are based on early ovarian follicle samples obtained from juveniles (fetal or new born). This allows an exact determination of major follicular stages during ontogenesis. In comparison to this, investigating ovarian folliculogenesis in the adult mammalian ovary can provide insight into the mechanisms involved in maintenance of the ovarian reserve and subsequently activation and developmental processes.

Overall, the aim of this study was to elucidate differential gene expression and biomolecular mechanisms in early preantral follicles from the domestic cat using RNA-sequencing data. To do so, PrFs, PFs, and SFs were isolated from domestic cat ovaries utilising a previously established ovarian follicle isolation method[21]. Two major developmental transitions were explored: PrF-PF and PF-SF. Distinct differences in gene expression levels between these two transitions were found. Functional annotation clustering analysis described biological processes (BPs), cellular components (CCs), and molecular functions (MFs) associated with preantral ovarian folliculogenesis in the domestic cat.

Materials and methods

All chemicals and materials were purchased from Merck KGaA, Darmstadt, Germany unless stated otherwise.

Sample collection. Ovaries were obtained from domestic cats after ovariectomy performed at animal shelters in Berlin. After excision, ovaries were stored in HEPES-MEM medium, supplemented with 3 g/L BSA and 1 × Antibiotic Antimycotic Solution in 50 mL tubes (Sarstedt AG & Co. KG, Nümbrecht Germany) at 4 °C and were processed within a 2–3 h time-frame. The collection of preantral ovarian follicles from domestic cat ovaries has been described previously[21]. In brief, ovaries were pressed through a cell dissociation sieve (60 mesh) in a Dulbecco's phosphate-buffered saline solution supplemented with BSA (DPBS-BSA—0.3 mg/mL) into a petri dish (Thermo Fisher Scientific, Dreieich, Germany). This cell suspension was pipetted through a series of nylon sieve 40 µm, 70 µm, and 100 µm cell strainers (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Flushing the 40 µm, 70 µm, and 100 µm sieves with 6 mL DPBS-BSA allowed for the enrichment of PrFs, PFs, and SFs, respectively. It should be noted that in each flushed sieve suspension, ovarian follicles from other stages could be found. Calibrated and siliconised glass pipettes (coated with Sigmacote) were utilised to collect ovarian follicles. Collected ovarian follicles were measured with an inverse microscope with a 40 x objective (Axiovert 100, Jenoptic, Jena, Germany) equipped with an RI camera and software system (CooperSurgical Fertility and Genomic Solutions, Germany) and ovarian follicle types were determined based on the following measured diameters: PrF ≤ 45 µm, PF 55–70 µm, and SF 85–110 µm, respectively (Fig. 1). Regarding SFs, only those exhibiting a visible zona pellucida were selected for sampling (Fig. 1c). Collected samples were lysed in 10 µL Lysis Buffer

1 from the NucleoSpin RNA Plus XS kit (Macherey–Nagel GmbH & Co. KG, Berlin, Germany) which immediately inactivated RNases. The samples were snap-frozen in liquid nitrogen and stored at − 80 °C until RNA extraction. Sample collection for RNA-sequencing included three biological replicates of pooled follicle samples: PrF (n = 180 follicles from 3 individuals collectively (n = 60 follicles each), N = 3 samples), PF (n = 45 follicles from 3 individuals collectively (n = 15 follicles each), N = 3 samples), and SF (n = 9 follicles from 3 individuals collectively (n = 3 follicles each), N = 3 samples), giving a total of 9 samples. For quantitative real-time PCR (qRT-PCR), additional pooled follicle samples were collected, measured, and stored accordingly: PrF (n = 180 follicles, N = 4 samples), PF (n = 45 follicles, N = 4 samples), and SF (n = 9 follicles, N = 4 samples).

RNA-sequencing. Total RNA was extracted with NucleoSpin RNA Plus XS (Macherey–Nagel GmbH & Co) from the 9 samples following the manufacturer's instructions. Total RNA sample integrity and concentration was measured by Agilent High Sensitivity RNA ScreenTape Assay (Agilent 2200 TapeStation system, Agilent Technologies, Inc., Santa Clara, US). Sample concentrations for quantification were determined by Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, USA). During extraction, genomic DNA was removed by gDNase during on-column DNA digestion. The purified RNA was dissolved in 20 µL RNase-free water and stored at − 80 °C. The cDNA was generated and amplified in 9 cycles with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio, Inc. Mountain View, USA). Libraries were generated (Nextera XT DNA Library Prep Kit, Illumina, San Diego, USA) and sample quality was checked by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA) and by Agilent High Sensitivity D1000 ScreenTape Assay (Agilent 2200 TapeStation system). Custom index primers were tagged to the libraries to allow for multiplexing during RNA-sequencing. Libraries were quantified, normalised based on measurements determined by Qubit 2.0 Fluorometer, and sequenced on the Illumina NextSeq 500 system (150 cycles). The raw RNA-Seq data was deposited and released with the BioProject accession number of PRJNA635095.

RNA-sequencing analysis. The analysis was performed in R (https://www.r-project.org/), R 3.4.4[22], with Bioconductor (https://www.bioconductor.org/) packages. The analysis workflow, scripts, and a list of acquired packages along with citations are available in the Github repository: https://github.com/kshauna/OvarianFollicleTranscriptomics-DomesticCat.

Raw data consisted of paired-end, double-indexed cDNA library sequencing reads. The PhiX adapter-ligated control was sequenced at a standard concentration of 5%. The de-multiplexed data received in fastq file format was quality checked with FastQC[23] and summarised with MultiQC[24]. Salmon[25] quantified transcripts from a FASTA file containing the reference transcriptome and FASTQ files containing the sequence reads. Transcript abundance, counts, and length were summarised by tximport[26]. The DESeqDataSet under three factor levels "type" determined differential gene expression for PrFs, PFs, and SFs with DESeq2[27]. Ovarian follicle type contrasts, PrF versus PF (PrF-PF) and PF versus SF (PF-SF), were designed. Differentially expressed genes (DEGs) were estimated from the un-normalised, paired-end fragments by the Independent Hypothesis Weighting[28] method with an alpha = 0.05, an adjusted P value < 0.05, and absolute $\log^2$ fold-change of 1 (Supplementary Data S1). For quality control, the log fold-change shrinkage estimates of normalised data were visualised with heatmaps of Euclidean distances and with a principal component analysis (PCA). To assign Entrez gene identifiers (IDs) BioMart[29,30] was employed. The Entrez IDs were input into the web-based portal "the database for annotation, visualisation and integrated discovery" (DAVID) Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/)[31,32]. The tool DAVID was chosen to: (1) identify major gene groups (functional classification); (2) to elucidate enriched annotation terms (functional annotation chart and clustering, respectively); (3) and to provide an overview of gene annotations (functional annotation table)[31]. Importantly, the last update, DAVID 6.8, occurred in October 2016 and the data analysed here was submitted to DAVID 6.8 at the beginning of 2019. Researchers interested in enrichment analysis should consider a combination of other tools such as gProfiler[33], Enrichr[34,35], and/or Metascape[36,37]. However, these portals and others such as, PANTHER[38], InterMine[39,40], and GeneTrail2[41] may be relying on old knowledge bases[37]. Here, the functional annotation and enrichment analysis workflow is summarised as follows: analysis with DAVID 6.8 output gene ontology (GO)[42] and KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology (KO)[43] terms which were categorised into functional annotation clusters. The functional annotation tool (FAT) was applied to the GO categories BP, CC, and MF which filtered out broad GO terms based on measured specificity. Default categories were unselected and "GOTERM_BP_FAT", "GOTERM_CC_FAT", "GOTERM_MF_FAT", and "KEGG_PATHWAY" were selected. An over-representative hypergeometric test on the resulting GO and KO terms was performed and the clustering options within DAVID were modified as follows: Similarity Term Overlap 3, Similarity Threshold 0.60, Initial Group Membership 3, Final Group Membership 3, Multiple Linkage Threshold 0.50, and an Enrichment Threshold EASE 0.2. The GO and KO terms with an arbitrary enrichment score (ES) > 0.1 were considered for further investigation. A false discovery rate (FDR), Q value (Q), corrected for multiple testing of P values of the enriched terms. A $Q < 0.05$ was considered as significant. For visualisation purposes the web-based portal Metascape (http://metascape.org) was utilised[36]. Metascape currently does not support the domestic cat but it does support human. As gene annotation databases are primarily compiled for human genes it is useful to use human orthologs for model organisms prior to analysis[36]. As a model organism, the domestic cat shares several female germ cell features to humans: (1) the oocyte proper and the germinal vesicle diameter is equivalent; (2) oocytes reach metaphase II (MII) stage of meiosis after 24 h in culture; and (3) the nuclear configuration is comparable[44,45]. Thus, g:Profiler g:Orth orthology (https://biit.cs.ut.ee/gprofiler/orth)[33] converted the *Felis catus* Entrez IDs into *Homo sapiens* Ensembl IDs (an orthologous species recognisable by Metascape). The "Express Analysis" in Metascape was selected which automatically removed ontology terms that did not satisfy the minimal statistical criteria. The analysis report produced functional annotation network cluster graphs and ontology enrichment bar plots which

were summarised in an Excel workbook, PowerPoint presentation, and Zip folder format. Metascape performed an additional gene annotation, membership search, and enrichment clustering on the gene list. Together, the multi-test corrected *Q* values (DAVID) and *P* values (Metascape) were considered when ranking the enriched terms. The DESeq2 function "plotCounts" plot RNA-sequencing normalised count data of selected genes on a log scale for diagnostic purposes only. For comparison to qRT-PCR results, normalised counts were fit with a negative binomial general linearised model and coefficients and intervals were extracted.

Gene expression by qRT-PCR. Total RNA extraction for qRT-PCR was performed as described previously. Reverse transcription of RNA into cDNA was performed as described by Hryciuk et al.[46]. Intron-spanning primers were designed according to gene sequences listed in GenBank (Supplementary Table S1). The cDNA was diluted 1:10 and analysed with the CFX96 Real-Time PCR detection system utilising the SsoFast EvaGreen Supermix (Bio-Rad Laboratories GmbH, Munich), at 98 °C and 8 s at different annealing temperatures. Quantification of qRT-PCR products was performed with CFX Manager Software 3.1 (Bio-Rad Laboratories GmbH). Serial dilutions of plasmid DNA carrying genes of interest sequences or of qRT-PCR products were utilised for calibration with β-actin (*BACT*) as a reference gene[47]. Statistical analysis was performed in R using R 3.4.4[22]. The Kruskal–Wallis rank sum test determined *P* values of gene expression. The Wilcoxon rank sum test determined post-hoc pairwise comparisons (*P* value adjustment: Benjamini–Hochberg). Sigma-Plot 10.0 (Systat Software Inc., San Jose, CA, USA) allowed for the visualisation of qRT-PCR statistical results through box plots.

Ethics declarations. Domestic cat ovaries were obtained from animal shelters in Berlin after routine ovariectomy for the purpose of permanent contraception primarily from stray domestic cat individuals. The surgical procedures were not related to the purpose of the experiment. The animal shelter agreed to donate the excised ovaries which we utilised as samples. Castrations are compliant with the "Protection of Animals Act" in Germany; no further guidelines had to be considered.

Results

RNA-sequencing. Raw data, 35 Gb, was obtained after Illumina NextSeq sequencing. FastQC analysis estimated the total sequence distribution between samples (Supplementary Fig. S1a). Sequence length ranged from 59.4 to 76 base pairs (bp) with a guanine-cytosine content of 48.94% on average. Fragment lengths were distributed at approximately 300 bp. The percentage of mapped reads (% aligned) and mapped reads (millions) (M aligned) was estimated with MultiQC (Supplementary Fig. S1a). Trimming of reads was not performed. Log count distributions between samples before and after normalisation were visualised (Supplementary Fig. S1b). The PCA of the nine samples explained sample variance by the first two principal components (PC1 versus PC2) which accounted for 37% and 16% variation, respectively whereby, PC1 separated PrF from PF and SF, whereas PC2 separated PF from SF. This emphasised the sample-to-sample relationships based on ovarian follicle type (Supplementary Fig. S2a). The PC1 versus PC3 for all genes accounted for 37% and 12% variation, respectively (Supplementary Fig. S2b). The inclusion of PC3 explained a reduced proportion of variation between the PF and SF samples. Collectively, the first three PCs explained approximately 65% of total variation. The PC1 versus PC2 for 500 genes accounted for 39% and 19% variation, respectively (Supplementary Fig. S2c). A hierarchical heatmap demonstrated that samples clustered together based on ovarian follicle type (Supplementary Fig. S3). Clustering of all PrF samples was identified with a high degree of correlation. An outlier PF sample was observed (sample_4_S4) but the remaining two samples were closely associated. All SF samples were highly correlated. Overall, the degree of correlation demonstrated a relationship between PF and SF follicle samples. These samples were either less correlated or not correlated at all with the PrF samples (Supplementary Fig. S3). Additionally, sample-to-sample relationships were visualised with hierarchical cluster dendograms (Supplementary Fig. S4).

Differential gene expression analysis. The highest number of DEGs was identified during the PrF-PF transition. This included 2,226 DEGs comprised of 1,206 down-regulated and 1,020 up-regulated genes. The lowest number of DEGs was found in the PF-SF transition whereby, the total 154 DEGs estimate included 122 down-regulated and 32 up-regulated genes (Fig. 2a, c). This revealed that the number of DEGs decreased when comparing the PrF-PF and PF-SF transitions. The patterns of gene expression changes across all samples were graphically depicted in heatmap format (Fig. 2b) and summarised with a diagram of ovarian follicle DEGs (Fig. 2c). All differential gene expression lists were combined into one DEG database (Supplementary Data S1). Collectively, 2,380 DEG transcripts including 1,328 down-regulated and 1,052 up-regulated genes were detected in early preantral ovarian follicles in domestic cat.

Functional annotation clustering analysis. To focus the study, the following aims were established for analysis of the domestic cat data: (1) identify signalling pathways conserved in mammalian ovarian folliculogenesis; (2) select signalling pathways that are most functionally annotated/enriched; (3) extract DESeq2 DEG values for the genes associated with the respective pathways and plot transcript expression patterns for some representatives of it. The main conserved pathways during ovarian folliculogenesis in mammals include: adenylate cyclase; mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk); phosphoinositide-3-kinase/protein kinase B (PI3K-Akt); phospholipase C; janus kinase/signal transducers and activators of transcription (JAKS/STATS); SMAD (*Caenorhabditis elegans Sma* gene and *Drosophila Mad*, Mothers against decapentaplegic); and nuclear receptors[48]. Additionally, any over-represented BPs not explicitly defined as "signalling pathway" were taken into consideration.

From 2,226 DEGs within the PrF-PF transition 1,916 DAVID IDs (an internal gene ID defining a unique gene cluster from a single gene entry) were identified resulting in 137 functionally annotated clusters

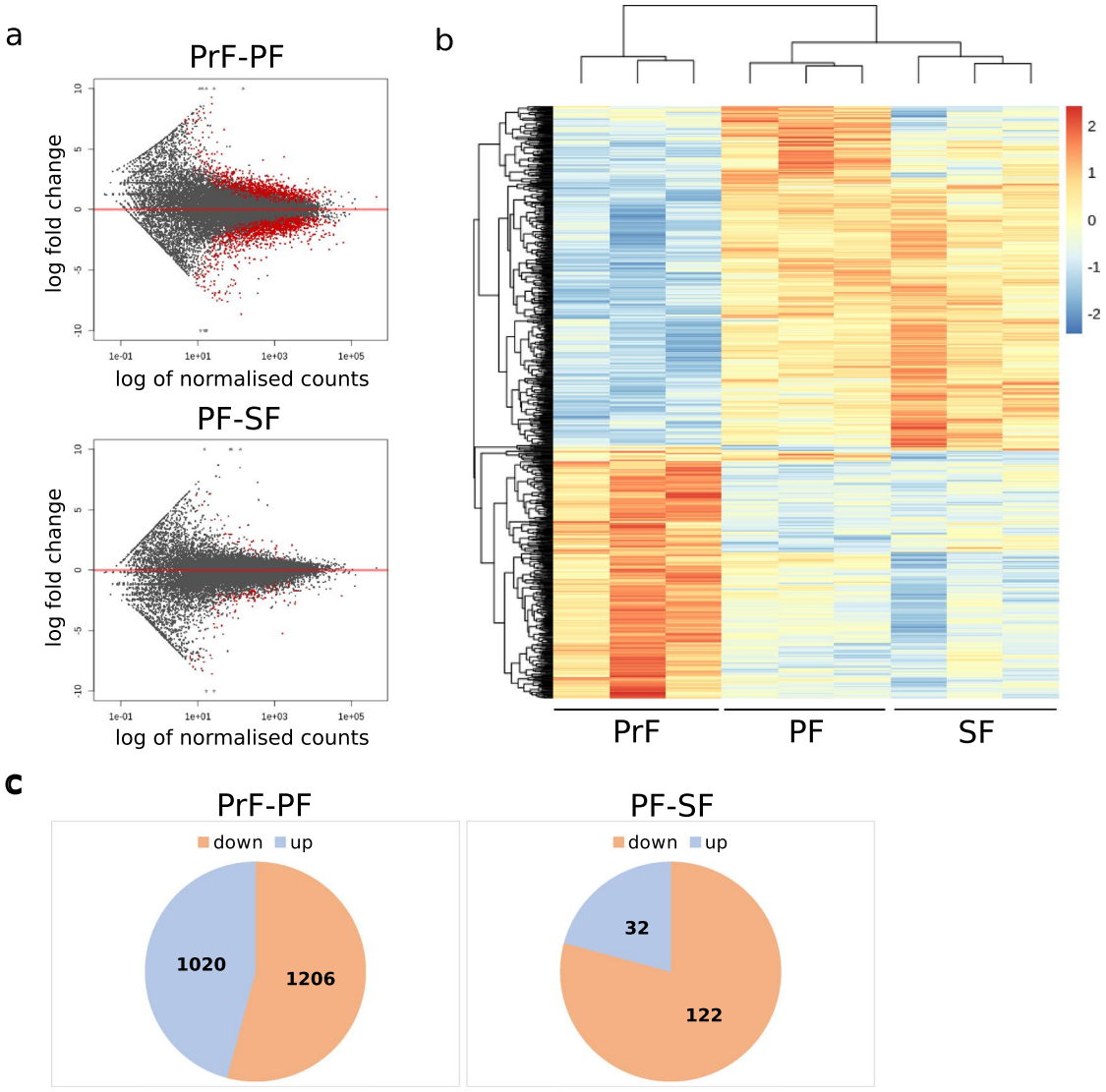


Figure 2. Differential gene expression during preantral ovarian folliculogenesis in domestic cat. **(a)** The estimated number of DEGs between ovarian follicle contrasts: primordial versus primary and primary versus secondary with an adjusted *P* value of < 0.05 and $\log^2$ fold-change ≥ 1 were visualised utilising MA plots whereby, non-significant genes (values not satisfying the *P* value and $\log^2$ fold-change threshold) are shown in black and significant genes (values satisfying the *P* value and $\log^2$ fold-change threshold) are shown in red, $\log^2$ fold-change is mapped to *y* and the normalised mean is mapped to *x* (transformed to $\log^{10}$ scale); **(b)** Patterns of gene expression changes are shown on a heatmap with an adjusted *P* value of < 0.01 and $\log^2$ fold-change ≥ 1. The colour red represents relative increase in expression, blue represents relative decrease, and pale orange (in the middle of the colour scale) represents no change. The columns are labelled with follicle type, respectively; **(c)** Pie charts summarise the estimated number of DEGs between the primordial versus primary and primary versus secondary contrasts with an adjusted *P* value of < 0.05 and $\log^2$ fold-change ≥ 1.

(Supplementary Data S2). Significantly enriched terms included "extracellular region part" ($Q = 2.10E{-}07$), "focal adhesion" ($Q = 5.30E{-}05$), "movement of cell or subcellular component" ($Q = 6.70E{-}04$), and "cell morphogenesis" ($Q = 3.80E{-}03$). The KO terms such as, "ECM receptor interaction" ($Q = 1.60E{-}06$) and "focal adhesion" ($Q = 1.80E{-}03$) were significantly enriched also. Other enriched terms and pathways were involved in "cellular response to growth factor stimulus" and the "phosphatidylinositol 3-kinase (PI3K)-Akt/Protein Kinase B (Akt)" and "transmembrane receptor protein serine/threonine kinase" signalling pathways (Table 1). Additionally, "platelet derived growth factor", "integrin-mediated", and "transforming growth factor (TGF)-β" signalling were observed during the PrF-PF transition (Supplementary Data S3). Interestingly, "axonemal dynein complex assembly" was identified in Annotation Cluster 32 and was coupled with "ciliary plasm" and "axoneme cellular components" (Supplementary Data S2).

Functional annotation clustering of the 154 PF-SF DEGs resulted in 128 DAVID IDs in 27 clusters (Supplementary Data S2). Identified terms included "reproductive process", "cellular response to chemical stimulus", "ovulation cycle process", and "epithelial cell proliferation" (Table 2). The KOs included terms involved in "focal adhesion" and "inositol phosphate metabolism" along with "sphingolipid", "phosphatidylinositol", "thyroid

Cluster	ES[1]	Description (DAIVD ID*)	Count[2]	FC[3]	BH[4]	FDR[5]
1	8.47	Extracellular region part	427	1.3	1.1E−7	2.1E−7
2	6.01	ECM-receptor interaction	31	3.4	3.3E−7	1.6E−6
		Focal adhesion	49	2.3	5.6E−6	5.3E−5
		PI3K-Akt signalling pathway	49	1.4	2.1E−1	2.2E1
3	5.23	Focal adhesion	64	1.9	9.9E−5	1.8E−3
4	5.01	Dilated cardiomyopathy	25	3.0	1.3E−4	1.8E−3
5	4.83	Endopeptidase inhibitor activity	31	2.5	3.9E−3	4.6E−3
6	4.29	Collagen metabolic process	14	3.5	2.8E−2	1.5E−1
7	4.01	Movement of cell or subcellular component	182	1.4	1.2E−3	6.7E−4
8	3.68	Glycosaminoglycan binding	27	2.1	4.9E−2	5.9E−1
9	3.07	Epithelium development	115	1.4	2.2E−2	1.0E−1
10	2.94	Cellular response to growth factor stimulus	62	1.5	8.7E−2	9.7E−1
		Transmembrane receptor protein serine/threonine kinase signalling pathway	38	1.5	4.4E−1	2.2E
11	2.91	Meiotic cell cycle	36	2.2	8.8E−3	2.4E−2
12	2.7	Meiosis I	20	2.5	6.1E−2	5.8E−1
13	2.62	Embryonic morphogenesis	75	1.5	4.3E−2	3.8E−1
14	2.6	Cellular response to amino acid stimulus	15	3.1	3.7E−2	2.8E−1
15	2.59	Cell morphogenesis	146	1.5	3.4E−3	3.8E−3
16	2.56	Negative regulation of cellular component movement	34	1.7	1.6E−1	3.1E0
17	2.56	ECM structural constituent	12	2.8	2.0E−1	3.6E0
18	2.48	Vasculature development	70	1.5	1.2E−1	1.7E0
19	2.38	Cilium organization	36	1.7	1.6E−1	2.7E0
20	2.38	Response to oxygen-containing compound	106	1.4	1.2E−1	1.6E0

Table 1. Functional annotation clusters identified during the PrF-PF transition in domestic cat using DAVID. The top 20 categories grouped by similar GO and KO terms are listed. The full list containing 137 identified clusters is found in Supplementary Data 2, "DAVID PrF-PF". [1]enrichment score, geometric mean of member's *P* values of the corresponding annotation cluster in -log10 scale of the annotation cluster; [2]number of gene counts, [3] Fold-change, [4]Benjamini-Hochberg value, and [5]false discovery rate (*P* value adjusted). *internal gene ID defines a unique gene cluster belonging to a gene entry.

hormone", "calcium", "vascular endothelial growth factor (VEGF)", "ErbB" and "hypoxia-inducible factor 1 (HIF-1)" signalling pathways (Table 2). Additionally, "β-catenin", "apelin", "extra-nuclear estrogen", "IL8 CXCR1", "RAS", and "TXA2" signalling were identified. Lastly, "E2F", "forkhead box O (FOXO)", "Hedgehog", "IL6 JAK STAT3", "insulin-like growth factor (IGF)", "KIT", "Notch", and "Wnt" were identified though not significantly enriched (Supplementary Data S3).

As described in the methods section, Metascape generated functional annotation network clusters and bar plots of enriched ontology terms for visualisation. The results from Metascape were extracted for PrF-PF (Fig. 3 and Supplementary Fig. S5) and PF-SF (Fig. 4 and Supplementary Fig. S6), respectively. Based on Metascape analysis, the functional annotation cluster and enrichment PrF-PF and PF-SF results were summarised in Excel format, respectively (Supplementary Data S3 and S4, respectively). The following pathways, processes, and regulators were selected for further analysis: PI3K-Akt; TGF-β; erythroblastoma (ErbB); HIF-1 signalling pathways; over-represented biological processes associated with the extracellular matrix (ECM); and potential structural regulators of ovarian folliculogenesis such as, the dynein complex.

Selected RNA-sequencing transcript expression. Supplementary Data S3 and S4 identified which genes were associated with the aforementioned selection, respectively. Several genes were selected (investigating both the significant and non-significant differential expression gene lists), transcript expression levels were plotted, and DESeq2 results were extracted for each (Fig. 5 and Table 3). Some genes were selected due to functional implications related to ovarian folliculogenesis in other species. Whereas, others such as, bone morphogenetic proteins (BMPs) and MMPs were selected due to the piling evidence describing the essential role of these factors during mammalian ovarian folliculogenesis. For each numbered section below the following genes are listed in order of decreasing "baseMean" values: (1) PI3K-Akt signalling pathway: TSC complex subunit 2 (*TSC2*); pyruvate dehydrogenase kinase 4 (*PDK4*); AKT serine/threonine kinase 2 (*AKT2*); epidermal growth factor (*EGF*); regulatory associated protein of mTOR complex 1 (*RPTOR*); and PI3K subunit delta (*PIK3CD*) (Fig. 5a). Transcript expression increased for each of these genes except *EGF* during the PrF-PF transition (Table 3, Fig. 5a). In contrast, the non-significantly differentially expressed gene *EGF* decreased sharply during the PrF-PF transition only to increase sharply during the PF-SF transition (Fig. 5a). (2) TGF-β signalling pathway: growth differentiation factor 9 (*GDF-9*); BMP and activin membrane bound inhibitor (*BAMBI*); *BMP15*; twisted gastrulation BMP signalling modulator 1 (*TWSG1*); SMAD specific E3 ubiquitin protein ligase 2 (*SMURF2*); *BMP4*; bone morphogenetic protein receptor type 1B (*BMPR1B*); and BMP binding endothelial regulator (*BMPER*) (Table 3,

Cluster	ES[1]	Description (DAIVD ID*)	Count[2]	FC[3]	BH[4]	FDR[5]
1	1.76	Reproductive process	15	2.5	5.8E−1	3.1E0
2	1.73	Phospholipase activity	4	8.0	8.7E−1	1.6E1
3	1.7	Cellular response to chemical stimulus	20	1.7	9.3E−1	2.3E1
4	1.52	Ion binding	31	1.5	7.8E−1	1.8E1
5	1.38	Sphingolipid signalling pathway	5	5.2	9.3E−1	1.7E1
		Fc gamma R-mediated phagocytosis	4	6.1	8.0E−1	2.8E1
		Choline metabolism in cancer	3	3.7	6.6E−1	9.2E1
6	1.31	Fc gamma R-mediated phagocytosis	4	6.1	8.0E−1	2.8E1
		Leukocyte transendothelial migration	4	4.4	5.3E−1	5.3E1
		Focal adhesion	5	3.0	5.5E−1	6.3E1
7	1.27	Cell adhesion	17	2.5	8.6E−1	1.8E0
8	1.25	Sphingolipid signalling pathway	5	5.2	9.3E−1	1.7E1
		Inflammatory mediator regulation of TRP channels	4	5.5	5.9E−1	3.4E1
		Phosphatidylinositol signalling system	4	5.1	6.1E−1	4.0E1
		Cholinergic synapse	4	4.7	5.8E−1	4.8E1
		Thyroid hormone signalling pathway	4	4.5	5.4E−1	5.2E1
		Oxytocin signalling pathway	4	3.5	5.7E−1	7.3E1
		Inositol phosphate metabolism	3	5.3	5.8E−1	7.5E1
		Calcium signalling pathway	4	2.9	6.3E−1	8.8E1
9	1.25	Cytosolic transport	4	5.4	9.2E−1	4.8E1
10	1.24	Extracellular region	34	1.4	9.4E−1	2.9E1
11	1.23	Sphingolipid signalling pathway	5	5.2	9.3E−1	1.7E1
		VEGF signalling pathway	3	6.3	5.4E−1	6.3E1
		HIF-1 signalling pathway	3	3.9	6.5E−1	9.1E1
12	1.17	Ovulation cycle process	3	8.7	9.3E−1	5.5E1
13	1.15	Serine hydrolase activity	5	4.3	7.6E−1	3.1E1
14	1.14	Regulation of angiogenesis	5	4.3	9.3E−1	4.0E1
15	1.14	Epithelial cell proliferation	6	3.3	9.3E−1	4.6E1
16	1.12	ErbB signalling pathway	4	5.8	6.7E−1	3.1E1
		Focal adhesion	5	3.0	5.5E−1	6.3E1
		Proteoglycans in cancer	4	2.6	6.6E−1	9.3E1
17	1.09	Serotonergic synapse	4	4.9	5.7E−1	4.4E1
		Cholinergic synapse	4	4.7	5.8E−1	4.8E1
		Glutamatergic synapse	4	4.6	5.3E−1	4.9E1
		Circadian entrainment	3	4.0	6.4E−1	8.9E1
		Retrograde endocannabinoid signalling	3	3.9	6.5E−1	9.1E1
18	1.09	Somatodendritic compartment	7	3.2	9.9E−1	2.4E1
19	1.07	Phosphorus metabolic process	22	1.6	9.3E−1	4.3E1
20	1.06	Regulation of angiogenesis	5	4.3	9.3E−1	4.0E1

Table 2. Functional annotation clusters identified during the PF-SF transition in domestic cat using DAVID. The top 20 categories grouped by similar GO and KO terms are listed. The full list containing 27 identified clusters is found in Supplementary Data 2, "DAVID PF-SF". *Internal gene ID defines unique gene cluster belonging to a gene entry. [1]Enrichment score, geometric mean of member's *P* values of the corresponding annotation cluster in − log10 scale of the annotation cluster. [2]Number of gene counts. [3]Fold-change. [4]Benjamini-Hochberg value. [5]False discovery rate (*P* value adjusted).

Fig. 5b). Overall, the genes associated with the TGF-β signalling pathway demonstrated the most dynamic transcript expression. The highest "baseMean" value was estimated for *GDF-9* however, this gene was not significantly differentially expressed (Table 3). Transcript expression of *GDF-9* was in a comparable range throughout each ovarian follicle stage (Fig. 5b). *BAMBI, BMP15, TWSG1*, *BMP4* and *BMPR1B* showed a significant increase from PrF to PF (Fig. 5b). There was an oppositional pattern observed for *SMURF2* and *BMPER* (Fig. 5b). Transcript counts of *BMP4* and *BMP15* increases towards SF too but this was not significant (Fig. 5b). (3) ErbB signalling pathway: Erb-B2 receptor tyrosine kinase 2 (*ERBB2*); SH3 domain containing kinase binding protein 1 (*SH3KBP1*); and ErbB factor neuregulin 2 (*NRG2*) (Fig. 5c). A sequential decrease all the way until the SF stage was observed for *NRG2*. In contrast, expression of *ERBB2* and *SH3KBP1* transcripts increases from PrF to PF stage (Fig. 5c, Table 3). (4) HIF-1 signalling pathway: phosphofructokinase, liver type (*PFKL*); hypoxia inducible factor 1 subunit alpha inhibitor (*HIF1AN*); egl-9 family hypoxia inducible factor 2 (*EGLN2*); basic

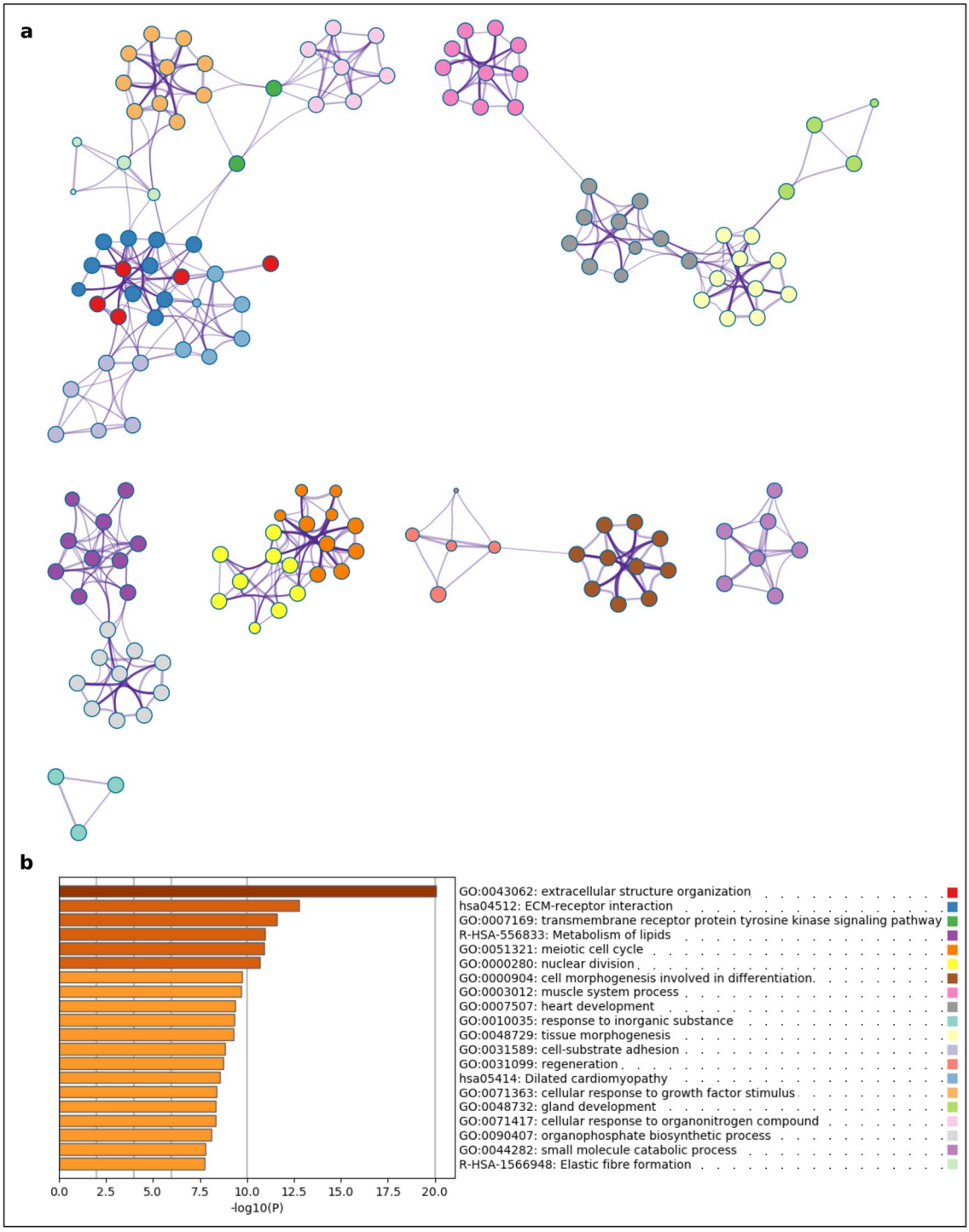


Figure 3. Functional enrichment analyses of differentially expressed genes (DEGs) during the primordial-to-primary ovarian follicle transition in domestic cat. **(a)** The enrichment ontology cluster graph represents each term as a circle, the size of the circle is proportional to the number of DEGs associated with that term, each cluster is coloured uniquely meaning that circles of the same colour are associated with the same cluster. The edges connect terms that have a similarity score of > 0.3 which influences the density of the edge line; **(b)** bar chart of GO and KO terms coloured by *P* values. Metascape (http://metascape.org) was utilised for visualisation.

helix-loop-helix family member e40 (*BHLHE40*); and furin, paired basic amino acid cleaving enzyme (*FURIN*) (Fig. 5d). Transcript counts for three of them (*PFKL*, *HIFNAN*, *EGLN2*) increased significantly during PrF-PF transition whereas the other two increased strongly in the PF-SF transition (Fig. 5d, Table 3). (5) Variable transcript expression patterns were found in the MMP group: *MMP12*, *MMP21* and *MMP7* expression decreased

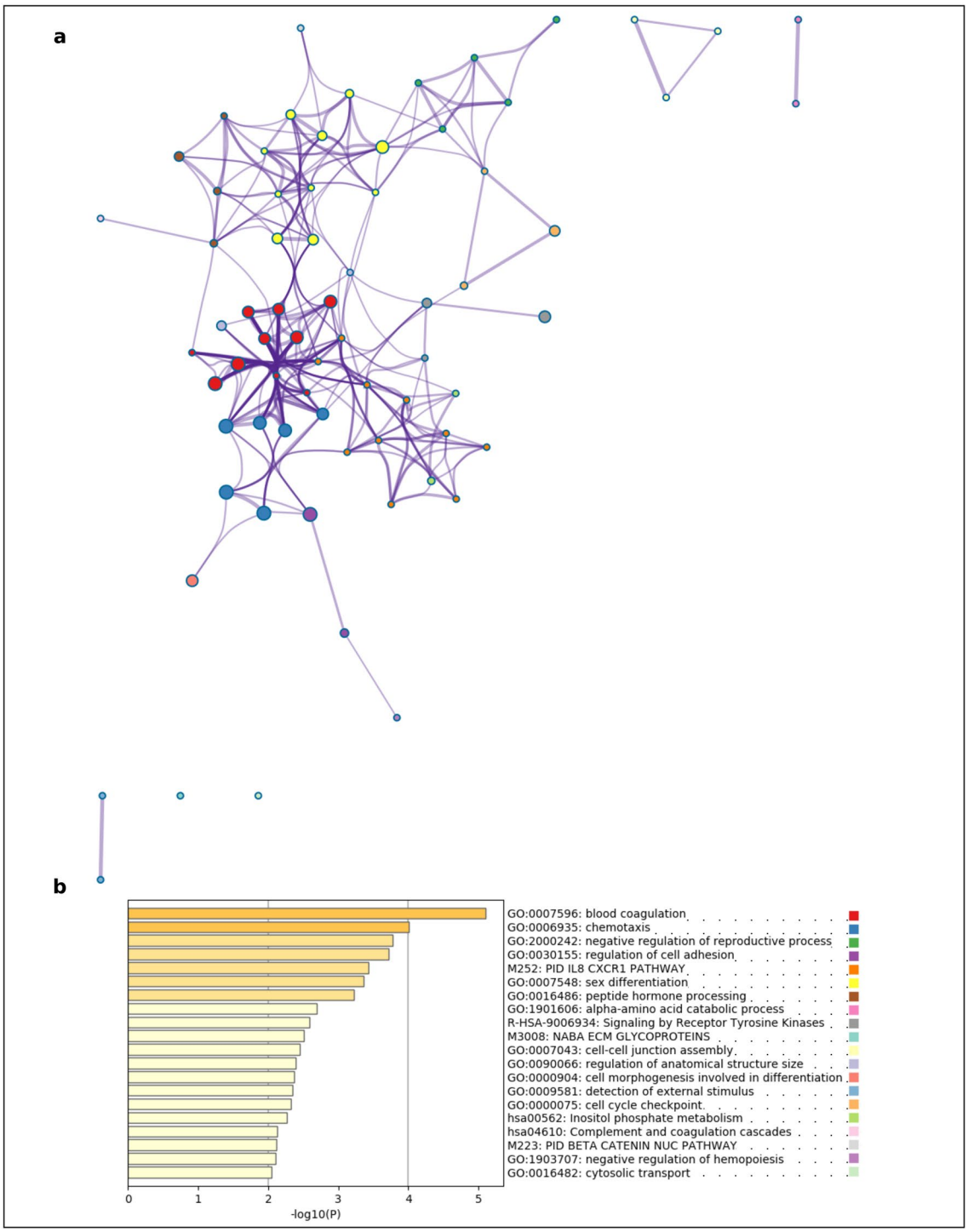
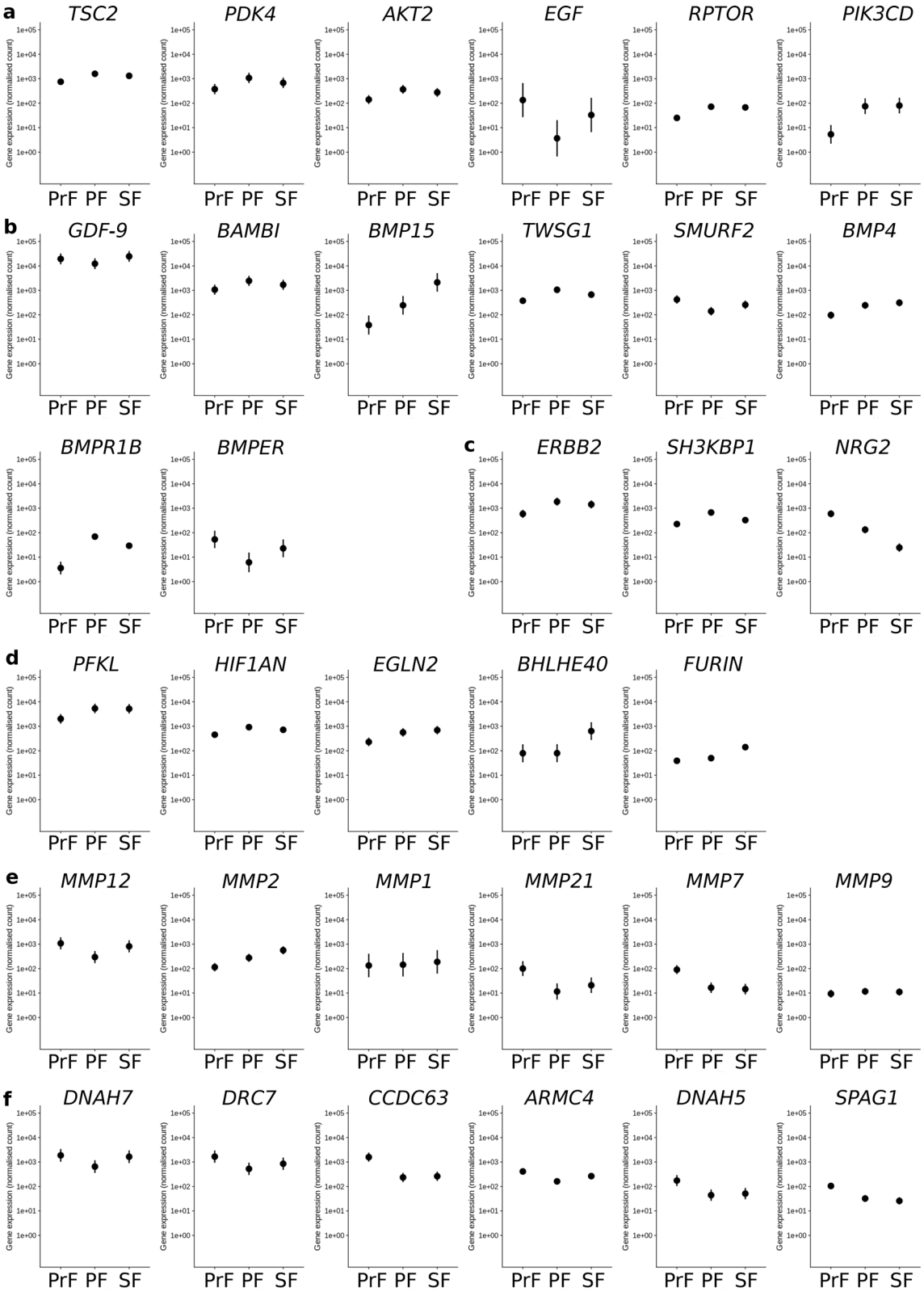


Figure 4. Functional enrichment analyses of differentially expressed genes (DEGs) during the primary-to-secondary ovarian follicle transition in domestic cat. **(a)** The enrichment ontology cluster graph represents each term as a circle, the size of the circle is proportional to the number of DEGs associated with that term, each cluster is coloured uniquely meaning that circles of the same colour are associated with the same cluster. The edges connect terms that have a similarity score of > 0.3 which influences the density of the edge line; **(b)** bar chart of GO and KO terms coloured by *P* values. Metascape (http://metascape.org) was utilised for visualisation.

in PrF-PF then increased in PF-SF; *MMP2* demonstrated an increase in expression in this transition; *MMP1* had a comparable transcript expression throughout each stage and was not significantly differentially expressed; and *MMP9* showed a low but unchanged expression level too (Fig. 5e). (6) The dynein genes included: dynein axonemal heavy chain 7 (*DNAH7*); dynein regulatory complex subunit 7 (*DRC7*); coiled-coil domain containing


a
TSC2
PDK4
AKT2
EGF
RPTOR
PIK3CD
b
GDF-9
BAMBI
BMP15
TWSG1
SMURF2
BMP4
BMPR1B
BMPER
c
ERBB2
SH3KBP1
NRG2
d
PFKL
HIF1AN
EGLN2
BHLHE40
FURIN
e
MMP12
MMP2
MMP1
MMP21
MMP7
MMP9
f
DNAH7
DRC7
CCDC63
ARMC4
DNAH5
SPAG1
Gene expression (normalised count)
PrF PF SF

◂**Figure 5.** Transcript expression plots of normalised counts for genes of interest from preantral ovarian follicles in domestic cat (RNA-sequencing data). **(a)** Genes associated with the PI3K-Akt pathway from left-to-right: TSC complex subunit 2 (*TSC2*); pyruvate dehydrogenase kinase 4 (*PDK4*); AKT serine/threonine kinase 2 (*AKT2*); epidermal growth factor (*EGF*); regulatory associated protein of mTOR complex 1 (*RPTOR*); and PI3K subunit delta (*PIK3CD*); **(b)** TGF-β pathway from left-to-right: growth differentiation factor 9 (*GDF-9*); BMP and activin membrane bound inhibitor (*BAMBI*); bone morphogenetic protein 15 (*BMP15*); twisted gastrulation BMP signalling modulator 1 (*TWSG1*); SMAD specific E3 ubiquitin protein ligase 2 (*SMURF2*); *BMP4*; bone morphogenetic protein receptor type 1B (*BMPR1B*); and BMP binding endothelial regulator (*BMPER*); **(c)** erythroblastoma (ErbB) pathway: Erb-B2 receptor tyrosine kinase 2 (*ERBB2*); SH3 domain containing kinase binding protein 1 (*SH3KBP1*); and ErbB factor neuregulin 2 (*NRG2*); **(d)** hypoxia-inducible factor 1 (HIF-1) pathway: phosphofructokinase, liver type (*PFKL*); hypoxia inducible factor 1 subunit alpha inhibitor (*HIF1AN*); egl-9 family hypoxia inducible factor 2 (*EGLN2*); basic helix-loop-helix family member e40 (*BHLHE40*); and furin, paired basic amino acid cleaving enzyme (*FURIN*); **(e)** matrix metalloproteinase (MMP): *MMP12*; *MMP2*; *MMP1*; *MMP21*; *MMP7*; and *MMP9*; **(f)** axonemal dynein complex: dynein axonemal heavy chain 7 (*DNAH7*); dynein regulatory complex subunit 7 (*DRC7*); coiled-coil domain containing 63 (*CCDC63*); armadillo repeat containing 4 (*ARMC4*); dynein axonemal heavy chain 5 (*DNAH5*); and sperm associated antigen 1 (*SPAG1*) in PrF, PF, and SFs. Gene expression of negative binomial general linearised model normalised data.

63 (*CCDC63*); armadillo repeat containing 4 (*ARMC4*); dynein axonemal heavy chain 5 (*DNAH5*); and sperm associated antigen 1 (*SPAG1*) (Fig. 5f). All counts were highest in PrFs in comparison to PFs. Values between PF and SF were comparable (Table 3).

qRT-PCR comparative analysis. The genes, *BMP15* and histone H1t (*HIST1H1T)*, were compared to the RNA-sequencing data by evaluating relative gene expression trends with qRT-PCR data. Diagnostic plots with RNA-sequencing data of selected genes demonstrated an increase in *BMP15* gene expression as ovarian follicles developed from PrFs toward SFs (Supplementary Fig. S7a). A decrease in *HISTH1T* gene expression was observed as ovarian follicles progressed from the PrF stage onward (Supplementary Fig. S7a). The negative binomial general linearised model of normalised RNA-sequencing data showed a similar increase in *BMP15* gene expression as the ovarian follicles developed (Supplementary Fig. S7b, above). Applying the same approach to *HISTH1T*, gene expression was detected in PrF only (Supplementary Fig. S7b, below). Analysis of *BMP15* by qRT-PCR revealed that gene expression was nearly undetectable in PrFs and PFs however, *BMP15* gene expression was observed in SFs (Supplementary Fig. S7b, above right). The Kruskal–Wallis test detected significant changes of gene expression throughout ovarian follicular development (P value = 0.04627), but the subsequent post-hoc test reported only a tendency for higher expression in SF compared to PrF and PF. In contrast, a high relative gene expression of *HIST1H1T* was found in PrFs which decreased considerably as ovarian follicles progressed toward PF and SF stages, the differences of PrF and PF as well as PrF and SF were confirmed as statistically significant (Supplementary Fig. S7b, below right).

Discussion

As far as we are aware, this is the first whole ovarian follicle transcriptomic analysis during primordial ovarian follicle (PrF) development and early follicular growth during the primary (PF) to secondary (SF) ovarian follicle transition in a non-rodent animal. In contrast to most gene expression studies performed on adult ovaries, in this study the ovarian follicle is considered as a functional unit. There was no differentiation made between oocytes and the surrounding ovarian follicular cells. This is primarily due to the fact that after isolation from the ovarian cortex, dissection of oocytes from early ovarian follicles is impossible without any severe damage. However, laser dissection microscopy (LCM) is an alternative method which may circumvent such problems and has thus far, been executed in human[20,49] and sheep[16,17]. Nevertheless, the use of whole ovarian follicles as a source for transcriptome analysis is essential to monitor follicular growth in vitro.

The initial RNA-sequencing analysis revealed a higher proportion of differentially expressed genes (DEGs) during the activation of dormant ovarian follicles (PrF-PF: 2,226 DEGs) in comparison to the growth phase (PF-SF: 156 DEGs) (Fig. 2). Analysing transcripts of early stages of human oocytes, 223 and 268 genes were detected as significantly expressed in oocytes from PrFs and PFs, respectively[50]. The differences in differential gene expression levels may be due to the use of whole ovarian follicles in comparison to the latter oocyte-specific approach. Another group analysed oocytes and granulosa cells (GCs) of ovine ovarian follicles separately[17]. In this study, less than 200 DEGs in oocytes and GCs during the PrF-PF transition were detected. In contrast to this data, a higher number of gene expression changes during the PF-SF transition were observed—around 500 DEGs in oocytes and 400 DEGs in GCs[17]. So far it is not clear if the discrepancies in the number of DEGs are species-specific or due to the particular analytical approach such as, intact ovarian follicles versus laser dissected ovarian follicles from tissue slices. Additionally, we must consider that in this study the ratio of expressed genes of oocyte origin is decreasing with ovarian follicular growth. An intact PrF contains < 20 follicular cells per oocyte, whereas, this ratio is shifted to 1:50 for PF and 1:200 SFs, respectively[51]. To account for the number of ovarian follicular cells the number of follicles per pool were adapted (PrF n = 180, PF n = 45, and SF n = 9). Therefore, differential gene expression in PF-SF is mirroring more GC expression than oocyte expression, maybe leading to a lower number of detectable DEGs than in PrF-PF. In the future, it will be worthwhile to consider single cell transcriptomics to investigate gene expression levels individually.

Gene name *symbol*	baseMean	PrF-PF		PF-SF	
		FC	padj	FC	padj
PI3K-Akt pathway					
TSC complex subunit 2 *TSC2**	1221.48	− 1.06	0.02	0.28	0.93
Pyruvate dehydrogenase kinase 4 *PDK4**	711.73	− 1.53	0.02	0.66	0.77
AKT serine/threonine kinase 2 *AKT2**	262.00	− 1.34	0.04	0.41	0.87
Epidermal growth factor *EGF*	56.92	5.21	0.12	− 3.18	0.75
Regulatory associated protein of mTOR complex 1 *RPTOR**	54.76	− 1.4	0.03	0.08	1.00
PI3K subunit delta *PIK3CD**	53.64	− 3.31	0.00	− 0.10	0.99
TGF-β pathway					
Growth differentiation factor 9 *GDF-9*	18,699.46	0.65	0.33	− 1.00	0.48
BMP and activin membrane bound inhibitor *BAMBI**	1734.87	− 1.22	0.05	0.53	0.81
Bone morphogenetic protein 15 *BMP15**	801.53	− 2.83	0.05	− 3.11	0.15
Twisted gastrulation BMP signalling modulator 1 *TWSG1**	701.81	− 1.47	0.00	0.66	0.58
SMAD specific E3 ubiquitin protein ligase 2 *SMURF2**	273.55	1.6	0.01	− 0.88	0.50
Bone morphogenetic protein 4 *BMP4**	218.04	− 1.2	0.04	− 0.35	0.88
Bone morphogenetic protein receptor type 1B *BMPR1B**	33.97	− 3.83	0.00	1.23	0.72
BMP binding endothelial regulator *BMPER**	27.34	3.03	0.03	− 1.91	0.55
ErbB pathway					
Erb-B2 receptor tyrosine kinase 2 *ERBB2**	1288.61	− 1.6	0.00	0.38	0.87
SH3 domain containing kinase binding protein 1 *SH3KBP1**	406.38	− 1.54	0.00	1.03	0.15
ErbB factor neuregulin 2 *NRG2**	250.12	2.12	0.00	2.36	0.00
HIF-1 pathway					
Phosphofructokinase, liver type *PFKL**	4193.36	− 1.4	0.01	0.05	1.00
Hypoxia inducible factor 1 subunit alpha inhibitor *HIF1AN**	698.78	− 1.04	0.00	0.35	0.76
Egl-9 family hypoxia inducible factor 2 *EGLN2**	496.63	− 1.22	0.04	− 0.30	0.93
Basic helix-loop-helix family member e40 *BHLHE40**	265.23	0.01	0.90	− 2.99	0.03
Furin, paired basic amino acid cleaving enzyme *FURIN**	76.47	− 0.3	0.83	− 1.49	0.04
Extracellular matrix					
Matrix metalloproteinase 12 *MMP12**	729.45	1.88	0.01	− 1.46	0.23
Matrix metalloproteinase 2 *MMP2**	316.92	− 1.28	0.04	− 1.02	0.35
Matrix metalloproteinase 1 *MMP1*	155.42	− 0.07	1	− 0.38	0.97
Matrix metalloproteinase 21 *MMP21**	44.25	3.06	0.01	− 0.85	0.90
Matrix metalloproteinase 7 *MMP7**	40.47	2.51	0.00	0.17	1.00
Matrix metalloproteinase 9 *MMP9*	10.73	− 0.11	1	0.05	1
Axonemal dynein complex					
Dynein axonemal heavy chain 7 *DNAH7**	1381.74	1.51	0.04	− 1.34	0.33
Dynein regulatory complex subunit 7 *DRC7**	1006.22	1.67	0.02	− 0.70	0.78
Coiled-coil domain containing 63 *CCDC63**	689.57	2.74	0.00	− 0.15	0.98
Armadillo repeat containing 4 *ARMC4**	277.97	1.35	0.00	− 0.71	0.48
Dynein axonemal heavy chain 5 *DNAH5**	89.48	2.01	0.02	− 0.24	0.99
Sperm associated antigen 1 *SPAG1**	54.02	1.66	0.02	0.37	0.94

Table 3. Transcript expression DESeq2 data from preantral ovarian follicles in domestic cat (RNA-sequencing data). The transcript expression data was extracted from DESeq2 analysis and were summarised in order of decreasing "baseMean" values. "PI3K-Akt pathway" genes: *TSC2*; *PDK4*; *AKT2*; *EGF*; *RPTOR*; and *PIK3CD*. "TGF-β pathway" genes: *GDF-9*; *BAMBI*; *BMP15*; *TWSG1*; *SMURF2*; *BMP4*; *BMPR1B*; *BMPER*. "ErbB pathway" genes: *ERBB2*, *SH3KBP1*, and *NRG2*. "HIF-1 pathway" genes: *PFKL*; *HIF1AN*; *EGLN2*; *BHLHE40*; *FURIN*. "Extracellular matrix" genes: *MMP12*; *MMP2*; *MMP1*; *MMP21*; *MMP7*; and *MMP9*. "Axonemal dynein complex" genes: *DNAH7*; *DRC7*; *CCDC63*; *ARMC4*; *DNAH5*; and *SPAG1*. "baseMean" is the average of the normalised count values, dividing by size factors, taken over all samples; "FC" fold-change is log2FoldChange, the effect size estimate. This value indicates how much the gene or transcript's expression seems to have changed between the contrasts reported on a logarithmic scale to base 2; "padj" is the adjusted *P* value for multiple testing for the gene or transcript; PrF-PF and PF-SF denote primordial versus primary and primary versus secondary DESeq2 contrasts; and * indicates differential gene expression significance with an adjusted *P* value of < 0.05 and log2 fold-change ≥ 1.

After differential gene expression analysis the gene lists were functionally annotated resulting in the identification of over-represented GO and KO terms. As described previously, conserved signalling pathways and biological processes (BPs) that were over-represented were considered. This included the two signalling pathways predominantly studied during ovarian folliculogenesis in other mammals: the PI3K-Akt and TGF-β pathways. Additionally, signalling pathways more ambiguously described though implicated in ovarian folliculogenesis such as, ErbB and HIF-1 pathways were given attention too. The PI3K-Akt signalling pathway within the oocyte is a key regulator of PrF activation and has been described during ovarian folliculogenesis in bovine, human, ovine, and porcine[52]. Currently, it is one of the major non-gonadotrophic growth factor pathways regulating ovarian follicles. As expected, the PI3K-Akt signalling pathway was identified during the PrF-PF transition in domestic cat (Table 2). The TGF-β signalling pathway was identified in the PrF-PF data under the parent GO term "transmembrane receptor protein serine/threonine kinase signalling pathway" (Table 2). Similarly to the PI3K-Akt pathway, the TGF-β signalling pathway has been studied during ovarian folliculogenesis in bovine, human, ovine, porcine, rodents, and rhesus monkeys[53]. For the domestic cat, the development of ovarian follicles in vitro with the supplementation of PI3K-Akt and TGF-β-associated factors has been investigated[9,54]. The studies focused on the effect of epidermal growth factor (EGF), its receptor (EGFR), and the growth differentiation 9 factor (GDF-9) supplementation either in combination or alone. Signalling via EGF and EGFR up-regulates the PI3K pathway[55]. In goat, EGF stimulates in vitro growth during the PrF-PF transition[56] and in rat, it is implicated in the growth of PrFs toward the SF stage[57]. The TGF-β factor, GDF-9, has been shown to have a similar influence on preantral ovarian follicles in several mammals. In vitro studies have implicated GDF-9 supplementation in PrF activation and ovarian follicle viability in human[58], goat[59,60], bovine[60], and hamster[61]. Additionally, GDF-9 has an over-arching function within the PI3K pathway in rat preantral ovarian follicles[62]. Interestingly, for the domestic cat, the culture of ovarian cortical slices in medium supplemented with EGF and/or GDF-9 have shown that EGF but not GDF-9 improved follicle viability[54]. Medium containing GDF-9 not only had no beneficial influence on ovarian tissues but negligibly impacted ovarian follicle viability[54]. Interestingly, although the transcriptomic data for *GDF-9* was determined with a high "baseMean" value it was not significantly differentially expressed (Table 3). Therefore, for the domestic cat it is likely to be more essential in later stages instead. This is the case in the rat where GDF-9 supplementation in vitro promotes ovarian follicular growth only after the PF stage[63,64]. Other functionally annotated factors have also been studied in other models during ovarian folliculogenesis. This includes BMP4 which is also described in rats to function as an ovarian follicle survival factor promoting PrF development[65] and in humans, where BMP4 is implicated in regulating ovulation via remodelling of the ovarian extracellular matrix (ECM) by facilitating in cumulus-oocyte-complex and mural GC separation[66]. A comparative analysis was performed on *BMP15* with the qRT-PCR method. The RNA-sequencing transcript counts of *BMP15* were shown to increase from the PF stage onward (Supplementary Fig. S7). Similarly, BMP15 mRNA increased in expression with ongoing follicular development (Supplementary Fig. S7). This supports previous studies in other species such as, human and rat where increased BMP15 expression promotes GC proliferation and theca layer development[67].

As mentioned, the ErbB and HIF-1 signalling pathways were included as mechanisms of interest. Currently, there are no studies into the role of these pathways during ovarian folliculogenesis in the domestic cat. However, there is evidence in other species of its involvement in ovarian follicle development. Briefly, the ErbB has recently been found to be down-regulated in human oocytes during the PrF-PF transition[20]. Previously, the ErbB factor neuregulin 1 (NRG1) has been investigated in mural GCs and theca cells from rodent periovulatory follicles[68–70]. In the domestic cat, *NRG2* transcript expression was observed along with other ErbB-associated factors such as, *ERBB2* and *SH3KBP1* (Table 3). In contrast to the rodent model, not only was a different variant found in the domestic cat but a sequential decrease of *NRG2* expression was observed all the way until the SF stage (Fig. 5c). Investigations into the HIF-1 pathway may also provide an alternative insight into preantral ovarian folliculogenesis in the domestic cat. Some factors identified had either similar or higher "baseMean" values than those identified for the TGF-β pathway (Table 3). For example, *PFKL* had the highest "baseMean" overall the factors (excluding *GDF-9*) (Table 3). Interestingly, each HIF-1 signalling factor demonstrated a tendency to increase in transcript expression during ovarian follicle development (Fig. 5d). In other species, increased HIF-1α expression in GCs is implicated in regulating follicular growth and development in rats[71]. Additionally, HIF-1 signalling in bovine primary GCs is suggested to be involved in steroidogenesis and cell proliferation during follicular development[72]. Although not extensively described here it will be interesting to study these pathways in relation to domestic cat ovarian folliculgenesis in the future.

Noteworthy, "ECM-receptor interaction" and "focal adhesion" were identified within the same functional annotation cluster as "PI3K-Akt signalling pathway" (Table 1). During the development of ovarian follicles the ECM undergoes significant compositional remodelling. Upon the initial activation of PrFs, GCs become cuboidal, forming a PF, and the ovarian follicle moves toward the medullar region and away from the cortical region of the ovary[73]. Previously, in vivo imaging windows in mouse ovaries have revealed that collagen fibers can function as migration tracks for infiltrating tumor cells[74]. Additionally, 3D imaging analyses have revealed that the inward movement of ovarian follicles is likely due to the stiffer cortical enclosure as compared with the softer inner medulla[75]. Furthermore, this analysis demonstrated that ovarian follicles are in close contact to each other[75]. Thus, the migration of activated PrFs and developing preantral ovarian follicles may occur through a collective collaboration based on collagen fiber tracking, morphological reactions, and interactions between neighbouring ovarian follicles. Regarding the ECM, degradation occurs via matrix MMPs. This allows for continued enlargement of the developing ovarian follicle. In the ovary, MMP2 and MMP9 expression has been observed in rat, sheep, mouse, rhesus macaque, horse, cow, and human; MMP1 and MMP13 in rabbit, rat, horse, and rhesus macaque; and MMP7 mRNA in macaque pre-ovulatory GCs[76]. In the domestic cat, MMP1, MMP2, MMP3, MMP7, MMP9, and MMP13 mRNA are detectable at every developmental stage, and the abundance and expression of these enzymes was consistently dynamic[76]. The domestic cat transcriptomic data also

demonstrated highly dynamic expression patterns throughout each MMP (*MMP2*, *MMP7*, *MMP12*, and *MMP21*) with *MMP2* showing a clear tendency to increase whereas, *MMP7*, *MMP12*, and *MMP21* decrease during ovarian folliculogenesis (Fig. 5e). Additionally, two MMP transcripts, *MMP12* and *MMP21*, not previously described for the domestic cat were identified. A higher level of transcript expression was observed for *MMP12* than *MMP21* (Fig. 5e). Additionally, the highest "baseMean" value overall was observed for *MMP12* (Table 3) therefore, it may be interesting to perform downstream mRNA analysis on this factor in the domestic cat. Subsequently, the changes that occur within the ECM subsequently affect nuclear dynamics through changes in lamina composition, membrane tension, and nuclear pore size which regulates chromatin configuration and gene expression[77]. Currently, these changes are not well understood for all species in respect to ovarian folliculogenesis. Screening for factors involved in oocyte dormancy with RNA-sequencing revealed that microenvironmental compression elicited a nuclear rotation response which was mediated by a motor protein called dynein in mice[73]. The inhibition of dynein significantly increased the number of growing follicles demonstrating its essential role in follicle dormancy in cultured murine ovaries[73]. Differentially expressed genes of the axonemal dynein complex were identified in the PrF-PF transition only (Supplementary Data S2). The heavy chain regions of the dynein complex contain the motor domain which is capable of producing movement in vitro[78]. In the transcriptomic data, the *DNAH5* and *DNAH7* heavy chains transcripts were identified along with the regulatory *DRC7* and structural factors *CCDC63* and *ARMC4* (Fig. 5f and Table 3). Thus, the two heavy chain transcripts, *DNAH5* and *DNAH7*, from the nine major phylogenetically classed dynein heavy chains may be involved in PrF dormancy and/or activation in the domestic cat. This, of course, needs further investigation in the future. Additionally, very high expression of histone *HIST1H1T* (H1.6) in PrFs was observed (Supplementary Fig. S7). H1 histones belong to the so-called linker histones, meaning that they are not part of the histone-octomer-centre of nucleosomes but connect to the ends of DNA that coils around such a centre leading to chromatin compaction. Furthermore, H1 also functions through interactions with other proteins that will in turn modify chromatin or take part in DNA-based processes like transcription[79]. Besides versions of H1 (H1.1 to H1.5) which are present in most somatic cells, other subtypes exist[79], some of which are germ-line variants[80]. HIST1H1T was so far described as a testis-specific variant[81], but could be detected in tumour cell lines, mouse embryonic stem cells and some normal somatic cells also[82]. To our knowledge, this is the first description of its presence in ovarian follicles, meaning that it could be present in female germ cells. Male H1 germ variants like H1T appear after spermatogonia stop proliferating and before transition proteins are detected and histones are replaced by protamines at late spermatid stages. H1T is the first variant to be expressed in meiotic spermatocytes, the presence in later germ cell stage up to elongated spermatids seems to be species-specific[80]. The dormant PrFs are arrested in meiosis I[83] and primary spermatocytes are in prophase of meiosis I[84]. It could be possible that H1T fulfils a specific function during this cell division phase, for example, on chromatin structure. It is discussed that H1T supports the highly decondensed chromatin stage in early spermatocyte[80]. However, is has been shown that H1T repressed at least ribosomal DNA transcription by condensing chromatin structure[82].

Conclusion

RNA-sequencing analysis of ovarian follicles in domestic cat contributed to the increasing knowledge on factors and processes which regulate the recruitment and growth of ovarian follicles. Many biological processes were comparable to known data obtained for species such as; human however, species-specific features for the domestic cat may be present. In this study, the analysis focused on signalling factors and pathways along with mechanistic cues associated with the ECM and nuclear dynamics. Overall, the results are relevant to fundamental ovarian follicle developmental biology with an outlook toward developing techniques such as, IVG of ovarian follicles in the future.

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Author contributions

Conceived and designed the experiments: K.J., B.C.B. Performed the experiments: S.K., B.C.B. Analysed the data: S.K., P.R.J., B.C.B. Contributed reagents/materials/analysis tools: P.R.J., S.M. Wrote the paper: S.K., K.J., B.C.B. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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Chapter 3: Early preantral follicles of the domestic cat express gonadotropin and sex steroid signalling potential

3.1 Summary

During the analysis of the first paper, we identified a GO term of interest related to steroid metabolism, “steroid metabolic process”. We also found genes of interest which are known drivers of ovarian steroidogenesis in ovarian follicles. We hypothesised that gonadotropin and sex steroid hormone signalling may be contributing to early follicle development and that early follicles could be a source of sex steroids in the domestic cat. The results were published in “Biology of Reproduction" (Kehoe et al., 2022). The methodological approaches included **(1)** the analysis of significant differential gene expression in candidate genes; **(2)** immunohistochemistry to localise protein expression in the oocytes and somatic cells of early follicles; and **(3)** measuring gene expression levels for genes of interest using qRT-PCR.

We verified gene expression in early follicles by examining the mean normalised gene count for the FSH and LH gonadotropin receptors (FSHR and LHCGR, respectively), sex steroid hormone receptors for progesterone, androgen and oestrogen and steroidogenic enzymes. We also determined which genes were significantly differentially expressed as follicles developed from primordial to primary and primary to secondary stages. Using the data obtained previously, we also presented the GO terms of these genes. Similarities to published work regarding gene expression patterns or localisation in cellular components were discussed. This study was the first to describe both gene and protein expression for enzymes of steroidogenesis (CYP11A1, CYP17A1, CYP19A1 and HSD3B1) as well as for sex steroid hormone receptors including the androgen receptor (AR), oestrogen receptor 1 (ESR1), progesterone receptor (PGR) and progesterone receptor membrane component 1 (PGRMC1) as well as the gonadotropin receptors in early follicles of any carnivore species. We discussed how early follicles exhibit gene expression of all enzymes necessary for sex steroid biogenesis and many sex steroid receptors as well as receptors for FSH and LH. Some of the analysed genes were significantly differentially expressed between development stages. Based on gene expression data and the immunohistochemistry results for protein expression, we concluded that gonadotropin and sex steroid reception may be contributing to the regulation of early follicle development. Protein expression of some sex steroid biogenesis enzymes in secondary follicles suggests that they could also be a source of sex steroids. The results illuminate which of the discussed molecular mechanisms, such as ovarian steroidogenesis, steroid metabolism, sex

steroid and gonadotropin signalling, operate during early folliculogenesis, a topic little explored in many mammalian species, including the domestic cat. In conclusion, we proposed suggestions for the next steps in the project that could optimise *in vitro* techniques such as IVG in the domestic cat model.

3.2 Author contributions

Shauna Kehoe: Collection of ovarian follicle samples, characterisation and imaging of ovarian follicles, isolation of RNA, qRT-PCR (unpublished data), RNA-sequencing analysis, writing scripts in R, documentation of experiments, experimental design of semi-quantitative immunohistochemistry analysis, analysis, discussing data, and preparing the manuscript.

Beate C. Braun and Katarina Jewgenow: Project design, supervision, discussing data, and preparing the manuscript.

Beate C. Braun: Immunohistochemistry and qRT-PCR.

Paul R. Johnston: Support in R scripts.

All authors were involved in correcting the manuscript.

Chapter 4: General Discussion

The publications included in this thesis (Kehoe et al., 2021, Kehoe et al., 2022) identified active genes, signalling pathways and molecular mechanisms relevant to development from primordial to primary and primary to secondary ovarian follicles in the domestic cat. The gene expression levels of some specific proteins that were studied could serve as developmental biomarkers for early folliculogenesis in this species. Below, I highlight the contribution made by both publications (Kehoe et al., 2021, Kehoe et al., 2022) toward identifying the general principles of early follicle development in mammals. At the same time, I provide a critique of the methodology while highlighting the next steps that could be taken in the future.

4.1 Molecular mechanisms during early ovarian folliculogenesis in the domestic cat

In the first publication (Kehoe et al., 2021), the most defining patterns within the data were those of enriched GO terms such as "ECM receptor interactions" and "extracellular structure organisation". Enrichment is defined by the over-representation of specific biological processes from our gene lists (Zhou et al., 2019, Huang et al., 2009, Sherman et al., 2022). In the ovary, the ovarian cortex is considered to be stiffer than the medullary region which creates a physical gradient that may be functional toward regulating follicle development (Woodruff and Shea, 2011). The mammalian ECM is composed of fibrous proteins (such as, collagens and elastin) and glycoproteins (Mouw et al., 2014). The structural re-organisation through enzymatic digestion by MMPs can alter cell proliferation, steroidogenesis, survival and differentiation (Bonnans et al., 2014). Along with the ECM-associated GO terms, gene expression patterns for a range of MMPs including *MMP1*, *MMP2**, *MMP7**, *MMP9*, *MMP12**, and *MMP21** were uncovered (Kehoe et al., 2021)[2]. Our results suggest that the ECM and MMPs may play a role during early follicle development in the cat ovary. Previously, gene expression levels had been determined for *MMP1*, *MMP2*, *MMP3*, *MMP7*, *MMP9*, and *MMP13* in primordial, primary and secondary follicles isolated from the domestic cat (Fujihara et al., 2016). MMP1, MMP2, MMP9 and MMP13 proteins were localised in the follicular compartments (Fujihara et al., 2016). When comparing both publications, there were similar gene expression patterns for *MMP1*, *MMP2*, *MMP7*, and *MMP9* (Kehoe et al., 2021, Fujihara

[2] a * denotes significantly differentially expressed genes during primordial to primary development

et al., 2016). In line with the hypothesis, ECM receptor interactions and the regulation of extracellular structure organisation by MMPs may have a regulatory role during early follicle development in the domestic cat. The results are consistent with previous observations made by Fujihara et al. (2016) and together build on existing evidence that the ovarian architecture may be involved in regulating early folliculogenesis in the domestic cat (Fujihara et al., 2016). Whereas the exact interactions remain unknown, these results should provide guidance for future gene and protein expression studies, where these results could be functionally examined during IVG studies.

The results from the first publication also revealed biological processes that were rather unexpected for this stage of folliculogenesis – ovarian steroidogenesis (Kehoe et al., 2021). Until now, the extent to which gonadotropins and the steroidogenic pathway were involved in early follicle development in the domestic cat was unknown. As described previously, ovarian steroidogenesis is popularly depicted through the two-cell, two-gonadotropin theory where LH stimulates theca cells to produce androgens, and FSH stimulates granulosa cells to produce oestrogens (Armstrong et al., 1979). The theory implies that ovarian follicles must contain theca cells to execute the signalling cascade (Hillier et al., 1994). This essentially excludes primordial and primary follicles as they contain only a single layer of squamous or cuboidal granulosa cells, respectively. Even so, our data still alluded to the molecular mechanism of ovarian steroidogenesis during early follicle development in the domestic cat (Kehoe et al., 2021).

In our second publication, we presented a variety of genes present in the early follicles of the domestic cat that are known players in ovarian steroidogenesis and steroid receptor activity (Kehoe et al., 2022). This included genes for the gonadotropin receptors, the cholesterol transporter *StAR*, enzymes of steroidogenesis and metabolism and steroid receptors (Kehoe et al., 2022). During primordial to primary follicle development, the genes for *CYP11A1* and *CYP17A1*, respectively, *HSD17B1* and 17-beta-hydroxysteroid dehydrogenase 7 (*HSD17B7),* respectively, steroid sulfatase (*STS*), *PGR*, *AR*, and *PGRMC1* were significantly differentially expressed (Kehoe et al., 2022). Protein expression for FSHR, LHCGR, AR, PGR, and ESR1 was detected in the oocytes of primordial and primary follicles. In the granulosa cells of primordial follicles, we detected protein expression for ESR1 and PGRMC1 whereas in the granulosa cells of primary follicles, protein expression for FSHR, LHCGR, ESR1, and PGRMC1 was observed (Kehoe et al., 2022).

During primary to secondary follicle development, the genes for *CYP17A1* and *AR* were significantly differentially expressed (Kehoe et al., 2022). By the secondary follicle stage, we could observe protein expression for both gonadotropin receptors, all analysed sex steroid receptors and selected steroidogenic enzymes in the oocytes, granulosa and/or theca cells, suggesting that steroidogenesis and steroid reception may be possible (Kehoe et al., 2022). In line with the hypothesis, ovarian steroidogenesis and sex steroid receptor activity may be functional in early follicles of the domestic cat. However, considering the tightly packed structure of the ovarian microenvironment, one plausible explanation is that early follicles may first need to move towards a more physically permissive space to take full advantage of gonadotropin and sex steroid hormone stimulation being delivered by the ovarian vasculature (Woodruff and Shea, 2011).

It is noteworthy that the results highlighted similarities and differences between studies performed in other species. In humans, sex steroid receptors are expressed in the primordial and primary oocytes isolated from adult ovaries, albeit at a low level (Markholt et al., 2012). AR, for example, was expressed at a low level in the primordial follicle oocytes (Markholt et al., 2012). Interestingly, in comparison to human foetal ovaries, AR was reported exclusively in the primordial somatic cells (Fowler et al., 2011). In contrast, only the oocytes of primordial, primary and secondary follicles expressed AR proteins in high amounts in the domestic cat (Kehoe et al., 2022). *PGRMC1* and progesterone receptor membrane component 2 (*PGRMC2*) were expressed at a low level in the primordial and primary oocytes in adult humans (Markholt et al., 2012). Expression of *PGRMC1* messenger RNA was also reported in the oocytes, granulosa and theca cells of immature rat ovaries (Peluso et al., 2006), whereas we reported granulosa-specific protein expression of PGRMC1 in primordial, primary and secondary follicles in the domestic cat (Kehoe et al., 2022). Overall, the data contribute to a clearer understanding of ovarian steroidogenesis and steroid receptor activity during early follicle development in the domestic cat. Moreover, it highlights species-specific characteristics, which could be explored more in-depth during IVG studies in the future.

In our first publication, we identified specific signalling pathways present during early follicle development in the domestic cat (Kehoe et al., 2021). The PI3K/Akt signalling pathway was profoundly involved during early follicle development (Kehoe et al., 2021). This was demonstrated through obvious GO terms such as “PI3K/Akt signalling pathway” and more implicit GO terms including the “transmembrane receptor protein kinase signalling pathway” (Kehoe et al., 2021). Several key genes from the signalling pathways increased significantly in

differential gene expression during primordial to primary development, including factors such as the tuberous sclerosis complex 2 (*TSC2*), pyruvate dehydrogenase kinase (*PDK*), protein kinase Akt-2 (*AKT2*), regulatory associated protein of mTOR complex 1 (*RPTOR*), and PI3K subunit delta (*PIK3CD*) (Kehoe et al., 2021). In line with the hypothesis, the PI3K/Akt signalling pathway may be essential to early follicle development in the domestic cat. In other species, the PI3K/Akt signalling pathway was reported to be crucial to ovarian folliculogenesis in genetically modified mouse (Adhikari and Liu, 2009, Reddy et al., 2008, Reddy et al., 2010, Zhang et al., 2014), bovine (Andrade et al., 2017) and human models (Grosbois and Demeestere, 2018, Kawamura et al., 2013, Wagner, 2020). As one of the best studied mechanisms in the ovary, these results build on existing evidence that the PI3K/Akt signalling pathway may be a very important molecular mechanism during early follicle development in mammalian species. The PI3K/Akt signalling pathway may be activated via several types of receptor interactions with growth factors, integrins, receptor tyrosine kinases, cytokine signalling and G-protein-coupled signalling (Hemmings and Restuccia, 2012). The response to these interactions can dictate the activation of mechanisms including protein synthesis, cell survival, apoptosis, migration and proliferation, meaning that activation of the PI3K/Akt pathway during early follicle development can have widespread downstream effects (Hemmings and Restuccia, 2012).

Other factors that are suspected to play an essential role during ovarian folliculogenesis in mammals include members from the TGF-β superfamily such as TGF-βs, inhibins, activins, BMPs and growth differentiation factors (GDFs) (Findlay et al., 2009). As far as I am aware, the aforementioned factors that were investigated in early follicles of the domestic cat include protein expression of the epidermal growth factor (EGF) and its receptor (EGFR) (Göritz et al., 1996), protein expression of TGF-ß types 1-3, TGF-ß receptor type 2, growth differentiation factor 9 (GDF-9), and BMP receptor type 2 (Bristol and Woodruff, 2004) and *in vitro* supplementation effects of EGF and GDF-9 (Fujihara et al., 2014). In our first publication, we also discussed the potential role of the TGF-ß signalling pathway during early follicle development in the domestic cat (Kehoe et al., 2021). Key TGF-ß signalling pathway genes increased significantly in differential gene expression during primordial to primary development, including BMP and activin membrane bound inhibitor (*BAMBI*), *BMP15*, twisted gastrulation BMP signalling modulator 1 (*TWSG1*), E3 ubiquitin protein ligase 2 (*SMURF2*), *BMP4*, BMP receptor type 1B, and BMP binding endothelial regulator (*BMPER*) (Kehoe et al., 2021). Collectively, these results contribute toward a clearer understanding of

two key signalling pathways, the PI3K/Akt and TGF-ß signalling pathways, which may be important to early folliculogenesis in the domestic cat. In the future, it may be important to evaluate these ligands and their receptors *in vitro* to better judge the complexity of both signal transduction systems and to enhance our perception of their interaction capabilities. Further down the line, this may have a beneficial impact on ARTs for mammalian species, such as the endangered feline species currently enrolled into captive breeding programs (Jewgenow and Zahmel, 2020).

4.2 Leaving no stone unturned – honourable mentions

While every attempt was made to provide a broad insight into as many molecular mechanisms as possible which may be involved in early follicle development in the domestic cat, there are some more that deserve an honourable mention. Beginning with hypoxia, the reduction of oxygen in tissues which induces reactive oxygen species - by-products of cellular aerobic metabolism (Juan et al., 2021). During the ovarian cycle, the blood flow to the ovary can change (Tan et al., 1996) which impacts the transport oxygen and other factors such as, hormones for instance (Brown and Russell, 2014). Regions of the ovary such as, the ovarian cortex, are less vascularised (Feng et al., 2017) which impacts the delivery of oxygen (Fraser, 2006). The ovarian follicle is suggested to be adapted to function in low oxygen and past studies have shown that hypoxia can even play a role in the regulation of follicle development (Lim et al., 2021). For example, hypoxia was shown to induce the dormant state in mouse oocytes from reconstituted ovaries that were derived from pluripotent stem cells (Shimamoto et al., 2019). Still the follicular environment must compensate for periods demanding high oxygen levels for metabolism (Lim et al., 2021). HIFs are transcription factors that play an essential role in cellular response to low oxygen with emerging studies showing that HIF-1 is a significant regulator of gene expression in ovarian compartments and plays a role in healthy follicle development (Lim et al., 2021). We found several HIF-1 factors were significantly differentially expressed during primordial to primary development including hypoxia inducible factor 1 subunit alpha inhibitor (*HIF1AN*), phosphofructokinase liver type (*PFKL*) and Egl-9 family hypoxia inducible factor 2 (*EGLN2*) (Kehoe et al., 2021). Two other HIF-1 factors, basic helix-loop-helix family member e40 (*BHLHE40*) and furin paired basic amino acid cleaving enzyme (*FURIN*), were significantly differentially expressed during primary to secondary follicle development (Kehoe et al., 2021). Additionally, GO terms suggested that early follicles were experiencing a “response to oxygen-containing compound” (Kehoe et al.,

2021). HIF-1 factors have not been extensively studied in early follicles however, a past study in rats revealed that HIF-1 alpha (HIF-1α) may participate in primordial follicle activation, as the increase in HIF-1α coincided with the development of ovarian follicles (Zhang et al., 2015). In the future, it would be worthwhile to examine the relationship between the HIF signalling pathway and early follicle development in the domestic cat.

The data also contribute towards a clearer understanding of other signalling pathways and molecular mechanisms that are not well-defined during early folliculogenesis in mammalian species. This includes the vascular endothelial growth factor signalling pathway, which was identified in our data through the enriched GO terms "regulation of angiogenesis" and "vasculature development" during the primary to secondary transition (Kehoe et al., 2021). In the ovaries, angiogenesis is suggested to provide nutrition to the developing follicles, which is why the vascular network must be constantly rebuilt (Nagamatsu et al., 2019).

Our data also provides insight into a biological process recently described in rodents, involving the regulation of early follicle development via the dynein complex (Nagamatsu et al., 2019). In our first study, we revealed key dynein complex genes, armadillo repeat containing 4 (*ARMCX4*), coiled-coil domain containing 63 (*CCDC63*), dynein axonemal heavy chain 5 (*DNAH5*) and 7 (*DNAH7*), dynein regulatory complex subunit 7 (*DRC7*), and sperm associated antigen 1 (*SPAG1*), which were all significantly differently expressed during primordial to primary follicle development (Kehoe et al., 2021). Much more future research will be required to gain a better understanding of the potential regulatory role these mechanisms may have in the domestic cat ovary.

Our publications did not discuss one of the major pathways responsible for mechanical stress: the Hippo signalling pathway (Gershon and Dekel, 2020, Vo and Kawamura, 2021). In human ovaries, ovarian tissue fragmentation leads to the disruption of Hippo signalling which subsequently promotes follicular growth (Kawamura et al., 2013). Other signalling pathways that were also not in the list of the most enriched biological processes included Wingless/Integrated, insulin, Notch and Hedgehog pathways (Kehoe et al., 2021). Yet they are found to play a role in follicle development at least in the mouse (Li et al., 2021)

4.3 The methodological challenges

Collecting early follicles by pressing ovaries through cell-dissociating mechanical sieves has previously resulted in up to 1,500 follicles being recovered from a single cat ovary (Jewgenow

and Göritz, 1995, Jewgenow et al., 1998). Similar results were sometimes observed in our studies using this technique. Indeed, different numbers of follicles from different follicular stages with a rough estimate of ~500-1,000 primordial follicles, <50 primary follicles and <20 secondary follicles per ovary could be observed. Surprisingly, on some occasions, very few follicles were recovered from 5 recorded individuals, and there were not enough follicles present to perform a sample collection of 60 primordial follicles, 15 primary follicles and/or 3 secondary follicles, the minimum number that should be collected. One possible explanation could be that these ovaries were more rigid, perhaps from scar tissue present due to repeated ovulations. Fortunately, it was not encountered too often as this technique is very time-consuming, and, in the end, enough samples were collected for our experiments. Other isolation methods for cat follicles are possible, including laser capture microdissection (LCM), using needles and/or scalpel blades from the cortical tissue or enzymatic digestion of the surrounding cortical tissue (Rojo et al., 2015, Nagashima et al., 2021). Methods such as LCM may not be possible without causing some damage to primordial follicles, as squamous granulosa cells very tightly surround cat oocytes (personal correspondence with Katarina Jewgenow). Enzymatic digestion may also cause damage to follicular cells through the enzymatic depletion of the surrounding somatic cells and degradation of the basement membrane (Demeestere et al., 2002). While it is beyond the scope of this study to consider other isolation methods as our method of choice successfully isolated whole follicle samples, it is worthwhile to highlight which alternatives exist for possible future experimentation.

An important aspect for both studies was to obtain a high RNA yield from our follicle samples. Initially, samples were collected in phosphate buffer saline solution supplemented with bovine serum albumin (PBS-BSA) as described above, snap frozen in LN_2, and then stored in the -80°C freezer for a brief period. While this method is suitable to collect and store follicle samples, we discovered downstream that RNA quality was low as measured by the RNA-integrity number (RIN) (TapeStation). The gold standard RIN value suitable for RNA-sequencing downstream is a RIN ≥ 7 (a value of 10 indicates the highest possible RNA quality) (Gallego Romero et al., 2014). Preliminary experiments revealed that when samples were lysed in chaotropic ion Lysis Buffer (LB1) (NucleoSpin®) then snap frozen, better RIN values were obtained (unpublished data). In these unpublished experiments, three replicates of 200 primordial follicle samples were collected per group, with three groups in total when three solutions were tested: PSA-BSA, LB1 lysis buffer, and an RNase inhibitor (RiboLock). Each sample was collected into droplets of their respective solutions and snap frozen in LN_2. After

a brief period of storage at -80°C the samples were thawed for RNA isolation. PBS-BSA and RiboLock had low RIN values that were < 5 on average, whereas LB1 achieved RIN values of 7 on average. With these results in mind, ovarian follicles were lysed in LB1 solution before snap freezing in LN_2 for both published studies (Kehoe et al., 2021, Kehoe et al., 2022).

The reliability of the data was affected by the unexpected qRT-PCR results in comparison to the RNA-sequencing results (Kehoe et al., 2021, Kehoe et al., 2022). This may be because the sample size (number of follicles from which RNA was isolated) was not sufficient. Values in qRT-PCR were measured often at the detection limit; therefore, standard deviations can become too large for the detection of a significant difference. With a larger number of follicles per analysed sample pool, the corresponding qRT-PCR results from both published studies may have been improved. As the collection of a sufficient number of follicles for further qRT-PCR would have taken too long, such a repetition was not done. Future studies examining the RNA-sequencing data should consider that an increase in the number of ovarian follicles used per sample for qRT-PCR analysis may strengthen the analysis downstream. One avenue for future research includes testing a larger selection of genes that were significantly differentially expressed - at least one or two genes per signalling pathway that we presented in our first study (Kehoe et al., 2021). Further research into the expression of the proteins, which are important because of their ability to carry biological functions, should also be considered.

4.4 Acknowledging the limitations

As no protein expression data were obtained in the first study, it was not possible to compare gene expression patterns with protein level-patterns or to clarify protein localisation in the early follicles of domestic cat. This provides an opportunity for future studies into the specific factors we discovered. For example, it would be of great interest to explore protein expression patterns of the newly described MMPs in early follicles of the domestic cat. If a particular MMP is implicated to be conspicuously present in early follicles, then the main substrates of the enzyme could be investigated too. For example, *MMP1* was detected in early follicles of the domestic cat using RNA-sequencing (Kehoe et al., 2021) and has been reported to be expressed at the protein level of each stage (primordial, primary and secondary) too (Fujihara et al., 2016). Fujihara et al., (2016) suggested that the effect of MMP1 expression levels on insulin growth factor 1 (IGF1) and its binding protein could be investigated, as MMP1-induced degradation of IGFBP3 may stimulate the release of IGF1, a factor known to influence ovarian follicles (Fujihara et al., 2016, Giudice, 1992).

It is beyond the scope of this study to describe the transcriptomics of the oocyte and somatic cells, as we chose to study whole follicle samples instead (Kehoe et al., 2021). To date, studies on early follicles are predominantly performed using the whole ovary (Herrera et al., 2005, Fowler et al., 2009), with single cells collected using an animal-age-specific (Herrera et al., 2005), using oocyte size-specific selection criterion (Markholt et al., 2012, Ernst et al., 2017, Chen et al., 2022), or in our case using whole follicle samples (Kehoe et al., 2021, Kehoe et al., 2022). These approaches preclude the ability to study cells in precise follicle stage-specific populations because both animal-age and size-specific cells are heterogeneous concerning follicle stages (Chen et al., 2022). In our case, it cannot provide insight into the potential interactions between oocytes and somatic cells. In the future, it would be helpful for identifying cell-specific molecular mechanisms if ovarian follicle cells could be sequenced separately by combining techniques such as LCM and single-cell RNA-sequencing (Hwang et al., 2018). Single-cell RNA-sequencing is a variation of RNA-sequencing where the source of total RNA for sequencing comes from a single cell (Hwang et al., 2018). In mice, it has been used to reveal which molecular mechanisms are present in the primordial follicle oocytes and somatic cells separately (Chen et al., 2022). Future studies could consider that it may be possible to perform similar transcriptomic analyses for each cell – this would represent another opportunity for further research into early folliculogenesis in the domestic cat.

Due to our methodological choices, the results also cannot confirm the impact of specific epigenetic modifications, or "tags," such as DNA methylation and histone modification, which can alter DNA accessibility and chromatin structure, thereby regulating patterns of gene expression (Sadakierska-Chudy and Filip, 2015). In oocytes, developmental competence is influenced by epigenetic factors that control gene expression and chromatin configuration in germinal vesicles, for instance the oocyte's nucleus that is arrested in prophase of meiosis I by the primary follicle stage (Laisk et al., 2019) in mouse (Zuccotti et al., 2002), domestic pig (Wu et al., 2006) and domestic cattle (Lodde et al., 2007). There are many kinds of histone modifications, including methylation, acetylation, phosphorylation, ubiquitination and sumoylation, which may influence ovarian folliculogenesis and female fertility (Sadakierska-Chudy and Filip, 2015). In mice, the loss of E2 SUMO-conjugating enzyme Ube2i in oocytes during folliculogenesis will lead to infertility (Rodriguez et al., 2019). To the best of my knowledge, the only epigenetic tag that has been studied in ovarian follicles (at the preantral and antral stage) in the domestic cat ovary is histone methylation (Phillips et al., 2012, Phillips et al., 2016). In the first study, histone lysine methylation changes were investigated through

immunostaining of preantral and antral follicles (Phillips et al., 2012). The second study interrogated nuclear versus cytoplasmic regulation on the previously identified histone tags again in preantral and antral follicles (Phillips et al., 2016). Further research is needed to establish epigenetic profiles during early folliculogenesis in the domestic cat.

While it is not the topic of this thesis, it is worthwhile to note that other "omics"-tools could be employed to measure the downstream consequences of activating signalling and metabolic pathways in the future (Jones et al., 2021). After transcriptomics, proteomics is the next step in the study of biological systems. Proteomics is the large-scale study of proteins which may be detected using immunoassays or mass spectrometry (Chandramouli and Qian, 2009). Proteomic profiles have already been elucidated in immature rat ovaries during primordial follicle development (Wang et al., 2009) and in mouse ovaries during primordial follicle activation and early follicle development (Xiong et al., 2019). In the domestic cat, proteomics was used to elucidate whether nuclear proteins in the germinal vesicle contribute to oocyte competence acquisition (Lee et al., 2018). To achieve this in the future, protein lysates will have to be prepared from early follicle samples for quantitative proteomic analysis downstream (Lee et al., 2018).

4.5 Conclusion

My studies provide insight into genes, signalling pathways and molecular mechanisms that may be key to regulating early follicle development in the domestic cat. Our characterisation of gene expression patterns by RNA-sequencing and additional protein localisation of gonadotropin receptors, sex steroid receptors and enzymes of steroidogenesis during early folliculogenesis sets the foundation for follow-up studies. As the first of their kind, these studies represent an effort to address the gap in our knowledge for this species and to deepen our understanding for other mammals too. Overall, the result of this thesis is consistent with concepts which suggest that early follicle development may require a combination of signals from the surrounding ovarian architecture in addition to hormonal cues and paracrine signalling.

4.6 Future perspectives

The next few years: Understanding temporal gene expression known to influence early follicle development could provide a biological basis for supplementing culture media in the future

(Jones et al., 2021). The recapitulation of such a complex process may require the asynchronous delivery of specific factors in an ordered cascade as originally described with "Eggbert" (Eppig and O'Brien, 1996, O'Brien et al., 2003). This may not be so simple, as recent studies on bovine oocytes revealed that the addition of PTEN/PI3K/Akt stimulators and inhibitors were deleterious to follicle quality (Maidarti et al., 2020, Maidarti, 2020) This emphasises how a full understanding of the requirements of follicular development is integral because if primordial follicles are inappropriately activated, DNA and gene expression may be compromised, which could lead to a decrease in oocyte quality, with potential ramifications in downstream ARTs (Amoushahi and Lykke-Hartmann, 2021, Maidarti, 2020).

Within the next decade? The ultimate goal of understanding primordial follicle activation and early follicle development in the domestic cat is to recapitulate this process *in vitro* so that we can learn how to generate a larger number of oocytes for assisted reproduction. Techniques that are established in the domestic cat model could be adapted for ARTs for endangered felid species in the future (Wildt et al., 1986). The work in progress includes: **(1)** slow freezing of ovarian cortex containing the dormant primordial follicles which has been successfully performed in the domestic cat (Wiedemann et al., 2013, Bosch et al., 2004), although feline oocytes may be more sensitive to cryopreservation protocols than other species (Fassbender et al., 2007, Kvist et al., 2006); **(2)** recapitulation of primordial follicle activation and early follicle growth *in vitro* through our acquaintance with potential *in vivo* molecular mechanisms (Kehoe et al., 2021, Kehoe et al., 2022); **(3)** production of developmentally competent oocytes during IVM which is considered as an established method in cats today with a success of 50-70% (Sowińska et al., 2020, Morselli et al., 2017); **(4)** IVF of oocytes which has been successfully established in the domestic cat over the last 30-40 years (Herrick et al., 2007, Pope et al., 2006b, Wolfe and Wildt, 1996) and is the basis for *in vitro* embryo production or vitrification of feline oocytes and embryos (Colombo et al., 2020, Pope et al., 2012, Ochota et al., 2017) - *in vitro* produced embryos can be cultured up to the blastocyst stage (Herrick, 2019, Zahmel et al., 2021); **(5)** and the transfer of embryos into a surrogate mother, currently not a standard method in the domestic cat, which has led to the production of several litters of live kittens (Pope et al., 2012, Pope et al., 2006a).

Collectively, these efforts aim to contribute toward establishing ARTs for critically endangered felid species (Jewgenow and Zahmel, 2020, Swanson, 2003) where there is a critical need to overcome mating difficulties and address infertility issues that some individuals face in captivity (Wildt et al., 2010, Comizzoli et al., 2018). This will also aid the maintenance of

genetic diversity through the preservation of heterozygosity to avoid inbreeding depression and support the persistence of genomic adaptability to environmental changes in the future (Wildt et al., 2010, Comizzoli et al., 2018). The Felid Gamete Rescue Project established in the Department of Reproduction Biology at the Leibniz Institute for Zoo & Wildlife Research aims to explore and improve current ARTs after gonads are collected from deceased individuals (Zahmel et al., 2019). Some achievements to date include IVM followed by intracytoplasmic sperm injection which has led to successful fertilisation and embryonic development up to the blastocyst stage in lion (*Panthera leo*) (Fernandez-Gonzalez et al., 2015), and the cryopreservation of lion oocytes for the first time when the oocytes survived vitrification, successfully matured *in vitro* and cleaved after IVF (Zahmel et al., 2021). Future studies into IVG methods of early follicles could consider the results presented in this thesis as a basis for initial experimentation if there are some spare ovarian cortex tissue pieces available.

While both publications have provided new insight into molecular mechanisms which may be key regulators of early follicle development in the domestic cat, there is still much more to be uncovered and many more questions yet to be answered. Although the remarkable feats conquered in the field of ARTs for the rodent model are yet to be achieved for the domestic cat, the next decade of research may bring paramount breakthroughs for this species. And with that deliver a renewed hope for the charismatic family of wild Felidae.

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List of Publications

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Kehoe S, Jewgenow K, Johnston PR, Braun BC. Early preantral follicles of the domestic cat express gonadotropin and sex steroid signaling potential. Biol Reprod. 2022 Jan 13;106(1):95-107. doi: 10.1093/biolre/ioab192. PMID: 34672344.
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