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Intercellular Signalling in the Human Pre-ovulatory Follicle: microRNA Expression in Granulosa Cells and Detection in the Follicular Fluid

Ilmatar Rooda

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ILMATAR ROODA

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TALLINN UNIVERSITY OF TECHNOLOGY
School of Science
Department of Chemistry and Biotechnology
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Supervisor: Associate professor Agne Velthut-Meikas, PhD
School of Science
Tallinn University of Technology
Tallinn, Estonia

Co-supervisor: Professor Andres Salumets, PhD
Institute of Clinical Medicine
University of Tartu
Tartu, Estonia

Opponents: Professor Christiani Andrade Amorim, PhD
Institute of Experimental and Clinical Research
University of Louvain
Brussels, Belgium

Professor Ana Rebane, PhD
Institute of Biomedicine and Translational Medicine
University of Tartu
Tartu, Estonia

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Ilmatar Rooda

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Rakkudevaheline kommunikatsioon inimese munasarja preovulatoorses folliikulis: mikroRNA-de ekspressioon granuloosrakkudes ja follikulaarvedelikus

ILMATAR ROODA

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II **Rooda I**, Kaselt B, Liivrand M, Smolander OP, Salumets A, Velthut-Meikas A.
Hsa-mir-548 family expression in human reproductive tissues.
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III **Rooda I***, Hasan MM*, Roos K, Viil J, Andronowska A, Smolander OP, Jaakma Ü, Salumets A, Fazeli A, Velthut-Meikas A.
Cellular, Extracellular and Extracellular Vesicular miRNA Profiles of Pre-Ovulatory Follicles Indicate Signaling Disturbances in Polycystic Ovaries.
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Author's Contribution to the Publications

Contributions to the papers in this thesis are:

I The author performed the majority of the experiments and analysed the results, wrote the manuscript draft and final manuscript.

II The author analysed all the data, wrote the manuscript draft and final manuscript.

III The author performed part of the experiments and analysed the data, wrote part of the manuscript draft and final manuscript.

Introduction

Human ovarian follicle growth is a highly controlled and regulated process which culminates with the ovulation of the oocyte. Ovarian follicles are formed during foetal development and are held in the ovarian reserve. Before and during the reproductive period follicles leave the reserve and start folliculogenesis. However, only when women reach puberty and sex hormones are produced does follicle development reach its final stage and the oocytes ovulate in a periodic manner. In addition to the hormones, microRNAs (miRNAs), well-characterised gene expression regulators, have an important role in follicle development. Disturbances in miRNA gene expression have an impact upon human fertility. Multiple miRNAs can regulate the same target gene, meaning that it is equally important to analyse miRNAs individually or as their co-expressed profiles. In spite of the known roles of miRNAs in follicle development, not all miRNAs present in ovarian follicles have been studied thoroughly. Furthermore, miRNAs can be present in extracellular space in the ovarian follicle and be detected from the follicular fluid. There is a lack of studies which address both the cellular and extracellular miRNAs of the ovarian follicle in parallel and in a genome-wide manner. Information obtained during these studies can potentially reveal how miRNAs are used in cell-to-cell communication. Moreover, comparing the miRNA profiles of follicles from healthy and fertile women to the follicles in ovary-related diseases can reveal the background of pathological follicle development.

The current thesis demonstrates that two miRNAs' – hsa-miR-548ba and hsa-miR-7973 – target genes are involved in ovarian follicle growth, activation and cell-to-cell interactions, respectively. In addition to hsa-miR-548ba other hsa-mir-548 family members have potentially important regulatory roles in the ovarian follicle. However, hsa-miR-548ba and other family members expressed in granulosa cells have a low degree of co-regulated pathways. Moreover, novel parallel datasets of miRNA expressions of the ovarian follicle cellular and extracellular fractions are provided. These miRNA profiles are disturbed in women diagnosed with polycystic ovary syndrome.

Abbreviations

AFC	Antral follicle count
AGO	Argonaute protein
AMH	Anti-Mullerian hormone
bFGF	Basic fibroblast growth factor
BMP-4	Bone morphogenic protein 4
CGC	Cumulus granulosa cell
CL	Corpus luteum
COC	Cumulus-oocyte complex
CX37	Connexin 37
CX43	Connexin 43
DE	Differentially expressed
DF	Dominant follicle
DGCR8	DiGeorge syndrome critical region 8
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EV	Extracellular vesicle
FDR	False discovery rate
FF	Follicular fluid
FGF7	Fibroblast growth factor 7
FOXL2	Forkhead box L2
FOXO1	Forkhead box protein O1
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GC	Granulosa cell
GDF9	Growth differentiation factor 9
GnRH	Gonadotropin-releasing hormone
GPHR	Glycoprotein hormone receptor
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IVF	*In vitro* fertilisation
KGF	Keratinocyte growth factor
KL	Kit ligand
Made1	Mariner-derived element 1
MGC	Mural granulosa cell
MITE	Miniature inverted-repeat transposable element
NOR	Normal ovarian reserve
PCOS	Polycystic ovary syndrome

PI3K	Phosphatidylinositol-3 kinase
PKB	Protein kinase B
POI	Premature ovarian insufficiency
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
RBP	RNA binding protein
RISC	RNA-induced silencing complex
RT-qPCR	Quantitative reverse transcription PCR
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
UTR	Untranslated region

1 Review of the Literature

Female reproduction relies on well-timed ovulation and correctly matured oocyte. The functional units in ovaries are ovarian follicles which are composed of the developing oocyte surrounded by somatic cells. Ovarian follicles are formed during foetal development and the number of follicles is then determined and represents the reproductive potential of the individual (Skinner, 2005). The quantity of germ cells is the highest at 20 weeks of gestation, totalling around 6 million follicles. By birth the number of follicles declines to approximately one million and continues decreasing (Charleston et al., 2007; Geber et al., 2012). Throughout the lifespan the number of follicles is primarily reduced by two pathways: dying in the process of atresia or due to the recruitment for folliculogenesis. The reproductive period ends when the primordial follicle pool reaches a critical number of follicles and is therefore unable to produce the necessary amount of hormones for follicle development and ovulation (Charleston et al., 2007; Gougeon, 1996). The highlight of folliculogenesis is oocyte ovulation. The choice of follicles reaching ovulation or undergoing atresia is controlled by several endocrine and paracrine factors (Gougeon, 1996; Richards et al., 1995). Communication between the oocyte and the somatic cells in the follicle is the key to successful follicle growth and ovulation (Hennet and Combelles, 2012). In the following chapters, an overview of the ovarian follicle development, the main functions of the somatic cells and known signalling and communication methods in the follicle is given.

1.1 Human ovarian follicle development

Follicles assembled during foetal development are termed primordial follicles. Resting in the ovarian cortex, primordial follicles form the ovarian follicle pool. During early gestation the primordial germs cells go through mitotic divisions with incomplete cytokinesis, resulting in an overabundance of interconnected oogonia. These oocytes commence meiosis during follicle formation, but arrest in prophase I (Grive and Freiman, 2015). The formation of primordial follicles requires the individual oocytes to segregate from the nest of oogonia and associate with pre-granulosa cells. To achieve this, random oocytes in the nest of oogonia go through apoptosis and as a result individual oocytes are isolated that associate with pre-granulosa cells to form the primordial follicles (Skinner, 2005). It has been shown in rat ovaries that progesterone has a negative effect on the primordial follicle formation through inhibiting oocyte apoptosis (Kezele and Skinner, 2003). In contrast, tumour necrosis factor-alpha (TNFα) induces oocyte apoptosis and allows primordial follicle formation (Marcinkiewicz et al., 2002). Activation and recruitment of the primordial follicles marks the beginning of further follicular development (McGee and Hsueh, 2000). Follicle recruitment can be divided into two: initial and cyclic. Initial recruitment starts right after formation of the primordial follicles and occurs throughout life until menopause. Initial recruitment is independent of gonadotropin hormones. When primordial follicles are recruited during initial recruitment, transition to the primary follicles begins. The first morphological change is granulosa cell (GC) shape from squamous to cuboidal and increased oocyte size (Figure 1) (Skinner, 2005). Furthermore, theca cell recruitment begins from the stromal-interstitial cell population (Skinner, 2005).

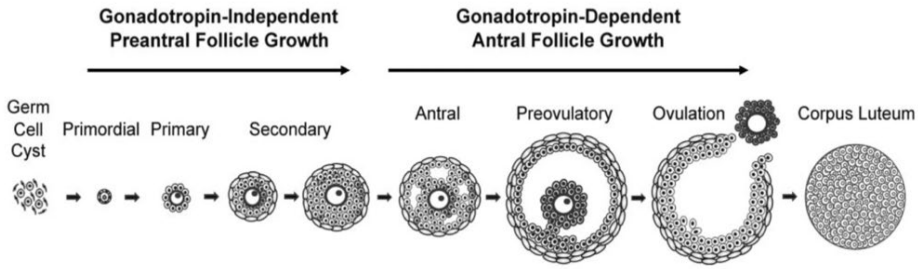

Figure 1. *Outline of the main stages of folliculogenesis. Folliculogenesis starts with the recruitment of a cohort of primordial follicles to develop into primary follicles. Proliferating granulosa cells (GCs) form several layers around the oocyte from the secondary to late preantral follicles. A layer of theca cells surrounds the follicle. The theca cells produce androgens, which are converted into estrogens in GCs. From the secondary/preantral stage the follicle growth is dependent on FSH stimulation. As GCs continue to proliferate, the antrum is formed separating two GC sub-types: cumulus and mural GCs. At this stage, the selection of dominant follicle (DF) occurs and only the DF continues to develop into the preovulatory stage while other follicles undergo atresia. After ovulation, triggered by a peak of LH, theca cells and mural granulosa cells luteinize and form corpus luteum to produce progesterone (adapted from Edson et al., 2009).*

Various factors have been associated with the primordial to primary transition: for example kit ligand (KL), basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF), keratinocyte growth factor (KGF), bone morphogenic protein-4 (BMP-4), forkhead box L2 (FOXL2) and anti-Mullerian hormone (AMH) (Edson et al., 2009; Skinner, 2005). It has been shown in the rat ovarian cultures that KL produced by GCs stimulates oocyte expansion and initiates follicle development. In addition, KL stimulates stroma and theca cell growth (Kezele and Skinner, 2003; Parrott and Skinner, 2000). bFGF produced by the oocyte acts on the GCs to influence primordial follicle development (Kezele and Skinner, 2003). The treatment of 4-day-old rat ovaries in culture with LIF resulted in a decreased number of primordial follicles and a consequent increase in the number of developing follicles (Nilsson et al., 2002). Similarly, KGF has been found to stimulate the primordial to primary follicle transition. KGF is located in the precursor theca cells which are in contact with the pre-granulosa cells. KGF is one of the first specific markers for developing precursor theca cells and supports the concept that precursor theca cells are a part of primordial follicle development (Kezele et al., 2005). The BMP family of growth factors belong to the TGFβ superfamily. BMP-4 has also been shown to promote the primordial to primary follicle transition. Moreover, BMP-4 is essential for oocyte survival (Nilsson and Skinner, 2003). Furthermore, FOXL2 is crucial for the transition from squamous to cuboidal GCs that occurs during the primordial to primary transition (Edson et al., 2009). When all the above-mentioned factors have a positive effect on the primordial to primary transition then AMH has the capacity to block primordial follicle development. AMH is not expressed in the primordial follicles, instead it is produced by the developing follicles (Skinner, 2005).

In the primary follicle stage GCs and theca cells continue proliferating. When entering into the secondary stage multiple layers of GCs are surrounding the oocyte (Figure 1). Moreover, the transition from primordial to secondary follicle (follicle size ~0.1–0.2 mm) is independent of gonadotropin hormones. The granulosa and theca cells are in their precursor stage, not yet fully differentiated and non-responsive to the gonadotropins.

In addition, at this stage pre-granulosa cells do not produce steroid hormones (Craig et al., 2007). When follicles increase in size (~0.2–0.4 mm) a fluid-filled cavity, also termed antrum, begins to form and follicles start to respond to gonadotropins (Figure 1) (Craig et al., 2007). Follicular fluid (FF) is derived from both the bloodstream through capillaries and from components secreted by somatic cells in the follicle. FF contains a selection of molecules including steroid and protein hormones and enzymes. Additionally, FF enables communication between different cell types in the follicle. GCs in the antral follicle differentiate into two separate populations: mural granulosa cells (MGCs) and cumulus granulosa cells (CGCs). CGCs surround the oocyte and together form a cumulus-oocyte complex (COC), while MGCs form the inner lining of the follicle (Figure 1) (Hennet and Combelles, 2012). Each antral follicle is surrounded by basal lamina that separates the internal follicle from the third somatic follicular cell type, the theca cells (Hennet and Combelles, 2012).

Cyclic recruitment starts when the individual enters puberty, and the hypothalamus-pituitary-gonadal axis matures. The increasing secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) production starts the cyclic follicle development of antral follicles which culminates in the ovulation of the oocyte. During cyclic recruitment a number of follicles from the growing antral cohort are recruited and rescued from atresia (McGee and Hsueh, 2000). The number of oocytes that ovulate in each cycle is species-specific and in humans a single oocyte ovulates during each menstrual cycle. This is accomplished by the dominant follicle (DF) selection. DF selection from a cohort of antral follicles begins when their size reaches above 2 mm (Gougeon, 1996). In this stage follicles develop rapidly: ~20 days compared to the ~290 days which primordial follicles take to develop into the secondary stage (Gougeon, 1996). Selectable follicles are highly responsive to gonadotropins which induce GC proliferation. However, the intrafollicular concentration of estradiol in selectable follicles is very low compared to androgens. In addition, the FF of selectable follicles contains high levels of transforming growth factor alpha (TGFα), epidermal growth factor (EGF), and insulin-like growth factor-binding proteins (IGFBPs) that inhibit FSH- and insulin-like growth factor II (IGF-II) that induces aromatase, respectively. Aromatase is an enzyme expressed in MGCs and its role is to convert androgens to estrogens and therefore inhibition of aromatase lowers estrogen levels (Simpson et al., 2002). To summarise, when follicles reach the selectable stage, their GCs become responsive to FSH in terms of proliferation but not in terms of estrogen production (Gougeon, 2010).

Each growing follicle possesses a threshold for FSH that represents the minimal FSH quantity to ensure ongoing pre-ovulatory development. It has been suggested that the DF, the one which is most rapidly growing in response to the rise of the FSH, is the follicle with the lowest FSH threshold (McNatty et al., 1983). The other follicles (subordinate) go through atresia and only the DF reaches the pre-ovulatory stage. During DF selection FSH production is decreasing due to the negative feedback of estradiol and inhibin on the pituitary. The DF produces the highest level of estrogens compared to other subordinate follicles and the FF of the DF has a lower androgen/estrogen ratio compared to other follicles (Baerwald et al., 2012). Rising serum estradiol levels suppress pituitary FSH secretion; however, in contrast increased estradiol production enhances pituitary LH production which results in the LH surge and ovulation of the oocyte together with CGCs from the DF which has reached the pre-ovulatory stage (Figure 1) (Edson et al., 2009). The pre-ovulatory follicles express high concentrations of LH receptor in MGCs which enables the cells to respond to the LH surge. This initiates a cascade of events leading to

the oocyte meiotic resumption, cumulus expansion, follicle rupture, and finally terminal differentiation of the remaining MGCs and thecal cells to create the corpus luteum (CL) – a highly differentiated endocrine structure (Edson et al., 2009).

Moreover, IGF stimulates human GC proliferation and differentiation along with the production of androgens by theca cells. Therefore, the IGF system is an important regulator of FSH action. It is proposed that the follicle with the lowest FSH threshold is the first to display an increased activity of IGF-II. This leads to an enhanced growth and differentiation of GCs. In addition, this follicle would be the first to produce estradiol, to differentiate LH receptors on its GCs, to grow despite decreasing levels of FSH, to inhibit the growth of less developed follicles and the first to undergo pre-ovulatory changes (Gougeon, 2010).

1.1.1 Somatic cells of the ovarian follicle

The main role of the somatic cells of the ovarian follicle is to support the development and maturation of the oocyte. Overall, GCs are the most abundant cell type in the ovarian follicle providing the physical support and microenvironment for the developing oocyte (Eppig, 2001; Matzuk et al., 2002). During antral folliculogenesis multiple fluid-filled spaces fuse to form a single antral cavity that separates two distinct GC populations. By the antral follicle stage two subpopulations of GCs (MGCs and CGCs) are formed (Hennet and Combelles, 2012). MGCs line the basal membrane of the follicle and are critical for steroidogenesis and ovulation, CGCs surround the oocyte and promote its growth and developmental competence (Edson et al., 2009). These two cell types appear to be defined by opposing gradients of FSH from the outside of the follicle and oocyte-secreted factors from the inside (Diaz et al., 2007). In addition, FSH becomes essential to prevent GC apoptosis and follicular atresia and to stimulate GC proliferation, estradiol production, and LH receptor expression (Edson et al., 2009).

Direct connections between GCs are essential for successful folliculogenesis. Gap junctions allow the transfer of ions, metabolites, and small molecules between bordering GCs. Two following connexins, the core proteins of the gap junction, are expressed in the mammalian ovary: connexin 43 (CX43; GJA1) and connexin 37 (CX37; GJA4) (Gershon et al., 2008). These two types of connexins have distinctive roles in the follicle, CX43 forms gap junctions between GCs throughout folliculogenesis and CX37 localises to oocyte-GC gap junctions at the beginning of the primary follicle stage (Gershon et al., 2008). CX43-deficient ovaries show a developmental block at the primary follicle stage with impaired GC proliferation and a blockade of oocyte growth (Juneja et al., 1999). Oocytes lacking CX37 demonstrate defects in meiotic competence and do not grow to a normal size, but follicular development proceeds to the later preantral stage (Simon et al., 1997). Moreover, CX37-deficient ovaries contain numerous small CL-like structures, suggesting that communication via gap junctions is a major mechanism regulating CL formation. If the oocyte-GC connection is disrupted, premature luteinization will occur (Edson et al., 2009).

The main role of MGCs is the biosynthesis of two important ovarian steroids: estradiol and progesterone (Hennet and Combelles, 2012). FSH promotes estrogen biosynthesis during follicle development. As the follicle reaches ovulation, the luteinized GCs obtain the capacity to synthesise and secrete progestins, which is regulated by LH (Skinner, 2005).

Estradiol production in the ovary by MGCs is necessary for folliculogenesis. It is demonstrated that aromatase-null mice ovaries contain all stages of follicles; however, these mice are infertile with absent corpora lutea. Deficient CL imply impaired ovulation

(Fisher et al., 1998). Moreover, antral follicles in these mice display abnormal morphology with uneven GC layers and increased apoptosis. In addition, consistent with the role of estradiol in the negative feedback regulation of gonadotropin production, serum FSH and LH are elevated in aromatase-null mice. Moreover, high LH is likely to contribute to increased serum testosterone levels (Britt et al., 2000). The effects of estradiol on folliculogenesis are mediated by two estrogen receptors, ERα and ERβ. ERs are members of the nuclear receptor superfamily of ligand-activated transcription factors (Prossnitz et al., 2008). ERβ is expressed in the GCs of growing follicles and is regulated by gonadotropins and ERα is predominantly expressed in thecal and interstitial cells (Britt and Findlay, 2003). Absence of ERα causes infertility with haemorrhagic follicles, no ovulation, and no CL. This phenotype is similar to that of the aromatase-null mice. Taken together, the ER and aromatase knock-out models suggest that, unlike FSH, estradiol is not essential for antral follicle formation although it is critical for the growth and differentiation of GCs to maintain the survival of the antral follicles and promote ovulation (Edson et al., 2009).

Progesterone is another important steroid hormone in human reproduction. In females progesterone is involved in oocyte ovulation, implantation of the embryo and maintaining the pregnancy (Graham and Clarke, 1997). Additionally, progesterone is involved in the lobular-alveolar development of the mammary gland and in the regulation of milk protein synthesis (Graham and Clarke, 1997).

The second subtype of GCs, CGCs, provide molecular and cytoplasmic components that support the maturation of the oocyte. CGCs form gap junctions named trans-zonal projections (TZPs) through the zona pellucida barrier of the oocyte. Gap junctions allow small molecular weight molecules to be transferred from CGCs to the oocyte (Hennet and Combelles, 2012). CGCs also provide developmental assistance to the oocyte by paracrine signalling. Paracrine signalling between the oocyte and the somatic cells of the follicle is bidirectional and essential for the oocyte development (Hennet and Combelles, 2012). It has been shown that the disruption of paracrine signalling between the mouse oocytes and their surrounding CGCs *in vitro* decreases the oocyte developmental competence (Yeo et al., 2009). Furthermore, oocytes are unable to metabolise glucose and can only generate ATP through oxidative phosphorylation. However, CGCs can consume glucose through aerobic glycolysis and then transfer the product (pyruvate) to the oocyte for oxidative phosphorylation as a substrate (Hennet and Combelles, 2012). In addition, CGCs provide the oocyte with certain amino acids, for example alanine and histidine (Pelland et al., 2009; Su et al., 2009). The transporter system for alanine and histidine is present in CGCs and not in the oocytes. Experiments in mice revealed that oocytes without the surrounding CGCs have lower levels of the above-mentioned amino acids compared to the COC (Pelland et al., 2009). Moreover, it has been shown that mice oocytes lack the ability to synthesise cholesterol (Su et al., 2008, p. 15). Therefore, CGCs assist oocytes in cholesterol biosynthesis. CGCs can synthesise cholesterol or take it up from FF with the help of apolipoprotein B-containing lipoproteins which are recognised by low-density lipoprotein receptors (Gautier et al., 2010).

In vivo pre-ovulatory oocyte development is accompanied by the differential expressions of proteins in the COC, particularly around the time of germinal vesicle breakdown (Dieleman et al., 2002). Expansion of the CGCs on a hyaluronan-rich extracellular matrix surrounding the oocyte is initiated by the LH surge and is required for normal ovulation and fertilisation. Regulation of this process is multifactorial and dependent on the activation of MAPK signalling, as well as oocyte-secreted paracrine

factors. After the LH surge, a number of genes involved in the formation and stabilisation of the extracellular matrix of the COC are upregulated (Edson et al., 2009). Receptors for LH are not expressed in CGCs and therefore the LH surge causes a rapid increase in the epidermal growth factor (EGF)-like family members, Areg, Ereg, and Btc (encoding amphiregulin, epiregulin, and betacellulin, respectively), in MGCs of mice pre-ovulatory follicles. These ligands are released from the cell surface by proteolytic cleavage of the ectodomain and then bind and activate EGF tyrosine kinase receptors (EGFRs). These growth factors stimulate CGC expansion and oocyte maturation *in vitro* in an EGFR-dependent manner. Moreover, within 4 h of human chorionic gonadotropin (hCG) stimulation, transcripts for Areg, Ereg, and Btc have also been detected in the COC, suggesting that an autocrine regulatory loop is established to maintain the EGF-like growth factor expression in CGCs. Thus, MAPK signalling is a critical target and effector of several events triggered by the LH surge in the pre-ovulatory follicles (Edson et al., 2009).

In addition to GCs another important somatic cell type in the follicle are theca cells. These are differentiated stromal cells that surround the follicle. Theca cell recruitment to the developing primordial follicles is essential in many mammals, including humans (Skinner, 2005). Theca cells form two distinct layers by the pre-ovulatory stage: theca interna and theca externa. The main function of the theca interna layer, separated by basement membrane from the outer layer of MGC, is the production and secretion of androgens (Gougeon, 2010). The production of androgens from cholesterol is induced by LH. In response to LH stimulation these thecal cells express key steroidogenic enzymes, including CYP11A1 and CYP17A1. In addition, LH promotes the up-regulation of STAR, which assists in the delivery of cholesterol to the inner mitochondrial membrane where CYP11A1 is located (Edson et al., 2009). Moreover, theca interna cells produce progestins under gonadotropin control. The theca externa, composed of fibroblasts, smooth muscle-like cells, and macrophages, are important during ovulation. (Gougeon, 2010; Hennet and Combelles, 2012). Because estradiol is not essential until the later stages of folliculogenesis and ovulation, thecal cells express the LH receptor from the secondary follicle stage, it is important to suppress the excess of androgen biosynthesis in the pre-antral and small antral follicles. To modulate the stimulatory effect of LH on theca androgen production in smaller follicles, GCs secrete factors, such as activins, that inhibit androgen production. KIT ligand from GCs may also up-regulate several proteins in thecal cells, including TGFα, TGFβ, fibroblast growth factor 7 (FGF7), and hepatocyte growth factor (HGF), that demonstrate autocrine inhibitory effects on androgen production. On the other hand, a certain threshold level of androgens from the theca cells may be necessary for pre-antral follicular growth and its production may be controlled by growth differentiation factor 9 (GDF9) (Edson et al., 2009).

1.2 Hormonal regulation of folliculogenesis

Several hormones control folliculogenesis and influence almost every aspect of somatic cell support to the oocyte. Some hormones like FSH and LH are produced externally. On the other hand, estrogens and androgens are produced within the follicle by MGCs and theca cells, respectively (Edson et al., 2009).

Gonadotropins FSH and LH are glycoproteins secreted from the pituitary gland. The synthesis and release of FSH and LH is controlled by the pulsative secretion of the hypothalamic gonadotropin-releasing hormone (GnRH) (Belchetz et al., 1978). Depending on the change in GnRH pulse frequency, different transcriptional processes

are engaged to stimulate the production and release of FSH or LH. FSH is activated preferentially by low GnRH pulse frequencies, whereas LH is stimulated preferentially by high GnRH pulse frequencies (Stamatiades and Kaiser, 2018). In addition, other factors such as activin, inhibin and steroid hormones contribute to gonadotropin production. When released from the pituitary LH and FSH enter the peripheral circulation and regulate folliculogenesis and ovulation in women and spermatogenesis and steroidogenesis in men (Burger et al., 2004). Estrogen produced by ovaries has a negative feedback on the GnRH, FSH and LH which results in the inhibition of the FSH and LH production. Moreover, the effect of estrogen is greater on the FSH compared to LH (Shaw et al., 2010). Taken together, hormonal control of the ovarian follicle development is regulated by hypothalamic-pituitary-gonadal axis (Stamatiades and Kaiser, 2018).

The regulation of intrafollicular hormone levels is important for the antral follicle microenvironment. For example, growth hormones in the FF enhance the FSH-dependent estradiol production by MGCs. Estradiol, in turn, upregulates androgen secretion by theca cells which is absorbed by MGCs for further estradiol production. Androgen production by theca cells is also stimulated by growth hormones and dependent on LH levels (Hennet and Combelles, 2012). MGCs synthesise inhibins that sensitise theca cells to LH, thus facilitating further estradiol synthesis through the supply of androgens until the LH surge (Knight and Glister, 2001; Kwintkiewicz and Giudice, 2009). A predominantly estrogenic environment is correlated with the oocyte developmental competence. In addition, locally produced hormones within the follicle, such as progesterone, AMH, and estradiol, are involved in signalling cascades and metabolite production (Hennet and Combelles, 2012).

1.2.1 Follicle stimulating hormone receptor

The binding of FSH to its receptor FSHR activates downstream signalling pathways that play a central role in mammalian reproduction like folliculogenesis in females and the maintenance of spermatogenesis in males (Banerjee et al., 2021). FSHR is a G protein-coupled receptor that belongs to the subfamily of glycoprotein hormone receptors (GPHRs) (Moyle et al., 1994). The other member of the GPHR family include, for example, the LH/chorionic gonadotropin receptor. The GPHRs are dimeric glycoproteins composed of a common α-subunit and a hormone-specific β-subunit (Pierce and Parsons, 1981).

There are several signalling pathways which are activated by FSH through FSHR. The canonical G protein/cAMP/protein kinase A (PKA) pathway is the main pathway by which FSH exerts its actions within target cells (Dattatreyamurty et al., 1987). The binding of FSH to its receptor results in the phosphorylation and activation of the transcription factor cAMP-response element-binding protein that regulates the expression of several target genes, including aromatase, inhibin, and LH receptor. However, a number of additional intracellular signalling pathways have been detected, some of which are PKA-independent. For example, the activation of protein kinase B (PKB/AKT) is an alternative signalling pathway of FSH-FSHR binding (Zeleznik et al., 2003). Moreover, FSH-activated phosphatidylinositol-3 kinase (PI3K)/AKT pathway targets forkhead box O1 (FOXO1) protein in GCs and therefore the FSH responsive genes are also regulated by FOXO1 transcription factor (Herndon et al., 2016). FSH regulates IGF-II expression similarly through the AKT-dependent pathway (Baumgarten et al., 2015). Furthermore, IGF-I and FSH signalling pathways interact with each other in GCs (Das and Kumar, 2018) as IGF-I also activates the PI3K pathway in GCs. FSH and IGF-I signalling pathways impact

GC proliferation, differentiation and survival partly by distinctly regulating the levels of FOXO1 (Richards et al., 2002). Moreover, experiments with Igf1- and Fshr-null mice showed that both mice were infertile with follicles arrested at the preantral follicle stage (Baker et al., 1996; Zhou et al., 1997). However, although Igf1 and Fshr mRNAs colocalise in healthy antral follicles, the expression of Igf1 is not altered in the ovaries of Fshr-null females, but Fshr and aromatase are reduced in Igf1 knock-out mice (Zhou et al., 1997). Therefore, IGF-I appears to enhance GC responsiveness to FSH by increasing the levels of FSHR (Edson et al., 2009).

Other than the well-known heterotrimeric G proteins, G protein-coupled receptor kinases (GRKs) and β-arrestins are two other classes of proteins shown to specifically interact with FSHR after FSH binding. GRKs and β-arrestins regulate FSHR stimulation by controlling the selective sensitisation, internalisation, and recycling of FSHR (Piketty et al., 2006). FSH also regulates the initiation of germ cell mitosis/meiosis in embryonic chickens through the involvement of progesterone and upregulation of miR-181a. This miRNA inhibits meiotic initiation by suppressing the Nuclear Receptor subfamily 6 Group A member 1 (NR6A1) transcript (He et al., 2013).

FSHR expression is not limited to the gonads. First of all, FSH has been shown to be involved in bone physiology: Fshr-null mice demonstrated significantly increased bone density (Sun et al., 2006). Moreover, FSHR is expressed in other parts of the female reproductive tract: for example the fallopian tube (Zheng et al., 1996) and endometrium (Ponikwicka-Tyszko et al., 2016) as well as in the developing placenta (Stilley et al., 2014). From pathological conditions FSHR expression has been found from tumour blood vessels (Radu et al., 2010) and from endometriotic lesions (Ponikwicka-Tyszko et al., 2016).

1.2.2 Aromatase

Cytochrome P450 enzymes are crucial for homeostasis. Aromatase belongs to this family of proteins and is coded by the gene CYP19A1. The role of aromatase is to convert androgens to estrogens by demethylation (Simpson et al., 2002). Aromatase is a crucial enzyme for a broad range of physiological functions such as glucose homeostasis, lipid homeostasis, brain function, follicular growth, bone mineralisation and regulation of the ovulatory process, which rely on estrogens (Agarwal et al., 1995). Aromatase expression and activity vary in the different parts of the body. The principal sites of aromatase expression in premenopausal women are GCs and the placental syncytiotrophoblasts. In addition, aromatisation of androgens takes place in adipose and skin tissues in both men and women (Blakemore and Naftolin, 2016).

Aromatase expression in the ovary, placenta, adipose tissue, and skin produces measurable amounts of estrogen in the circulating blood. The substrate for aromatase is synthesised in a different cell type than the one expressing the aromatase. This precursor steroid-producing cell may be distal or adjacent. For example, in the case of adipose tissue and skin, the precursor is distal as the androgens are derived from the adrenal cortex. On the contrary, in ovarian GCs the precursor is primarily derived from the adjacent theca cells that surround the GCs in the ovarian follicle (Blakemore and Naftolin, 2016; Simpson et al., 2002).

Moreover, in the brain tissue several cell types including neurons and astrocytes express aromatase. The estrogen replacement used for postmenopausal women for the prevention of dementia has demonstrated positive effects and therefore suggests that brain aromatase may modify cognitive functions (Nelson and Bulun, 2001). Furthermore,

studies in animals suggest that aromatase activity may also be important in sexual behaviour (Nelson and Bulun, 2001).

In addition, hsa-miR-7973, which was discovered by small RNA deep sequencing of the MGCs and CGCs, is located in the intronic region of the aromatase gene (Velthut-Meikas et al., 2013).

1.3 Cell-to-cell communication in the ovarian follicle

A key aspect of cellular and organismal homeostasis in the higher eukaryotes is intercellular communication, where cells are required to communicate with each other in order to maintain the vital functions of the body (Fritz et al., 2016). An optimal intrafollicular environment is critical for the development, maturation, and quality of the oocyte. By the pre-ovulatory stage, the follicle diameter expands up to 20 mm in size and follicle antrum is filled with FF (Hennet and Combelles, 2012). The content of the FF reflects the local secretory activity of the oocyte, MGC, CGC and theca cells (Hennet and Combelles, 2012). Moreover, this fluid-filled environment enables long-distance cell communication between different cell populations (Figure 2) (Andrade et al., 2019). The composition of FF is distinct from that of serum, suggesting that it may be uniquely shaped to fit the needs of the developing oocytes. FF contains various components: reactive oxygen species and antioxidants, hormones, metabolites, proteins, (lipo)proteins, RNA binding proteins (RBPs), and extracellular vesicles (EVs) containing nucleic acids (Hennet and Combelles, 2012).

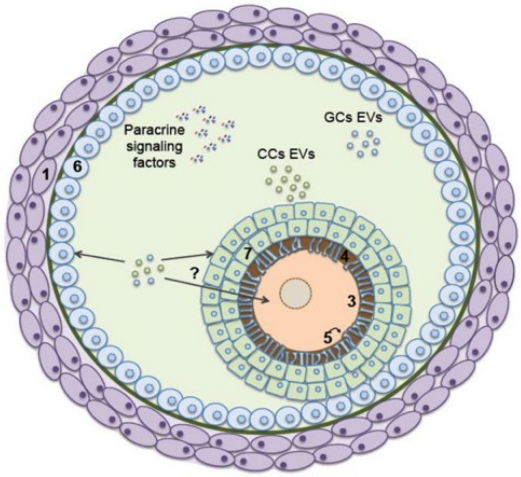

Figure 2. *Cell-to-cell communication in the ovarian follicle. In antral follicles a follicular fluid (FF) barrier is formed between follicular cells. Bilateral communication is maintained by paracrine signalling and extracellular vesicle (EV) transfer. Follicle development and ovulation are regulated by paracrine signalling of oocyte-secreted factors and transactivation of the epidermal growth factor (EGF) receptor by LH. EVs are secreted into the FF and are taken up by different cell types. The direct transfer of EVs to the oocyte remains undetermined. CCs – cumulus cells; EVs – extracellular vesicles; GCs – granulosa cells; 1. Theca cells; 2. Granulosa cells; 3. Oocyte; 4. Zona pellucida; 5. Transzonal projections; 6. Mural granulosa cells and 7. Cumulus cells (adapted from Andrade et al., 2019).*

1.4 Ovarian pathologies

Infertility is a condition characterised by the failure to achieve a pregnancy after 12 months of regular and unprotected sexual intercourse. It is estimated to affect around 15% of reproductive-aged couples worldwide (Jose-Miller et al., 2007; Vander Borght and Wyns, 2018). The causing factors can be various abnormalities in the male or female reproductive system and are distributed relatively equally between the genders (Jose-Miller et al., 2007). The disease-related infertility may affect both genders and/or be specific to one gender. The factors affecting the fertility of both males and females are, for example: infections, systemic diseases, and lifestyle-related factors. Factors affecting only female fertility are for example premature ovarian insufficiency (POI), polycystic ovary syndrome (PCOS), endometriosis, uterine fibroids and endometrial polyps (Vander Borght and Wyns, 2018). The aetiology of infertility remains unknown for around 30% of the cases (Ray et al., 2012).

From the ovarian side the two main infertility-causing syndromes in females are POI and PCOS. POI occurs in around 1% of women and is defined as the ending of menstrual cycles <40 years of age in the presence of an elevated serum FSH (Vander Borght and Wyns, 2018). The main causing factors of POI are, for example genetic, environmental, infectious, and subsequent to cancer therapy or surgery (Vander Borght and Wyns, 2018).

PCOS is a heterogeneous condition and is the most prevalent endocrine disorder in women, affecting up to 20% of females worldwide (Ajmal et al., 2019). PCOS is typically described by the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004) as a syndrome involving two of the three following criteria: infrequent or absent ovulation, polycystic ovaries and hyperandrogenism. Hormonal balance plays an important role in the ovarian function and the regulation of the menstrual cycle that maintains fertility (Ajmal et al., 2019). Imbalance in the estrogen/androgen levels leads to cysts in the ovarian antral follicles. A cyst is a water-filled sac containing the oocyte that in normal conditions would have ovulated for fertilisation. In addition, as ovulation is blocked, it results in the disruption of the menstrual cycle. If hormonal imbalance continues multiple cysts are formed and then the condition is characterised as PCOS (Vander Borght and Wyns, 2018). In normal conditions the ovarian theca cells provide support to the growing follicle; however, theca cells in PCOS patients are hyper-responsive to the stimulatory effects of insulin. As a result, theca cells proliferate causing ovarian hyperthecosis. Furthermore, insulin resistance amplifies the androgenic potential in the theca cells which further exaggerates PCOS (Patel, 2018). Moreover, PCOS patients have increased LH levels and elevated LH:FSH ratio (Taylor et al., 1997). Theca cells are highly sensitive to gonadotropin stimulation and therefore elevated LH levels advance androgenism in PCOS (Patel, 2018). Taken together, elevated plasma LH and insulin levels stimulate theca cells to produce androgens which leads to hyperandrogenism (Broekmans and Fauser, 2006).

The exact cause of PCOS is unknown. However, there are multiple potential causing factors. Firstly, genetic background can be involved in PCOS development. First-degree relatives of PCOS women are more likely to develop PCOS compared to women from non-PCOS families (Kahsar-Miller et al., 2001). Moreover, variations like single nucleotide polymorphisms (SNPs) in *TLR2*, *ICAM1* and *MTHFR* genes have been observed in PCOS patients (Patel, 2018). Moreover, obesity has been associated with impaired metabolic and ovulatory dysfunction linked to PCOS. Weight loss by obese women has been found to restore ovulation and reduce hyperandrogenism in some cases (Crosignani et al.,

2003). In addition, insulin resistance (reduced cellular ability to respond to normal or elevated levels of insulin), plays a role in the development of metabolic disturbances in PCOS (Shaikh et al., 2014).

1.5 Controlled ovarian stimulation and *in vitro* fertilisation

In vitro fertilisation (IVF) is defined as the fertilisation of an oocyte by a spermatozoon outside the body. The first baby born from *in vitro* fertilisation (IVF) was in England in 1978 following a natural cycle without ovarian stimulation (Steptoe and Edwards, 1978). Since then, major advances have been made to improve the safety and success rates of the procedure. IVF has become the main treatment of infertility when conventional therapy fails or is unlikely to be successful (Niederberger et al., 2018). Modern IVF is combined with controlled ovarian stimulation. This allows for the possibility to stimulate multiple follicles which increases the number of oocytes available for fertilisation and the production of good-quality embryos (Arslan et al., 2005). The optimal number of oocytes after controlled stimulation is 8–14. A higher number of oocytes significantly increases the risk of ovarian hyperstimulation syndrome (Nyboe Andersen et al., 2017). Several parameters can be used to predict a woman's response to stimulation: AMH and FSH levels or the antral follicle count (AFC) (La Marca and Sunkara, 2014).

The IVF cycle begins with the controlled ovarian stimulation. Ovarian stimulation has three basic steps. Firstly, injections of the exogenous gonadotropins to stimulate multi-follicular development. Secondly, cotreatment with either GnRH agonist or antagonists to inhibit pituitary function. This prevents a premature endogenous LH surge and improves IVF success rates. Thirdly, triggering of final oocyte maturation 36–38 hours before oocyte retrieval. In a natural cycle the LH surge resumes meiosis to metaphase II, causes rupture of the dominant follicle and formation of the CL. In IVF this is usually induced by an injection of hCG as it has a longer metabolic half-life than LH (Gallos et al., 2017; Howie and Kay, 2018; Rao, 1979).

Women with PCOS are at an increased risk of excessive ovarian response and ovarian hyperstimulation. Metformin pre-treatment significantly reduces the risk of ovarian hyperstimulation in women with PCOS (Tso et al, 2014). Pre-treatment increases pregnancy rate; however, it has no effect on live birth rate when compared to no treatment group (Tso et al., 2014).

1.6 miRNA genes and biogenesis

miRNAs are a class of small non-coding RNA molecules of on average ~22 nt length with an important role in post-transcriptional gene expression regulation in both animals and plants (Bartel, 2004). miRNAs were first discovered in the studies of the development of *C. elegans* larvae where it was revealed that *lin-4* gene product does not encode for a protein, but instead small RNAs with complementary sequences in the 3' untranslated region (UTR) sequences of the *lin-14* gene (Lee et al., 1993). Moreover, it was shown that *lin*-4 reduces LIN-14 protein levels (Olsen and Ambros, 1999). Years later miRNAs have been described as an abundant class of regulatory RNAs with known functions including the control of cell proliferation and cell death (Bartel, 2004).

miRNAs can be located in the genomes as separate genes with dedicated promotor sequences or in the intronic regions of protein coding genes. Intronic miRNAs with the same orientation as the mRNA genes are likely to be processed from the mRNA introns of the host gene and do not possess separate regulatory promoters. The same orientational

arrangement provides a possible mechanism of the co-expression of a miRNA and an mRNA (Bartel, 2004). Moreover, miRNA genes can be located in the genome as gene clusters. The arrangement and expression of clustered miRNAs indicate the transcription of a multi-cistronic primary transcript (Lagos-Quintana et al., 2001; Lau et al., 2001). The miRNAs within a genomic cluster are often conserved in evolution, however, not all conserved miRNAs are clustered (Bartel, 2004). One example of clustered miRNAs is the mir-15a- mir-16 cluster, which locates on human chromosome 13 (Lagos-Quintana et al., 2001). The transcriptions of miRNA genes that are located in intergenic regions are probably regulated by their own promoters (Lee et al., 2002).

The biogenesis of miRNAs goes through chronological processes to generate mature miRNA molecules. Primary miRNAs (pri-miRNAs) are typically transcribed by polymerase II and are capped and polyadenylated (Kim, 2005). The length of the pri-miRNA can be longer than 1 kb and a typical animal pri-miRNA contains an imperfectly paired stem of ~33 bp, with a terminal loop and flanking segments (Figure 3) (Bartel, 2004). The first processing step takes place in the nucleus where the stem-loop sequence is cleaved from the remainder of the transcript creating a precursor miRNA (pre-miRNA) sequence. In animals the first cleavage is performed by RNase III endonuclease Drosha. Drosha cleaves both strands of the stem at the sites near the base of the primary stem loop, leaving a 5' phosphate and ~2 nt 3' overhang to the pre-miRNA sequence. In addition to Drosha, for precise and efficient pri-miRNA processing the help of a cofactor DiGeorge syndrome critical region 8 (DGCR8) is necessary. DGCR8 contains two dsRNA-binding domain and stably associates with the ribonuclease (Bartel, 2018). However, the above-mentioned pri-miRNA cleavage is not the single pathway for generating pre-miRNAs in animals. An alternative pathway uses the splicing of pre-mRNA transcripts to excise introns that possess the structural features of pre-miRNAs. These RNA molecules are named mirtrons and continue the miRNA processing pathway without using the Drosha-DGCR8 (Figure 3) (Okamura et al., 2007). Mirtrons are found in the animal kingdom; however, this pathway is not frequent (Carthew and Sontheimer, 2009). Next, in animals, pre-miRNA sequences are transported from the nucleus to the cytoplasm by an active transport pathway using the export receptor Exportin-5. In the cytoplasm the pre-miRNA sequence is further processed by the Dicer enzyme (Bartel, 2004). Similar to Drosha, Dicer is an RNase III endonuclease which was first discovered as a generator of the small interfering RNA (siRNA) and has later been shown to be a necessary enzyme for miRNA maturation (Bernstein et al., 2001). Dicer recognises the double-stranded part of the pre-miRNA and cuts both strands of the duplex at about two helical turns away from the base of the stem loop. This cleavage by Dicer cuts off the terminal base pairs and the loop of the pre-miRNA. Dicer leaves 5´ phosphate and ~2 nt 3' overhang to the cleaved sequence. As a result, imperfect duplex that comprises the miRNA guide strand and a similar-sized fragment derived from the opposing arm of the pre-miRNA is produced (Bartel, 2004; Ha and Kim, 2014). The fragment of the opposing arm, called the miRNA passenger strand, is found less frequently from miRNA sequencing experiments. This indicates that miRNA duplex is less stable and more short-lived compared to the guide strand (Bartel, 2004).

The guide strand from the miRNA duplex is incorporated into the RNA-induced silencing complex (RISC). This involves the unwinding of the miRNA duplex and the association of the guide strand with the argonaute (AGO) effector protein. Mammals have four AGO proteins (AGO1-4); however, only AGO2 has catalytic activity (Meister, 2013). Incorporated guide strand directs target recognition by Watson-Crick base pairing

to a target mRNA molecule, while the passenger strand is discarded (Figure 3) (Carthew and Sontheimer, 2009). Leading strand selection is based on the relative thermodynamic stability of the ends of the duplex. The 5' terminus of the guide strand is the less stable base-pair end of the duplex (Kim, 2005). However, there are always some exceptions and the passenger strand can also be detected in some AGO complexes (Okamura et al., 2008).

miRNAs incorporated into RISC can downregulate gene expression by either of the two post-transcriptional mechanisms: mRNA cleavage or translational repression. The mRNA cleavage pathway is initiated if the mRNA has sufficient complementarity (perfect or near perfect) to the miRNA. In such a case an miRNA-guided mRNA cleavage is generated between the nucleotides pairing to residues 10 and 11 of the miRNA (Elbashir et al., 2001; Hutvágner and Zamore, 2002; Kasschau et al., 2003; Llave et al., 2002). When cleavage of the mRNA is completed, the miRNA still remains intact and can guide the recognition and destruction of additional mRNA molecules (Bartel, 2004).

Translational repression occurs if the mRNA does not possess sufficient complementarity to be cleaved but demonstrates a suitable miRNA complementary site. Compared to plants, animal miRNAs have a lower degree of complementarity to their corresponding target mRNAs. Perfect or near-perfect complementarity is assumed to be necessary for RISC-mediated cleavage but not for translational repression. This suggests that translational repression is more dominant in animals compared to plants (Bartel, 2004). Moreover, multiple miRNA complementarity sites are identified in target mRNAs, therefore, more than one RISC complex can associate with the target mRNA. The cooperative action of multiple RISCs tends to provide a more efficient translational inhibition (Doench and Sharp, 2004).

miRNAs are known to function in the RISC complex as post-transcriptional repressors. However, additional functions are proposed for miRNAs, for example targeting of the DNA for transcriptional silencing. AGO proteins and siRNAs have been associated with DNA methylation and silencing in plants (Hamilton et al., 2002; Mette et al., 2000; Zilberman et al., 2003) and heterochromatin formation in fungi (Hall et al., 2002; Reinhart and Bartel, 2002; Volpe et al., 2002). These examples suggest the existence of a nuclear RISC-like complex and their association with miRNAs (Bartel, 2004).

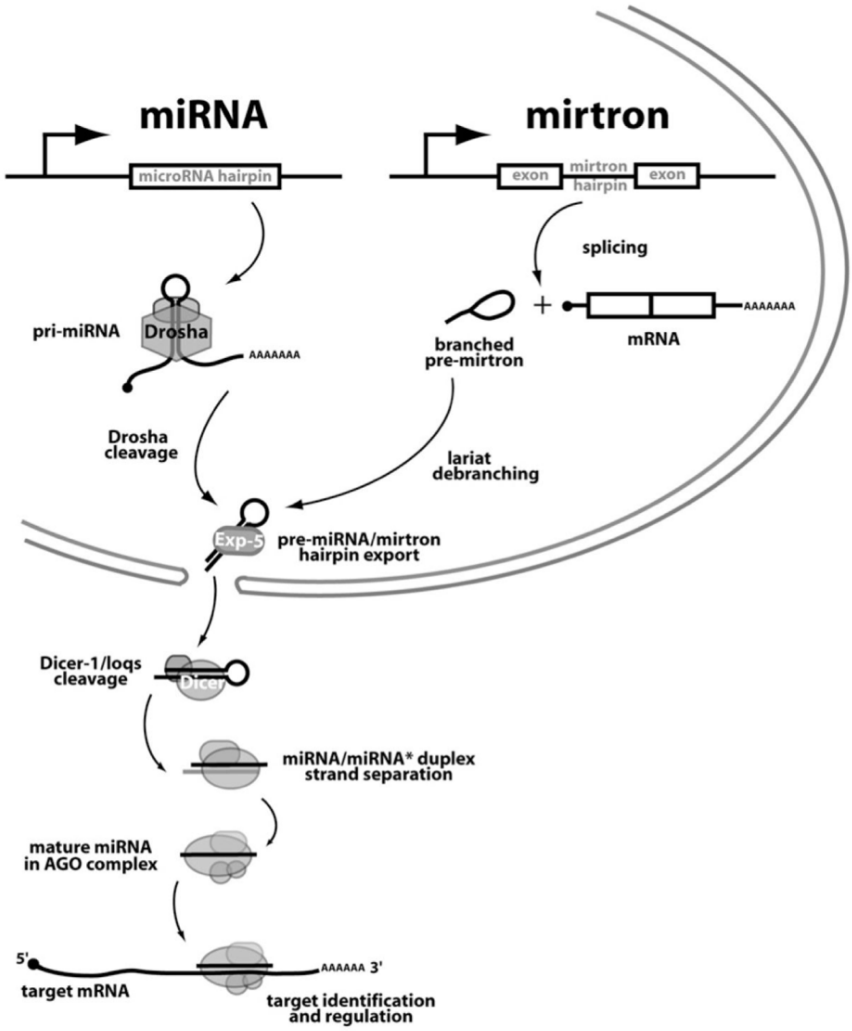

Figure 3. *According to the canonical miRNA pathway the Drosha/DGCR8 complex recognises and cleaves the pri-miRNA transcripts to produce pre-miRNA hairpins (left). Non-canonical pathway in which introns with hairpin potential are spliced and debranched to produce mirtron hairpins (right). Both pre-miRNA and mirtron hairpins are exported from the nucleus by Exportin-5 and cleaved by Dicer-1 to generate ~22-nt-long miRNA duplexes. The guide miRNA is incorporated into the AGO protein and guides the regulation of the target transcripts (taken from Okamura et al., 2007 by permission of Cell Press).*

1.6.1 miRNA targeting

The highest number of miRNA binding sites on animal mRNAs is located in the 3'UTR and they are often present in multiple copies. Most animal miRNAs bind with mismatches and bulges; however, an important role in precise targeting relies on the miRNA nucleotides 2–7 from the 5´ end, which represents the seed region (Lewis et al., 2005). Residues in the seed region possess perfect complementarity to the 3'UTR elements of the target mRNA. Other regions in the miRNA sequence supplement the target

specificity (Grimson et al., 2007). In contrast to animals most plant miRNAs bind with near-perfect complementarity to sites within the coding sequence of their targets. Moreover, residues 2–8 (7 nt seed) of the miRNA are the most conserved among homologous metazoan miRNAs (Bartel, 2018; Kim, 2005). To amplify the 6 nt seed match (nucleotides 2–7) most of the regulatory sites have an additional complementarity to miRNA nucleotide in position 8 or an adenosine across the miRNA nucleotide in position 1, or both, to bind 7 or 8 nt sites in total, respectively. These 7–8 nt sites mediate the majority of the transcriptional repression and are the sites identified by the most effective target-prediction tools (Agarwal et al., 2015; Bartel, 2009). Nevertheless, the 6 nt sites that either match only the seed or are offset by one nucleotide in either the 5′ or 3' direction can in some cases mediate detectable repression. Complementarity of the 3′ region of the miRNA, particularly nucleotides 13–16, can supplement the repression in addition to the seed region complementarity. However, the 3′ complementarity does not have a significant influence on the site affinity and efficacy. Only around 5% of the seed-matched regulatory sites appear to include this additional pairing (Bartel, 2018; Friedman et al., 2009). Still, these sites falling outside the seed region offer a potential advantage by a mechanism that avoids the regulatory redundancy often observed for co-expressed miRNAs from the same seed family (Bartel, 2018).

The seed sequences were initially thought to be fully accessible for target recognition (Bartel, 2004). However, AGO binding leaves only miRNA nucleotides 2–5 suitably preorganised and accessible for the target search (Elkayam et al., 2012; Nakanishi et al., 2012; Schirle and MacRae, 2012). Moreover, single-molecule analyses demonstrate that this sub-seed segment is indeed the most critical sequence for target association (Chandradoss et al., 2015; Salomon et al., 2015).

The short length of the miRNA provides an opportunity to bind to multiple sites in the 3′UTR regions of the target mRNAs (Friedman et al., 2009). Moreover, some 3′UTRs possess multiple target sites for the same miRNA. Furthermore, the average number of conserved targets per miRNA family is above 400, and 3′UTRs of around 60% of the human mRNAs are evolutionarily conserved targets of miRNAs (Friedman et al., 2009). If conserved sites in other mRNA regions (e.g. ORFs) (Bartel, 2018) and sites observed for evolutionarily recent miRNA families are added, the estimation of the proportion of targets exceeds 60% of human mRNAs (Friedman et al., 2009). This means that all developmental or physiological processes are likely to be influenced by miRNA regulation (Bartel, 2018).

The effective endogenous target abundance is defined by the number of sites that must be added to achieve the half-maximal de-repression of targets, meaning competing sites reach an effective concentration matching that of the endogenous sites (Denzler et al., 2016). In the cells, the effective target abundance is typically higher compared to the miRNA abundance, and this occurs even with highly expressed miRNAs. The high effective target abundances have important effects. For example, when high numbers of sites are competing for miRNA binding then few if any sites are overloaded. As a consequence, even the most repressed targets are still sensitive to increased miRNA levels (Denzler et al., 2016). However, this declines the competing endogenous miRNA hypothesis which proposes that the changes in the expression of individual cellular targets influence the repression of other cellular targets by modulating the amount of free miRNA. Moreover, the high effective target abundances may explain why low

expression miRNAs have small effects and as well as challenges the idea that the miRNAs delivered by exosomes reach significant levels in recipient cells (Bartel, 2018).

Several algorithms have been developed to bioinformatically predict the mRNA targets for specific miRNAs. The first step in target prediction is to search for 7–8 nt long seed region complementary sites in the 3´UTRs of potential target mRNAs. However, not all mRNAs with a 7–8 nt seed region complementary site respond to the miRNA and therefore additional steps are necessary for more accurate prediction. For example, the selection of 3´UTRs that are conserved in evolution is often considered (Bartel, 2009). On the other hand, some of the most responsive targets are not among those with the most confidently detected site conservation. Moreover, the miRNAs that emerged more recently have too few targets under selective conservation (Friedman et al., 2009). Therefore, models have been developed that use the features of the sites and their contexts within 3´UTRs to predict the targeting efficacy: the type of site (6 nt, 7 nt and 8 nt site), the number of sites, the potential for 3´ supplementary complementarity, and site context (Grimson et al., 2007). Regarding the site context, the more effective sites tend to be near the edges of UTRs and within more structurally accessible regions (Bartel, 2009). Also, features of miRNA, such as predicted seed-pairing stability and the number of its transcriptome sites competing for binding, as well as features of the mRNAs such as UTR length, ORF length, presence of alternative 3´UTR isoforms, and the presence of additional marginal sites in 3´UTRs and ORFs are all associated with efficacy (Agarwal et al., 2015). With the additional features, bioinformatic prediction programs are becoming increasingly accurate at predicting the mRNAs most responsive to specific miRNAs (Agarwal et al., 2015), although there is still room for improvement (Bartel, 2009).

1.6.2 miRNA families

miRNA genes are widely expressed in animals, plants, protists and viruses (Griffiths-Jones et al., 2008). Known miRNA sequences are concentrated into the miRBase database (http://www.mirbase.org/) (Kozomara et al., 2019) which categorises the detected sequences into families according to the sequence similarity of the mature miRNA and/or the structure of their pre-miRNAs (Kamanu et al., 2013). miRNA families are of interest because they suggest a common sequence or structure configuration in sets of genes and consequently a common function (Kaczkowski et al., 2009). Moreover, miRNA genes in a family can exhibit a full sequence conservation of the mature miRNA or a partial conservation of only the seed sequences at positions 2–8. Furthermore, it has been observed that miRNA genes in the same miRNA family are non-randomly co-localised and well organised around genes involved in infectious, immune system, sensory system and neurodegenerative diseases, and the development of cancer (Kamanu et al., 2013).

In many species, there are multiple miRNA loci with related sequences that mainly evolved from gene duplication. For instance, let-7 family contains 14 paralogous loci (encoding miRNA sisters) in the human genome. Furthermore, 34 miRNA families are phylogenetically conserved in metazoan and 196 miRNA families are conserved among mammals (Chiang et al., 2010; Wheeler et al., 2009). miRNAs of one family may have a common origin; however, they diverge in their seed region. For example, miR-141 and miR-200c are members of a conserved miR-200 superfamily and differ by one nucleotide in their seed regions. Experiments with miRNA knock-outs displayed that these two miRNAs only possess a small amount of common targets (Kim et al., 2013). This result

demonstrates the importance of the miRNA seed sequence in miRNA function (Ha and Kim, 2014).

One large miRNA family, mir-548, originates from the mariner-derived element 1 (Made1) transposable elements (Piriyapongsa and Jordan, 2007). Made1 is one of the primate-specific short miniature inverted-repeat transposable elements (MITEs) that form almost perfect palindromes. Made1 preferred genomic loci for insertion are transcriptionally active regions, therefore Made1 elements are mostly located either close to or within genes (Fattash et al., 2013). The secondary structure of Made1 RNA contains highly stable hairpin loops that are recognised by the miRNA processing machinery. Genes of the hsa-mir-548 family members are located on almost all of the human chromosomes and the highest numbers of their genes are found on chromosomes 6, 8, and X (Liang et al., 2012). Moreover, some family members are present as multicopy pre-miRNAs in the genome. For example, hsa-miR-548f and hsa-miR-548h have 5 multicopy pre-miRNA genes that are located on different chromosomes. Compared to some other large miRNA families, for example hsa-let-7, the hsa-mir-548 family is poorly conserved. In addition, hsa-mir-548 family members have undergone several seed-shifting events, leading to changes in their seed sequences and hence the increased variability of their mRNA targets (Liang et al., 2012).

One hsa-mir-548 family member, hsa-miR-548ba, was discovered during high-throughput small RNA sequencing of human MGCs and CGCs. Similar to many other hsa-mir-548 family members, the location of hsa-miR-548ba in the genome is intronic region of the protein coding gene, more specifically the FSHR gene (Velthut-Meikas et al., 2013).

1.6.3 miRNAs in reproduction

miRNAs play well-established roles in gene expression regulation in normal and pathological conditions (Bartel, 2018). Moreover, different tissues have variable miRNA expression patterns of importance in tissue characteristics, differentiation and function (Ludwig et al., 2016).

The functions of human reproductive organs are also under the regulatory control of miRNAs. Several studies have shown that miRNA expression regulates different processes in the human ovary and the expression of miRNAs diverges between cell types and the stages of the menstrual cycle. Independent of the animal model (mouse, bovine, sheep, porcine) let-7 family, miR-21, miR-99a, miR-125b, miR-126, miR-143, miR-145, and miR-199b have been found to be the most abundant miRNAs in the ovary, suggesting their important roles in ovarian functions (Hossain et al., 2012). Moreover, the gender of the studied animal is an important factor, meaning that the ovary and the testis express different miRNA profiles (Mishima et al., 2008).

The Dicer knock-out mice have been a useful model for miRNA function analysis in the ovary. When Dicer expression was eliminated from GCs, reduction in the ovary weight, lower rates of ovulation, abnormal response to gonadotropins, accelerated follicle recruitment leading to the increased exhaustion of the primordial follicle pool, and higher follicle degeneration rates were observed (Hong et al., 2008; Nagaraja et al., 2008). Moreover, the CL functions were impaired (Otsuka et al., 2008). In addition, miRNA expression studies performed separately in the oocytes or CGCs revealed the presence of distinct sets of miRNAs which are reliant on the bilateral communication of the two cell types (Abd El Naby et al., 2013; Miles et al., 2012). Furthermore, several studies have identified differentially expressed miRNAs between different

oocyte maturation stages. The changes in the expression of miRNAs were also observed in the CGCs matured with or without the oocyte cytoplasm. Dependency of the two cell types on each other for miRNA expression may be involved in the regulation of genes associated with the nuclear maturation of the oocyte. This implies that miRNA transcription or the transcriptional regulation by miRNAs in the oocyte or CGCs is controlled by their secreted factors acting in paracrine manner (Hossain 2012).

GCs are the most commonly studied cell type of the ovarian follicle. Several miRNAs have been shown to be involved in regulating the proliferation and apoptosis of cultured GCs (Hossain et al., 2012). miR-224 regulates mouse GC proliferation by regulating the TGF-β/Smad pathway through inhibiting the TGF-β superfamily type I receptors (Yao et al., 2010). Additionally, it has been shown that gonadotropins LH and hCG regulate miRNA expression. miR-21 expression was induced by LH in mouse MGCs and therefore miR-21 is suggested to play a role in MGC luteinization (Fiedler et al., 2008). However, miRNAs themselves can inhibit or activate estrogen and progesterone production in GC (Hossain et al., 2012).

Altered miRNA expression has been linked to infertility, including PCOS. For example, the GCs isolated from PCOS patients express lower levels of hsa-miR-145 (targets insulin receptor substrate 1 (IRS1)), hsa-miR-126-5p, hsa-miR-29a-5p (Mao et al., 2018) and hsa-miR-92b (Xu et al., 2015) (associated with apoptosis regulation) compared to the fertile control group. Therefore, miRNAs altered in PCOS have been suggested to regulate cell apoptosis (Chen et al., 2019). Moreover, two miRNAs, hsa-miR-320 and hsa-miR-509-3p, are differentially expressed in PCOS patients compared to fertile controls. These miRNAs target RUNX2 and MAP3K8, respectively, both of those genes are involved in the regulation of estrogen production (Huang et al., 2016; Zhang et al., 2017). The expression of miRNAs described above is altered in polycystic ovaries and these are therefore associated with the subsequent development of PCOS. A high level of insulin is a common feature of PCOS, and this may also induce the alteration of hsa-miR-145 and hsa-miR-93 expression (Chen et al., 2019).

Ovarian theca cells are the main sites of androgen production in the ovaries (Gougeon, 2010). Theca cells of PCOS women express lower levels of hsa-miR-92a and hsa-miR-92b. GATA6 is one of the targets of hsa-miR-92a and IRS-2 is regulated by both hsa-miR-92a and hsa-miR-92b (Lin et al., 2015). GATA6 and IRS2 are expressed at significantly higher levels in PCOS theca cells compared to fertile women, which is in accordance with the regulatory miRNA expression patterns (Ho et al., 2005; Yen et al., 2004). GATA6 is an important androgen production-related protein that stimulates the activity of human CYP17A1 promoter (Ho et al., 2005). IRS2 is an insulin receptor substrate that is involved in the increased PI3K activity and promotes androgen production by regulating the activity of thecal CYP17A1 upon insulin binding to its receptor (Yen et al., 2004). Taken together, lower levels of hsa-miR-92a and hsa-miR-92b in theca cells influence the dysregulation of androgen biosynthesis in PCOS women (Chen et al., 2019).

1.7 miRNAs in the extracellular space

Some of the important mediators of cell-to-cell communication are cell-free RBP and nanoparticles, including EVs that contain a variety of biomolecules: proteins, miRNAs, mRNAs, DNA, and lipids (Fritz et al., 2016). The release of EV and RBP has been extensively studied and attributed to all cell types in the human body. Moreover, cell-free RNAs in RBP and EVs have been detected in all investigated body fluids, including FF

(Sang et al., 2013; Santonocito et al., 2014). EVs are lipid bilayer-coated nanoparticles of varying size range. EVs are classified into three major subtypes based on their size, shape, membrane proteins and origin: exosomes (30–100 nm), microvesicles (100–1000 nm), and apoptotic bodies (500 nm–2 μm) (Fritz et al., 2016).

While small RNAs in EVs have attracted more attention, more than 90% of the circulating miRNAs are present outside of EVs associated with AGO2 (Arroyo et al., 2011; Turchinovich et al., 2011), nucleophosmin 1 (Wang et al., 2010), or high-density lipoproteins (Vickers et al., 2011; Wagner et al., 2013), as well as other proteins (Fritz et al., 2016). The secretion of RNA molecules via EVs is at least partly controlled by the releasing cells as the EV content varies and reflects the cell of origin (Sork et al., 2018). However, AGO2-miRNA complexes may also be released non-specifically into the extracellular space after cell death by apoptosis (Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012). In addition, some specific miRNAs have a higher abundance of EVs compared to the parent cells. Therefore, it is proposed that specific mechanisms are available in cells for precise miRNA loading into EVs (Fritz et al., 2016).

The RNA content of both EVs and RBPs can be taken up by recipient cells from the same or another cell population (Valadi et al., 2007). Examples of such cell-to-cell communication have been described in cancer studies and immune regulation (Yang et al., 2011). In reproductive studies, EVs isolated from FF were fluorescently labelled and demonstrated to be taken up by ovarian GCs in an equine *in vitro* model. This suggests that the exchange of RNA is potentially an important means of communication in the normal ovarian physiology (da Silveira et al., 2012). Furthermore, the extracellular RNAs have the potential to be used as a diagnostic marker for disease detection as several cell populations have been demonstrated to change the repertoire of their released cell-free RNAs upon external stimulus or disease (Bellingham et al., 2012; Driedonks et al., 2018).

1.7.1 miRNAs in the ovarian follicular fluid

The sources of EVs in the ovarian follicle are all follicular cells and hence they may be an important means of intercellular communication in the ovary (Andrade et al., 2019). It has been shown in the bovine model that EVs promote changes in cellular gene expression and support COC expansion *in vitro*. Moreover, the effect on the cell proliferation varied between EVs obtained from the different sizes of ovarian follicles indicating that the content of the EVs may be different and/or the size of the follicle influences the EV release and/or uptake (Hung et al., 2017). Additionally, FF contains miRNAs involved in the regulation of steroidogenesis and the levels of those miRNAs are different in PCOS patients compared to the control group admitted for IVF due to male factor infertility (Sang et al., 2013). Moreover, miRNAs present in FF are associated with IVF outcome and embryo quality. For example, hsa-miR-92a and hsa-miR-130b were over-expressed in the FF containing oocytes that failed to fertilise compared to normally fertilised oocytes. In addition, hsa-miR-888 was more abundant and hsa-miR-214 and hsa-miR-454 were less abundant in EVs obtained from FF samples that resulted in impaired day 3 embryo quality compared to good-quality embryos (Martinez et al., 2018).

miRNA expression levels are changed in the FF of PCOS patients compared to the control group. Differentially expressed miRNAs were correlated to serum AMH levels and free androgen index in PCOS patients (Butler et al., 2019). Hsa-miR-182 expression level was decreased in the GCs of PCOS patients compared to the control group, but was

up-regulated in the FF of PCOS patients (Naji et al., 2018). Moreover, the expression of hsa-miR-93 and hsa-miR-21 was downregulated in the FF of hyperandrogenic PCOS patients, which was not the case in non-androgenic PCOS women, meaning that the miRNA expression profile in the follicle may differ between PCOS patients with different underlying factors (Naji et al., 2017).

2 Aims of the study

The main aim of the thesis was to describe the miRNA expression profile and investigate their importance in the granulosa cells and in the follicular fluid of the pre-ovulatory ovarian follicles. The more specific aims of the thesis were the following:

- Determine the roles of hsa-miR-548ba and hsa-miR-7973 in the ovarian granulosa cells by validating their respective mRNA targets and dependency of miRNA expression on that of their host genes *FSHR* and *CYP19A1*, respectively.
- Determine the potentially overlapping functions of the hsa-mir-548 family members in the female reproductive tract that would help to interpret the reproductive tissue-specific importance of individual miRNAs.
- Compare the genome-wide cellular and extracellular miRNA profiles of pre-ovulatory follicles in PCOS women and in healthy/fertile women.

3 Materials and Methods

The following methods, described in more detail in the respective publications, were used in this study:

- Culture of the ovarian tumour cell line KGN – Publication I
- Isolation of primary granulosa cells – Publication I
- Culture of primary cumulus cells – Publication I
- Transfection of the KGN cell line with miRNA mimics – Publication I
- Cytotoxicity analysis of the KGN cell line – Publication I
- mRNA extraction, cDNA synthesis and RT-qPCR analysis – Publication I
- miRNA extraction, cDNA synthesis and RT-qPCR analysis – Publication I and III
- Gene expression analysis using Affymetrix GeneChip Human Gene 2.0 ST Array – Publication I
- Gene expression analysis using high-throughput small RNA-seq – Publication II and III
- Validation of miRNA binding to its mRNA target by the luciferase reporter assay – Publication I
- Targeted LC/MS/MS analysis – Publication I
- Collection of FF samples – Publication III
- Isolation of EV from the FF – Publication III
- Nanoparticle tracking analysis, Western blot, and transmission electron microscopy analysis of extracellular vesicles – Publication III
- Bioinformatic analysis of small RNA-seq data – Publication II and III
- Bioinformatic prediction of miRNA targets and gene ontology analysis of targeted pathways – Publication I, II and III
- Statistical data analysis – Publication I, II and III

4 Results and Discussion

4.1 The role of miRNAs hsa-miR-548ba and hsa-miR-7973 in the gene expression regulation of human granulosa cells

miRNAs hsa-miR-548ba and hsa-miR-7973 were discovered in our research group by deep sequencing of MGCs and CGCs isolated from pre-ovulatory follicles of women undergoing controlled ovarian stimulation and IVF (Velthut-Meikas et al., 2013). The genomic locations of hsa-miR-548ba and hsa-miR-7973 genes lie within the intronic regions of FSHR and CYP19A1 genes, respectively. Both of those genes are essential for successful follicle development (Ulloa-Aguirre et al., 2007). As several miRNAs have been shown to be important regulators in follicle development (Maalouf et al., 2016) the locations of those particular miRNAs raised a hypothesis that these miRNAs play a similarly important role in the ovarian follicle growth. As newly discovered miRNAs their targets and hence their functions were not known. Therefore, the first study focuses on the miRNA target detection and validation.

To determine the potential targets of hsa-miR-548ba and hsa-miR-7973, the miRNA sequences were overexpressed in the GC tumour cell-line KGN. Total RNA extracted from the transfected KGN cells was used as an input for the Affymetrix GeneChip Human 2.0 ST Array. As a result, a list of differentially expressed (DE) genes between hsa-miR-548ba or hsa-miR-7973 transfected cells compared to control transfection was detected (1,474 and 1,552 DE genes, adjusted p-value <0.01, respectively, Supplementary Table IIA, C, Publication I). From those genes 1,015 were regulated by both miRNAs, 459 and 537 genes only by hsa-miR-548ba or hsa-miR-7973, respectively. Despite the overlapping part of the DE genes, cluster analysis displayed that cells transfected with different miRNAs form separate clusters (Figure 2, Publication I).

However, the overexpression of miRNAs in a cell can lead to secondary target genes (Tu et al., 2009). Secondary effects of miRNA targeting may appear through miRNA regulation of transcription factors which can lead to additional transcriptional changes (Cloonan, 2015). To distinguish between the primary and secondary targets bioinformatical target prediction was performed. To reduce the number of false positive results among the predicted targets, a combination of different prediction programs were used: DIANA microT v 3.0 (Maragkakis et al., 2009), microT CDS v 5.0 (Reczko et al., 2012), TargetScan 7.1 (Agarwal et al., 2015) and miRDB (Chen and Wang, 2020). A gene was considered a potential target if it was predicted by at least two programs out of four and its gene expression fold change according to microarray analysis was $\geq \log_2(|0.3|)$.

By combing the DE genes from the Affymetrix array and bioinformatically predicted targets a list of 76 and 58 potential targets was formed for hsa-miR-548ba and hsa-miR-7973, respectively. One of them, TGFBR2, is a common target for both miRNAs. For further validation the potential target list was filtered according to published data linking gene function to the ovarian function and/or follicle development (Table 1, Publication I). Genes that demonstrated a statistically significant gene expression change by quantitative reverse transcription PCR (RT-qPCR) were further validated by luciferase reporter array. This method is based on the knowledge that miRNAs function as translational repressors of their targets and allows us to validate the binding of the miRNA to the 3'UTR sequence of its target mRNA (Oh et al., 2013). The full length of the target 3'UTR sequences was used in the assay as using only a fragment of 3'UTR sequence can lead to false results due to the different secondary structure

compared to the full length (Grimson et al., 2007). This discrepancy was also confirmed when the shorter PTEN 3'UTR was used compared to the longer 3'UTR version (Figure 4A, Publication I). Despite the fact that the predicted target sites were present in the shorter version of the 3'UTR the direct binding of the hsa-miR-548ba occurred only to the longer version (Figure 4A, Publication I). Accessibility of the miRNA to its binding site may be aided or prevented by mRNA secondary structure. Studies with both animal and plant cells have shown that site accessibility is just as important as individual nucleotide complementarities in the seed region for effective suppression (Kertesz et al., 2007; Long et al., 2007; Zheng et al., 2017). Moreover, it is reported that miRNAs can bind to seed-like motifs and this type of non-canonical binding may contribute to the miRNA targeting (Cloonan, 2015). For example, experiments with miR-155 have displayed that around 40% miR-155 targeting is through non-canonical targeting (Loeb et al., 2012). Therefore, unsuccessful translational inhibition of hsa-miR-548ba with shorter PTEN 3'UTR may be caused by a different secondary structure which enables the miRNA target site accessibility or absence of some non-canonical target sites that may be present in longer PTEN 3'UTR sequence. However, this hypothesis still needs to be validated.

The confirmed targets for hsa-miR-548ba were proven to be the following: *LIFR, PTEN, NEO1* and *SP110*. *LIFR, PTEN* and *NEO1* all play a part in the early stages of follicle development (Hagihara et al., 2011; Nilsson et al., 2002; Reddy et al., 2008). Follicle activation and the recruitment of primordial follicles is a highly controlled and regulated process where the PI3K-Akt signalling pathway plays a major role (Ernst et al., 2017). PTEN negatively regulates the PI3K-AKT pathway by dephosphorylating PIP3 (Adhikari et al., 2009). Oocyte-specific mouse knock-out of Pten results in the premature activation of the follicle pool (Reddy et al., 2008). Therefore, PTEN is extremely important for maintaining the ovarian pool and hence the female reproductive lifespan. However, in the later stages of follicle growth PTEN appears to have a negative effect on the oocyte quality as the decreased PTEN expression and increased expression of its downregulating miRNAs correlate with high-quality oocytes in the bovine system (Andrade et al., 2017). Therefore, a time-specific regulation of PTEN during follicle development is necessary. Hsa-miR-548ba expression levels are currently only measured from pre-ovulatory follicles and therefore there is no information on how this miRNA is expressed during earlier follicle developmental stages. Leukaemia inhibitory factor (LIF), ligand to LIFR, promotes the first step of follicle development: the primordial to primary transition in rat ovaries (Nilsson et al., 2002). Moreover, NEO1, which is a receptor for netrins, is also involved in the BMP signalling pathway by directly binding BMPs, and regulates RAC1-PI3K-AKT pathway in human gastric cancer cells (Hagihara et al., 2011; Qu et al., 2018). In addition to the mentioned PI3K-AKT pathway, BMPs are involved in the oocyte-somatic cell communication and in the formation and expansion of the COC (Chang et al., 2016). Compared to PTEN, LIFR and NEO1 also play roles in later stages of follicle development.

The confirmed targets for hsa-miR-7973 were the following: *ADAM19, PXDN* and *FMNL3.* All the genes have been shown to participate in the extracellular matrix (ECM) modulation and cell-to-cell interactions (Qi and Sang, 2009, p. 19; Zheng and Liang, 2018). Therefore, the two miRNAs of interest are involved in different processes of follicle development. From hsa-miR-7973 targets, ADAM19 cleaves the ECM proteins, but also cytokines such as neuregulin (Qi and Sang, 2009, p. 19). The proper timing of meiosis is important for oocyte quality and it has been shown that the time of oocyte meiotic resumption is regulated by the expression of neuregulin 1 in COC (Noma et al.,

2011). Similar to hsa-miR-548ba, the hsa-miR-7973 expression levels have only been measured from the pre-ovulatory follicles. However, the target genes of this particular miRNA indicate that it may be involved in the later stages of follicle development when the ECM must be modulated for oocyte ovulation and meiosis is resumed (Hennet and Combelles, 2012).

The principle for selecting potential targets for further validation was the differential expression in Affymetrix microarray and positive bioinformatic prediction result. However, relying on the bioinformatical predictions can lead to false negative results as not all real target genes are predicted. One example is hsa-miR-21-5p, which has been shown to target PTEN mRNA experimentally (Zhou et al., 2010). However, PTEN is not a predicted target for this miRNA according to the programs used in this study. Moreover, hsa-miR-21-5p was used as a positive control in the luciferase assay, where the binding to the PTEN 3'UTR sequence was confirmed by the reduced luciferase levels (Supplementary Figure 3, Publication I). Different approaches for miRNA target detection may give improved efficiency. One possible alternative would be the combination of cross-linking RISC to target mRNA (Cambronne et al., 2012) followed by high-throughput sequencing to analyse mRNAs which are enriched in RISC compared to non-enriched RNA pool.

Work with hsa-miR-7973 also confirms that not all potential targets can be bioinformatically predicted, and different methods may validate targets with dissimilar results. *FSHR* mRNA was downregulated by hsa-miR-7973 according to the results from the Affymetrix gene chip and from RT-qPCR, however the gene expression changes were not statistically significant (Figure 4A and 4C, unpublished results). In the luciferase assay hsa-miR-7973 exhibited direct binding to the 3´UTR of FSHR compared to the control. Moreover, the results of the mirTrap analysis, which is based on the trapping RISC to target mRNA (Cambronne et al., 2012), confirmed the enrichment of *FSHR* mRNA in the RISC of hsa-miR-7973 transfected cells (Figure 4B and 4D, unpublished results). *INHBA*, another potential target for hsa-miR-7973, exhibited statistically significant results with all four methods (Figure 4A-D, unpublished results). Interestingly, *INHBA* mRNA displayed upregulation after transfection with hsa-miR-7973 according to Affymetrix microarray and RT-qPCR (Figure 4A and 4C, unpublished results), while protein levels were diminished according to the luciferase assay (Figure 4D, unpublished results). miRNAs are mostly known as the negative regulators of gene expression; however, an upregulation in the expression of miRNA target mRNAs has also been reported (Valinezhad Orang et al., 2014). The upregulation of target mRNA can be influenced by cellular state (quiescence, cancer or normal cells) (Lin et al., 2011; Vasudevan, 2012) or by the presence of other factors, for example Fragile-X mental retardation protein 1 interaction with AGO can lead to upregulation of the miRNA targets (Valinezhad Orang et al., 2014). Moreover, it has been shown that miRNA binding site location can lead to a different outcome of miRNA regulation (5' vs 3' site of target mRNA) (Jopling et al., 2008). This may also explain the results with hsa-miR-7973 and *INHBA*. In luciferase assay only 3' end of *INHBA* is used compared to microarray and RT-qPCR where *INHBA* is in its natural structure. The upregulation of *INHBA* by hsa-miR-7973 can be through alternative 5' site which is not present in luciferase assay and therefore the downregulation of INHBA is mediated through 3' target sites.

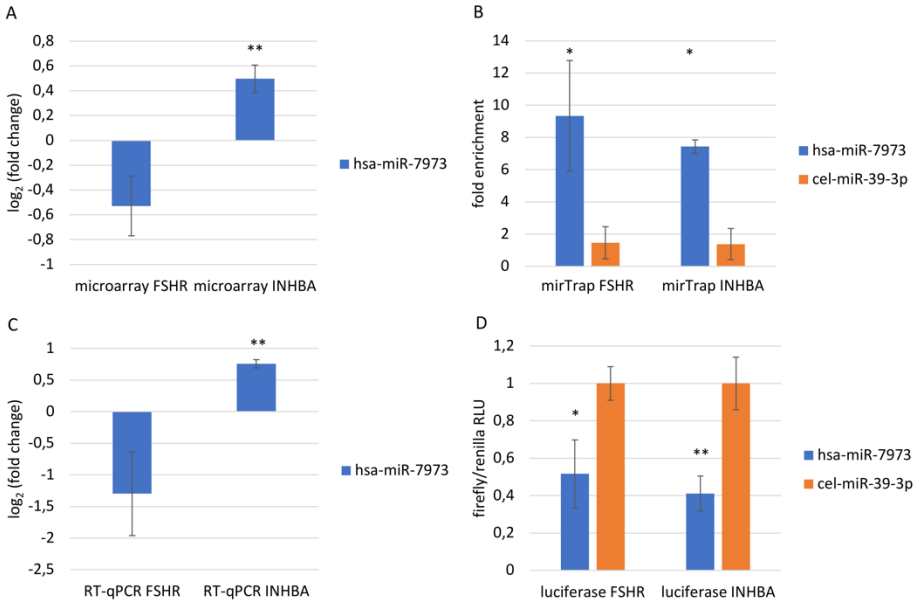

Figure 4. *FSHR and INHBA gene expression change after transfection of KGN cells with hsa-miR-7973. A: Affimetrix microarray (n=4), B: mirTrap (n=2), C: RT-qPCR (n=3) and D: luciferase assay (n=3). Results are displayed as average ± standard deviation (*p < 0,05; **p < 0,01, Student t-test), unpublished results. RLU- relative light unit.*

To conclude, the list of the reported potential target genes is not final and new targets can be discovered with different experimental and computational approaches. Moreover, the expression of the two miRNAs of interest is analysed in ovarian GCs; however, they can be expressed outside of the ovary where they may possess other tissue-specific targets.

4.2 Hsa-mir-548 family expression and targeted pathways in FSHR-positive reproductive tissues

miRNAs are grouped into families according to their mature sequence and/or the structure of their pre-miRNA (Kamanu et al., 2013). Hsa-miR-548ba is a member of a large hsa-mir-548 family with 86 members according to the mature miRNA sequences and as reported by miRbase v 22.1 (Figure 1A, Publication II). The previous section of the thesis demonstrates that hsa-miR-548ba targets important genes involved in ovarian follicle activation and growth. However, the members of the hsa-mir-548 family contain similar nucleotide sequences and can potentially target the same mRNAs. This may result in false interpretations of the functions of a single member of the family, in our case hsa-miR-548ba, as the potentially overlapping roles of other members have not been considered. Therefore, this study focuses on how the miRNA family is expressed in the ovarian follicle and which genes and pathways are targeted by the members of the hsa-mir-548 family collectively. In addition, as there is no information regarding hsa-miR-548ba expression in other reproductive tissues, these were also analysed in the study. The justification for selecting only FSHR-expressing reproductive tissues was based on the hsa-miR-548ba genomic location in the intronic region of the FSHR gene, its

expression level correlating with that of its host gene in primary GCs and the fact that FSH exerted positive influence on hsa-miR-548ba expression (Figure 5A, Publication I).

Firstly, hsa-mir-548 family sequence similarity analysis was performed to study their evolutionary conservation. Four members displayed the shortest distances on the phylogenetic tree: hsa-miR-548m, hsa-miR-548ag, hsa-miR-548d-5p, hsa-miR-548ay-5p and hsa-miR-548ad-5p (Figure 2C and Supplementary Figure 1, Publication II). Moreover, three family members (hsa-miR-548ag, hsa-miR-548ai and hsa-miR-570-5p) share the critical seed sequence with hsa-miR-548ba (Figure 2D, Publication II). Hsa-miR-548ai and hsa-miR-570-5p demonstrate dissimilarities in their 3' part of the sequences and therefore reside more distantly in the phylogenetic tree (Supplementary Figure 1, Publication II).

Small RNA high throughput sequencing results from female FSHR-positive reproductive tissues (Figure 5) (Table 1, Publication II) displayed that hsa-miR-548ba expression is the highest in GCs, both CGCs and MGCs. Additionally, high levels of hsa-miR-548ba expression were observed in the myometrium samples. Hsa-miR-548ba was expressed at borderline levels in all the other analysed tissue samples (Figure 2B, Publication II). Family members with the same seed sequence to that of hsa-miR-548ba are not co-expressed in GCs and in myometrium (Figure 2E, F, Publication II). However, a number of other family members expressed in GCs share a common seed sequence between themselves, but not with hsa-miR-548ba (Figure 2G, H, Publication II). Therefore, possible co-regulation of pathways by some of the hsa-mir-548 family members may occur in GCs.

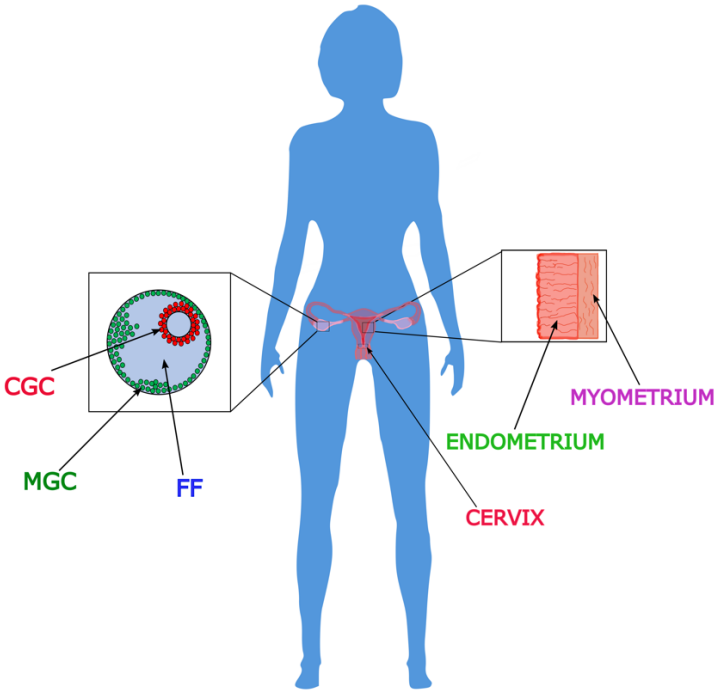

Figure 5. *Overview of female reproductive samples analysed in this study. Ovarian samples: CGC- cumulus granulosa cells, MGC-mural granulosa cells and FF-follicular fluid and uterine samples: endometrium, myometrium, and cervix.*

Next, the target gene prediction and pathway enrichment analysis were performed for the co-expressed hsa-mir-548 family miRNAs. The focus was emphasised on the granulosa and myometrium samples as the hsa-miR-548ba expression was the highest in those samples. In addition to hsa-miR-548ba, twelve and one other family members were expressed in GCs and myometrium tissue, respectively (Figure 2E, F, Publication II). Despite the fact that miRNAs which share a seed sequence with hsa-miR-548ba were not present in GCs, some overlapping targeting was still predicted for hsa-miR-548ba, and other family members collectively expressed (Figure 4, Publication II). It is known that several miRNAs can target the same mRNA molecule (Wu et al., 2010). Moreover, the miRWalk program, which was used in this study, accounts for additional features for target predictions in addition to the seed sequence (Sticht et al., 2018). Pathway enrichment analysis displayed that hsa-miR-548ba co-regulates the "RAB geranylgeranylation" pathway together with hsa-miR-548b-5p in MGC (Supplementary Table 3B, Publication II). This pathway is required for Rab protein activation: inhibition of this pathway disturbs Rab27a geranylgeranylation in mice oocytes. It is suggested that Rab27a has a role in oocyte protein secretion, and therefore the normal functioning of this pathway is necessary for the oocyte-granulosa cell communication (Jiang et al., 2017).

The co-regulation of pathways also occurs between family members other than hsa-miR-548ba. The "PIP3 activates AKT signaling" pathway is targeted by hsa-miR-548d-5p and hsa-miR-548i in CGCs and MGCs and is additionally targeted by hsa-miR-548w and hsa-miR-548b-5p in CGCs and MGCs, respectively. The "PI5P, PP2A and IER3 regulate PI3K/AKT signaling" pathway is collectively targeted by hsa-miR-548d-5p and hsa-miR-548b-5p in MGC. Both pathways hold an important role in the follicles. The "PIP3 activates AKT signaling" as a regulator of the follicle dormancy and activation and GC differentiation and proliferation (Makker et al., 2014). The second pathway is involved in GC survival during follicle development (Jin et al., 2017). Interestingly, additional miRNAs with the same seed sequence as the miRNAs targeting those mentioned pathways are expressed in GCs but do not contribute to the co-targeting process. This can be explained by the nucleotide substitutions outside of the miRNA seed sequences as these have changed potential targeting features (Ding et al., 2016) and have led to different target genes.

Pathway enrichment analysis of the miRNAs co-expressed in the myometrium exhibited the co-regulation of "Signaling by BRAF and RAF fusions" by hsa-miR-548ba and hsa-miR-548o-3p. BRAF and RAF fusion is a result of chromosomal rearrangement events and is detected in distinct cancer types (Lavoie and Therrien, 2015). Therefore, in normal myometrial tissue this pathway is not expected to be present. However, hsa-miR-548o-3p displayed targeting of the "PI5P, PP2A and IER3 regulate PI3K/AKT signaling" and "PPARA activates gene expression" pathways. The first pathway is involved in smooth muscle contractions (Butler et al., 2013) and the second pathway has a potential role in pregnancy maintenance (Dong et al., 2013). In addition, hsa-miR-548o-3p expression was detected in all the other uterine samples (myometrium, endometrium, and cervix) but not in ovarian samples, indicating an organ-specific expression (Supplementary Table 2, Publication II).

Lastly, the analysis of FF samples revealed that seven members of the hsa-mir-548 family are also present in the FF of the ovarian pre-ovulatory follicle (Figure 2J, Publication II). Some of the miRNAs were only detected in FF and not in GCs, for example hsa-miR-548o-5p and hsa-miR-548c-5p. As FF is derived from both the bloodstream

through capillaries and from components secreted by GCs and theca cells (Hennet and Combelles, 2012), miRNAs present in FF may infiltrate into the FF from blood plasma and that could partly explain the lack of their expression in the GCs. Moreover, the oocyte has not been investigated as the source of miRNAs secreted into the FF due to the lack of human data. Therefore, the study concludes that hsa-mir-548 family members are secreted into FF by other cell types as well as by ovarian GCs. The exact sources and mechanism of how hsa-mir-548 family members are selected for secretion remains unknown.

To conclude, from all of the analysed FSHR-positive samples, hsa-miR-548ba can be detected in ovarian GCs and myometrium with the highest expression levels. In addition to hsa-miR-548ba, twelve and one other hsa-mir-548 family members are expressed in granulosa and myometrium samples, respectively. Moreover, hsa-mir-548 family members are detectable in extracellular FF. miRNA target pathway enrichment analysis revealed one commonly regulated signalling pathway with hsa-miR-548ba and co-expressed family members: hsa-miR-548ba and hsa-miR-548b-5p co-regulate RAB geranylgeranylation in MGC.

4.3 Genome-wide miRNA expression in the human ovarian follicle

miRNAs play important roles in cellular gene expression regulation (Sereno et al., 2020). In addition to cells, miRNAs are present in various body fluids including FF (Sang et al., 2013). miRNAs can be secreted from the cells in the complexes of RBP or as loaded into EVs (Fritz et al., 2016). Many possible sorting mechanisms have been proposed for loading miRNAs into EVs involving the sequence characteristics, post-transcriptional modifications, subcellular location and intracellular concentration of the miRNA (Fritz et al., 2016). The secretion of miRNAs into FF has mostly been studied in the bovine model (da Silveira et al., 2012; Sohel et al., 2013). The whole FF and/or EV extracted from the FF have been analysed separately for their miRNA content, therefore there is a lack of parallel data of follicular cells, FF and EVs from the same individual. Moreover, as the above-described study discussing the diversity of expression of hsa-mir-548 family members in reproductive tissues and FF has demonstrated, each individual miRNA may be enriched in cells or FF and thus indicates the potential role of miRNAs in gene regulation also between cells (Figure 2F and J, Publication II). Therefore, a comparison between parallel samples provides an opportunity to unravel the profiles of cellular and extracellular miRNAs of the ovarian follicle and present new information about potential intrafollicular signalling.

For the modelling of intrafollicular signalling fertile oocyte donor samples were used. Three types of samples were collected from the same ovarian follicle: MGC, cell-depleted FF and EVs purified from the cell-depleted FF (Figure 1, Publication III). From all three sample types small RNA sequencing was performed. The results revealed that the sample types contain different profiles of small RNA sequence lengths (Supplementary Figure 3, Publication III). Size peak at 17-24 nt representing the miRNA population is present in all sample types (MGC, FF and EV). Additional peak in the length of 28-36 nt is present in MGC and FF samples, but not in the EV samples. This is reflected in the shorter average sequence length in the EV samples compared to FF and MGC (p-value <0.005, Supplementary Table 1, Publication III). miRNAs are a well-studied class of small RNAs, however, other small RNA biotypes are present in FF and EV samples (Figure 6, unpublished data) which is in accordance with the previous studies of extracellular RNA sequencing (Navakanitworakul et al., 2016; Nolte-'t Hoen et al., 2012; Sork et al., 2018).

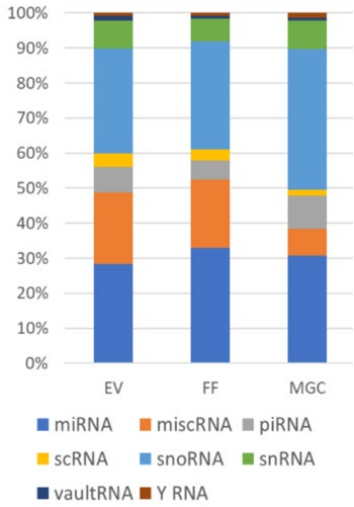

Figure 6. *The proportion of small RNA biotypes detected from the sequencing data. Ribosomal and transfer RNAs are not depicted due to multiple repeated loci of their genes in the human genome. scRNA- small conditional RNA, miscRNA- miscellaneous RNA, snoRNA- small nucleolar RNA, piRNA- piwi-interacting RNA and snRNA- small nuclear RNA, unpublished results.*

The functionality of extracellular RNAs can roughly be divided into three: known function, predicted function and unknown function (Min et al., 2019). miRNAs belong into the RNA group with a known function and therefore investigation continued with the miRNA profiles of the samples. Altogether, 1525 unique miRNAs were detected by at least one read: 1381 in MGC, 1060 in FF and 658 in EV. The three sample types form separate clusters according to their miRNA content, meaning that the miRNA profile in the ovary is sample type specific (Figure 3A, B, Publication III). Compared to the MGC samples, FF and EV samples cluster closer to each other. This is an expected result as EVs are purified from the corresponding FF samples. Comparisons of the three sample types (MGC, FF and EV) showed that 172 miRNAs are common to all three sample types (>5 reads observed in >50% samples per sample type). A group of 124 miRNAs was detected from MGC samples, indicating that those miRNAs are specific to cells and not secreted to the extracellular space at detectable levels. As expected, the EVs did not contain any unique miRNAs compared to the FF and MGC. However, three miRNAs (hsa-miR-374a-5p, hsa-miR-190a-5p and hsa-miR-196a-5p) were present only in the MGC and EV samples. This may be a result of the specific enrichment of these miRNAs into EVs and in FF samples those miRNAs remained below the detection limit (Figure 4A, Publication III). FF samples contain 23 miRNAs that are not present in any other sample types. Comparison to the serum and plasma small RNA sequencing results (Srinivasan et al., 2019) showed that 10 of those miRNAs may have reached FF by plasma infiltration into the follicle through peri-follicular capillaries (Supplementary Table 3, Publication III). Moreover, neither CGCs, nor the oocyte were not analysed in this study and the remaining 13 unique miRNAs in FF samples may originate from those cell types.

Next, miRNAs which are present in FF or EV samples were compared with the aim to reveal if miRNA loading into EVs serves a specific functional purpose in the follicle. A list of 175 miRNAs was detected from the EVs. FF samples contained 113 miRNAs which were

absent from the EV samples, which indicates another mechanism of secretion for those miRNAs into extracellular space. Targets were predicted for those two lists of miRNAs and analysed pathway over-representation for the predicted targets to investigate if there is a potential overlap in the targeted pathways between miRNAs inside or outside of the EVs. As a result, only three pathways were commonly targeted by miRNAs present in FF samples outside of the EVs (Figure 4B, Supplementary Table 4A, Publication III). However, 436 targeted pathways were over-represented for miRNAs detected in EVs (Figure 4C, Supplementary Table 4B, Publication III). This indicates that miRNAs loaded into EVs carry directed molecular signals that can potentially be used for intercellular communication. Furthermore, miRNAs in EV samples potentially target the following Reactome pathways which, in the context of the ovarian follicle, play essential roles: "Estrogen receptor-mediated signaling", "Signaling by Nuclear Receptors", and "PTEN Regulation", among others. It is considered that cargo segregation into EVs is not a random event: for example, the human bone marrow- and adipose-mesenchymal stem-cell-derived exosomes contain distinctive small RNA molecules linked to their differentiation status (Baglio et al., 2015). Moreover, studies in the equine and bovine models have demonstrated that follicular fluid EVs were taken up by GCs *in vitro* and this process affected the expression of genes involved in follicle development (da Silveira et al., 2012; Hung et al., 2017). Furthermore, some miRNAs in the follicular EVs may also regulate oocyte growth, as alterations in their expression were observed between follicles with different oocyte maturation stages (Sohel et al., 2013). The pathway enrichment analysis and previous studies led to the conclusion that EVs in FF most likely play important regulatory roles in the ovarian function.

To summarise, miRNA profile analysis of samples from fertile women indicates specific miRNA segregation into vesicles with various targeted pathways downstream. At the same time, miRNAs secreted by non-EV mediated pathways play less specific roles in the ovarian functions. It is possible that a high proportion of these miRNAs have been released by cellular degradation and not by specific secretion as it is shown that AGO2-miRNA complexes may be released after cell death (Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012).

RNA sequencing data can be useful for predicting novel, yet unannotated, miRNAs. After filtering candidate novel miRNA sequences predicted by the miRDeep2 algorithm, this study proposes one potential new miRNA (mature sequence: CCUGGGCAUGGGACUGG, predicted stem-loop sequence in Figure 8A, Publication III) that was expressed in all three sample types (EV, FF and MGC). However, it was most frequently detected in EV samples demonstrating significantly higher expression levels compared to FF and MGC samples (Figure 8B, Publication III). Hence, the study proposes a novel EV-specific miRNA sequence detected from the FF. Its importance in the ovary and detection potential in other biofluids still needs to be determined.

The EVs analysed in this study contain microvesicles as well as exosomes. Analysing the two EV sub-types separately would provide a better insight into miRNA secretion mechanisms. The obstacle to performing such an analysis lies in the overlapping size and density of the EV subtypes (Yang et al., 2020) making them challenging to be separated with high purity. However, new methods are continuously in development which allow improved separation. For example, immunoaffinity capture is based on specific binding of surface markers and antibodies and can be used on microfluidic chips allowing the automation of purification process (Yang et al., 2020).

Follicular environment affects the oocyte quality; this connection provides an opportunity to evaluate the integrity of the oocyte by analysing its surrounding milieu (Dumesic et al., 2015). The cellular and extracellular miRNA profiles of a single pre-ovulatory follicle may provide valuable information for the evaluation of the oocyte competence during IVF treatment. During this study we had no data regarding the fertilisation status of the oocytes. However, in the future this would be a promising approach to select top-quality oocytes for fertilisation and the corresponding developing embryos for uterine transfer.

4.4 The impact of PCOS on the miRNA expression in the ovarian follicle

PCOS results in dramatic biochemical and morphological changes in the ovary: disturbances in steroidogenesis in these patients often lead to the formation of follicular cysts and anovulation (Patel, 2018). It was hence hypothesised that these changes should be well reflected in the intercellular communication in the follicle as well. We were further interested in whether the ovarian miRNA expression, secretion, or segregation into EVs are compromised by PCOS. It was observed that each of the studied follicular compartments (MGC, cell-depleted FF and EV) is affected differently by PCOS.

Several miRNAs have been shown to be DE in the GCs and/or FF between fertile and PCOS women (Butler et al., 2019; Cirillo et al., 2019; Roth et al., 2014; Sang et al., 2013). In the current study, the highest number of differences in miRNA expression was detected from cellular and FF samples between the PCOS and oocyte donor groups: 30 and 10 DE miRNAs, respectively (FDR <0.1, Figure 6A, B, Supplementary Table 7A, B, Publication III). Due to a higher variation of miRNA expression levels across patients in the EV samples (Figure 3A, Publication III), no miRNAs reached the same FDR cut-off level. However, 7 miRNAs were DE in EV samples between the two groups without considering the FDR (p-value <0.05, Figure 6C, Supplementary Table VII, Publication III). Fewer differences in EVs may be caused by the more complex processing of EV samples before RNA extraction compared to MGCs and FF.

Hsa-miR-200c-3p was the only commonly up-regulated miRNA in the extracellular samples of FF and EV. It has been shown that hsa-miR-200c-3p is expressed higher in the GCs of PCOS patients compared to fertile controls (He et al., 2018). Moreover, hsa-miR-200c-3p expression is related to pregnancy complications such as pre-eclampsia and preterm labour (Mayor-Lynn et al., 2011). Similarly, in this study, hsa-miR-200c-3p displayed higher expression in the EV and FF samples of PCOS patients. However, an expression change of this miRNA was not detected in the MGC samples.

Overall, several miRNAs were identified as DE between the patient groups which have not been previously associated with PCOS but are involved in the regulation of gene expression in follicles or in other ovary-related disorders. For example, the levels of four DE miRNAs (hsa-miR-203a-3p, hsa-miR-195-5p, hsa-miR-486-3p, and hsa-miR-484) are altered in the GCs of women with diminished ovarian reserve (Woo et al., 2017). Interestingly, all of the miRNAs listed above are expressed in the MGCs of the PCOS group according to the same pattern as in the normal ovarian reserve (NOR) patients. It has been noted that PCOS women have a slower age-related decline in AFC compared to non-PCOS patients (Wiser et al., 2013). These miRNA expression results could possibly be explained by a slower decline in AFC in PCOS women. Moreover, hsa-miR-224-5p, which was up-regulated in the MGCs of PCOS women in this study, downregulates SMAD4, which is involved in the regulation of apoptosis of GC (Du et al., 2020, p. 4)

The expression differences of hsa-miR-486-5p (Shi et al., 2015) in MGCs have previously been associated with PCOS compared to women undergoing IVF due to male factor infertility with same directional expression as in the current study. Moreover, hsa-miR-200a-3p (Xu et al., 2015) and hsa-miR-30a-3p (Yao et al., 2018) have previously been linked to PCOS, but in other follicular compartments. In these studies, hsa-miR-200a-3p in CGCs and hsa-miR-30a-3p in FF were less abundant in PCOS women which is the opposite of the current MGC results. Differential expression of hsa-miR-509-3-5p (Butler et al., 2019) and hsa-miR-200c-3p (Butler et al., 2019; He et al., 2018) in FF have previously been associated with PCOS with mutual expression direction to the current FF results. Hsa-miR-1307-3p (Li et al., 2015) and hsa-miR-223-3p (Xu et al., 2015) are also altered in the CGCs of PCOS patients, but with an opposite direction to the FF results of this study. These comparisons demonstrate the dependence of miRNA expression on the cell population. By the pre-ovulatory follicular stage, when the samples have been obtained, CGCs have differentiated from MGCs and have significant dissimilarities in gene expression and post-transcriptional regulation patterns (Kõks et al., 2010; Velthut-Meikas et al., 2013). From the EV samples hsa-miR-200c-3p (Butler et al., 2019; He et al., 2018) and hsa-miR-17-5p (Liu et al., 2020) have previously been shown to be altered in PCOS women in line with the results of the current study.

Validation of the small RNA sequencing results was performed by RT-qPCR for miRNAs with the highest fold change and that have been previously related to ovarian functions (Table 2, Publication III). Since oocyte donors are generally young women the average age difference between the two study groups in the RNA sequencing experiment was statistically significant (p-value=0.002, Table 1, Publication III). As there is previous evidence that the expression of some miRNAs can be affected by age (Diez-Fraile et al., 2014; Moreno et al., 2015), a validation cohort of age-matching women undergoing IVF due to male-factor infertility was added to the oocyte donor samples used for RNA sequencing. The average age difference between the PCOS and validation control group was therefore not statistically significant (p-value=0.626, Table 1, Publication III). Validation results revealed that the higher expression of hsa-miR-205-5p in PCOS patients compared to donors is more likely to be caused by the age than the PCOS syndrome as adding the age-matched control samples changed the miRNA expression level and direction (Supplementary Figure 5, Publication III). However, hsa-miR-205-5p expression has previously been measured from lung cancer patients in both cancer tissue and blood samples without detecting correlation with the patient age (Li et al., 2017; O'Farrell et al., 2021). Therefore, age-related change in hsa-miR-205-5p expression may be different between ovary and other organs.

The results of the study clearly demonstrate that the effects of miRNA expression differences brought upon by PCOS lead to different molecular outcomes depending on the investigated sample type. For example, cytokine-mediated signalling was affected in the cellular compartment and by non-EV-mediated RNA secretion, while EV-mediated signalling potentially affects the IGF1R pathways in PCOS patients. These results emphasise the importance of studying the follicle as a system to better understand inter-cellular signalling and possible molecular disturbances in the PCOS ovary.

In this study the cells of individual ovarian follicles were sequenced. In addition to MGCs there are additional cell types present in the follicles. However, the majority of the cells captured by this method as used in this study are MGCs. In addition, CGCs are one group of possible cells that can be collected and used in this type of analysis. Furthermore, sequencing methods have developed drastically during the last decade and

single-cell RNA sequencing methods are now available (Hwang et al., 2018). This method allows us to study all cell populations separately and at a single-cell level. Different cell types in the ovarian follicle may have diverse effects on the development of PCOS. Moreover, the proportion of cells in each population may also be different in polycystic ovaries. miRNA sequencing at single-cell level may give more specific insights which cells specifically contribute to the formation of PCOS. Furthermore, differentiated GCs (MGCs and CGCs) have been shown to contain further sub-populations (Dong et al., 2019). Analysis of GC RNA at the single cell level may reveal if there is a specific sub-population responsible for the development of polycystic ovaries.

In conclusion, the current study proposes novel miRNAs and their regulated signalling pathways underlying the infertility of patients with PCOS.

5 Conclusions

The main results of the thesis are the following:

- The two studied miRNAs, hsa-miR-548ba and hsa-miR-7973, play an important role in folliculogenesis.
- miRNAs, hsa-miR-548ba and hsa-miR-7973, potentially regulate different functions in the ovarian follicle. Targets of hsa-miR-548ba are involved in the regulation of ovarian follicle development and hsa-miR-7973 regulates ECM modulation and cellular interactions.
- miRNAs hsa-miR-548ba and hsa-miR-7973 directly target *LIFR, PTEN, NEO1, SP100* and *ADAM19, PXDN, FMNL3* in granulosa cells, respectively.
- From FSHR-positive reproductive tissues hsa-miR-548ba is expressed in GC and myometrium samples at the highest level.
- In addition to hsa-miR-548ba, granulosa and myometrium tissues express twelve and one other hsa-mir-548 family member, respectively.
- Hsa-mir-548 family members co-expressed in GCs do not demonstrate a high degree of co-regulated pathways with hsa-miR-548ba. Overall, one common pathway "RAB geranylgeranylation pathway" was predicted to be co-targeted between hsa-miR-548ba and hsa-miR-548b-5p in MGC.
- miRNA profile analysis of FF and EV samples of healthy women indicates specific miRNA segregation into vesicles with various targeted pathways downstream ("Estrogen receptor-mediated signaling" and "PTEN Regulation", among others).
- Comparison of MGCs, FF and EVs isolated from healthy follicles to samples obtained from PCOS patients displayed that all three sample types are affected differently by PCOS. miRNAs DE between PCOS and controls display regulation of diverse pathways: cellular and non-EV miRNAs affect cytokine-mediated signalling, in contrast EV-mediated signalling potentially affects the IGF1R pathways in PCOS patients.

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Abstract

Intercellular Signalling in Human Pre-ovulatory Follicle: microRNA Expression in Granulosa Cells and Detection in the Follicular Fluid

Human reproduction relies on the well-timed and balanced development of the oocyte. During female foetal development the oocytes are surrounded with somatic granulosa cells and together these two cell types form primordial ovarian follicles. The number of primordial follicles is finite and represents the reproductive potential of a woman. Primordial follicles are activated and recruited from the ovarian pool before and throughout the reproductive period. These follicles go through major morphological and transcriptional changes during folliculogenesis to ovulate a mature oocyte with the potential to fertilise and commence the embryonic development. Gonadotropins and steroid hormones play a role in the process. The first steps of folliculogenesis (primordial, primary, and secondary stage) are independent of the gonadotropins. However, later stages (antral and preovulatory) are highly dependent on the hormonal regulation of the follicle stimulating hormone (FSH) as well as the luteinizing hormone (LH). Moreover, follicles start to produce estrogens with help from the aromatase enzyme. At this stage an antrum forms which is filled with follicular fluid (FF). The antrum separates the granulosa cells into two sub-populations: mural granulosa cells (MGCs) and cumulus granulosa cells (CGCs) with distinctive roles in the follicle. At the same time FF in the antrum enables paracrine signalling between the cell types. The ovarian follicle development is highly dependent on the communication between the oocyte and granulosa cells as well as between MGCs and CGCs. Disturbances in the follicular cell signalling and communication lead to lower oocyte quality or even to infertility.

In addition to hormones and growth factors, cell-to-cell signalling involves other types of molecules. For example, miRNAs as post-transcriptional regulators play additional roles in cell-cell communication. In addition to cells miRNAs are found in FF in RNA binding protein (RBP) or as packed into extracellular vesicles (EVs). These extracellular miRNAs can be taken up by follicular cells, where they also regulate their gene expression.

The aim of the current thesis was to investigate miRNA expression in the human ovarian pre-ovulatory follicle and to study the possible cell-to-cell signalling mediated by those small RNA molecules. Firstly, the study focused on two miRNAs: hsa-miR-548ba and hsa-miR-7973, both expressed in MGCs and CGCs in the pre-ovulatory follicle and expressed from the FSH receptor (FSHR) and aromatase gene intronic regions in the human genome, respectively. The role of these two miRNAs was not previously known. By investigating miRNA targets, the results revealed that hsa-miR-548ba directly targets *LIFR, PTEN, NEO1* and *SP110*. From those target genes PTEN is an important inhibitory regulator of the primordial follicle activation. LIFR and NEO1 have additionally proposed roles in follicle development. Therefore, hsa-miR-548ba potentially plays a regulatory role in the early stages of folliculogenesis. Hsa-miR-7973 displayed direct targeting of the *ADAM19, PXDN* and *FMNL3.* Known roles of those proteins involve extracellular matrix (ECM) modulation and cell-to-cell interactions. In the pre-ovulatory follicle ECM modulation is highly important for successful ovulation. The target genes of these two miRNAs indicate that they possess distinctive regulatory roles in the ovarian follicle.

It is previously known that hsa-miR-548ba is a member of a large hsa-mir-548 miRNA family. The other members may potentially intervene with the hsa-miR-548ba regulation

of ovarian functions by co-regulating common targets in a cell. In addition, as the hsa-miR-548ba gene is located in the intronic region of FSHR and is co-expressed in concordance with its host gene in GCs, all FSHR-positive reproductive tissues were studied with an aim to reveal the expression patterns of the hsa-mir-548 family members and their possibly overlapping targeted pathways. The results showed that hsa-miR-548ba is expressed at the highest in GCs and myometrium tissue together with twelve and one other family members, respectively. It was predicted that hsa-miR-548ba and hsa-miR-548b-5p co-regulate "RAB geranylgeranylation pathway" in MGCs and this pathway is involved in granulosa cell-oocyte signalling. Overall, the hsa-mir-548 family miRNAs expressed in GCs display only a small number of overlapping target mRNAs and hence a low degree of co-regulation of pathways with hsa-miR-548ba.

In the final part of the thesis the importance of cell-to-cell signalling by extracellular miRNAs in the pre-ovulatory follicles was investigated. Three sample types were collected: MGCs, cell-free FF and EVs extracted from the same FF samples and analysed their small RNA content. The results revealed the expression patterns of miRNAs in the cellular and extracellular fraction of the ovarian follicle. Moreover, it was demonstrated that miRNAs detected in EVs have a potentially better targeted regulatory purpose as these miRNAs may regulate over 400 pathways, for example: "Estrogen receptor-mediated signalling" and "PTEN Regulation", among others. On the contrary, miRNAs present in FF and not in EVs were predicted to regulate only three pathways in total.

In addition, the ovarian follicle miRNA profiles between fertile and healthy women (oocyte donors) and patients with polycystic ovary syndrome (PCOS) were compared. Several miRNAs which had not previously been associated with PCOS were detected as DE, for example hsa-miR-224-5p, hsa-miR-203a-3p, hsa-miR-195-5p, hsa-miR-486-3p, and hsa-miR-484. Interestingly, some miRNAs which were formerly associated with PCOS demonstrated an opposite direction of expression in the obtained results; however, prior studies were performed with different sample types (for instance CGCs). This indicates that the same miRNA may have variable expression patterns in different ovarian cell types and hence may be differently affected by disturbances such as PCOS. It was concluded that miRNAs disturbed in MGCs, FF or EVs of PCOS women regulate separate pathways: while cytokine-mediated signalling was affected in the cellular compartment and by non-EV-mediated RNA secretion, EV-mediated signalling potentially affects the IGF1R pathways in PCOS patients. This highlights that it is important to analyse the ovarian follicle as a whole to achieve a better understanding of how PCOS affects cell-to-cell communication mediated by miRNAs.

Altogether, the studies included into the current thesis increase the knowledge of miRNA expression in human pre-ovulatory follicles both at the cellular and extracellular level. New insights help us to better understand the importance of miRNA secretion in EVs compared to other macromolecular compartments in potential cell-to-cell communication in the human pre-ovulatory follicle. As the molecular signalling between the follicular cells is vital for successful oocyte ovulation, a comparison of the ovarian follicle miRNA profiles between fertile women and PCOS patients provides valuable information to understand this condition and may provide new possibilities for therapeutic approaches for PCOS patients.

Lühikokkuvõte

Rakkudevaheline signaliseerimine inimese munasarja preovulatoorses folliikulis: mikroRNA-de ekspressioon granuloosrakkudes ja follikulaarvedelikus

Inimese reproduktsioon põhineb tasakaalustatud ja õigesti ajastatud munaraku küpsemisel. Loote arengu käigus ümbritsetakse munarakk somaatiliste granuloos-rakkudega, mis koos moodustatavad primordiaalsed folliikulid. Primordiaalsete folliikulite arv on lõplik ja moodustab naise reproduktiivse potentsiaali. Reproduktiivse elueani jõudmiseni ja selle jooksul primordiaalsed folliikulid aktiveeruvad ja lahkuvad munasarja reservist. Oma arengu käigus läbib folliikul suured morfoloogilised ja transkriptsioonilised muutused, et ovulatsiooni käigus väljastada küps munarakk, mis on võimeline viljastuma ja toetama embrüo arengut. Folliikuli areng on suuresti sõltuv gonadotropiinidest ja stereoidhormoonidest. Esimesed etapid folliikuli arengus (primordiaalne, primaarne ja sekundaarne) on gonadotropiinidest sõltumatud, hilisemad etapid (antraalne ja preovulatoorne) on seevastu sõltuvad folliikuleid stimuleeriva hormooni (FSH) kui ka luteiniseeriva hormooni (LH) regulatsioonist. Lisaks toodavad folliikuli granuloosrakud aromataasi abil stereoidhormoone östrogeene. Nendes etappides moodustub folliikulisse follikulaarvedelikuga (FF) täietud õõs eraldades diferentseerunud granuloosrakud kaheks alampopulatsiooniks: muraalse (MGC) ja kumuluse granuloosrakkudeks (CGC), millel on folliikuli arengus kanda erinevad rollid. Kuigi tekkinud õõs eraldab granuloosrakkude populatsioonid, võimaldab FF rakkudevahelist parakriinset suhtlust, millest sõltub munasarja folliikuli areng. Kui rakkudevaheline suhtlus folliikulis on häirunud, siis võib see põhjustada nii munaraku kvaliteedi langust kui ka viljatust.

Lisaks hormoonidele ja kasvufaktoritele kasutatakse rakkudevahelises signaliseerimises ka teisi molekule, näiteks mikroRNA-sid (miRNA-sid). miRNA-d on tuntud post-transkriptsioonilised regulaatorid. miRNA-d avalduvad rakkudes, kuid neid on tuvastatud ka kehavedelikes RNA-valk kompleksidena (RBP) või pakituna rakuvälistesse vesiikulitesse (EV). Ühest rakust vabanenud miRNA-d võivad siseneda teistesse rakkudesse ja reguleerida sealset geeniekspressiooni.

Käesoleva väitekirja eesmärgiks oli uurida miRNA-de ekspressiooni preovulatoorse folliikuli granuloosrakkudes ning nende olulisust rakkudevahelises molekulaarses signaliseerimises. Töö esimese osa fookusseeritud kahele miRNA-le: hsa-miR-548ba ja hsa-miR-7973, mis mõlemad paiknevad vastavalt inimese genoomis FSH retseptori (FSHR) ja aromataasi geenide intronites ning nende ekspressioon on eelnevalt tuvastatud nii MGC kui CGC rakkudes. Nende kahe miRNA rolli granuloosrakkudes ei olnud eelnevalt teada. Töö tulemusena selgus, et hsa-miR-548ba otsesed sihtmärkgeenid on *LIFR, PTEN, NEO1* ja *SP110*. Eelnimetatud sihtmärkidest on PTEN oluline primordiaalsete folliikulite aktiveerumise regulatsioonis. LIFR ja NEO1 mängivad samuti rolli folliikulite arengus. Teine miRNA, hsa-miR-7973, näitas otsest regulatsiooni *ADAM19, PXDN* ja *FMNL3* geenide puhul. Teadaolevalt on need valgud seotud rakuvälise maatriksi (ECM) ja rakkudevaheliste interaktsioonide ümberkorraldustes, mis on mõlemad vajalikud edukaks ovulatsiooniks. Kokkuvõttes viitab töös läbi viidud sihtmärkide analüüs nende miRNA-de erinevatele rollidele munasarja folliikulis.

On eelnevalt teada, et hsa-miR-548ba kuulub suurde hsa-mir-548 miRNA-de perekonda ja potentsiaalselt võib esineda ühiste sihtmärkide ko-regulatsiooni perekonna

liikmete vahel. Kuna hsa-miR-548ba asub FSHR geeni intronis ning miRNA ekspressioon näitab ühist mustrit oma peremeesgeeni avaldumisega, siis uuriti edasises töös FSHR-positiivseid reproduktiivkudesid. Tulemused näitasid, et hsa-miR-548ba on kõrgeimalt avaldunud granuloosrakkudes ja müomeetriumis, vastavalt koos 12 ja ühe muu liikmega samast miRNA perekonnast. MGC-des leiti ühe signaaliraja kattuv regulatsioon erinevate hsa-mir-548 perekonna liikmete poolt. Nimelt on just nendes rakkudes nii hsa-miR-548ba kui hsa-miR-548b-5p ühiseks sihtmärgiks RAB valgu geranüülgeranüülimise signaalirada, mis on oluline granuloosrakkude ja munaraku suhtluses. Teistes rakutüüpides ei leitud kattuvate signaaliradade regulatsiooni hsa-miR-548ba ja ülejäänud perekonna miRNA-de vahel.

Doktoritöö viimases osas uuriti miRNA-de potentsiaali osaleda preovulatoorse folliikuli rakkudevahelises suhtluses. Selleks koguti MGC, rakuvaba FF ja FF-st eraldatud EV-de proovid ning kirjeldati nende miRNA-de sisaldus. Tulemused osutasid, et miRNA-d, mis esinevad EV-desse pakituna, omavad potentsiaalselt olulisemat regulatoorset rolli võrrelduna FF-is väljaspool EV-sid esinevate miRNA-dega. EV-des tuvastatud miRNA-d reguleerivad kokku üle 400 signaaliraja, sealhulgas näiteks "Östrogeeni retseptori vahendatud signaliseerimine" ja "PTEN regulatsioon". Seevastu miRNA-d, mida tuvastati FF-is, kuid väljaspool EV-sid, reguleerivad ühiselt vaid 3 munasarja toimimise seisukohast mittespetsiifilist signaalirada.

Lisaks võrreldi tervete viljakate naiste (munarakudoonorite) MGC, FF ja EV miRNA profiile polütsüstilise munasarja sündroomi (PCOS) patsientide omadega. Tulemustest selgus, et PCOS mõjutab erinevalt rakkudesisest ja -välist miRNA profiili. Uuringu käigus tuvastati miRNA-sid, mida ei ole varem PCOS-iga seostatud, näiteks hsa-miR-224-5p, hsa-miR-203a-3p, hsa-miR-195-5p, hsa-miR-486-3p ja hsa-miR-484. Mõned eelnevalt PCOS-iga seostatud miRNA-dest CGC-s näitasid käesolevas töös erinevat ekspressioonitaseme muutuse suunda, mis viitab, et sama miRNA võib mängida erinevaid rolle rakutüübist sõltuvalt. Käesolevas töös selgus, et PCOS-i poolt mõjutatud miRNA-d reguleerivad erinevaid protsesse eri tüüpi proovides. Näiteks PCOS patsientides erinevalt ekspresseeritud rakulised ja väljaspool EV-sid paiknevad rakuvälised miRNA-d reguleerivad tsütokiinide poolt vahendatud signalisatsiooni, samal ajal kui EV-desse pakitud miRNA-d reguleerivad IGF1R signaalirada. See toob omakorda välja tõsiasja, et on oluline analüüsida folliikulit kui tervikut, kui eesmärgiks on modelleerida miRNA-de poolt vahendatud rakkudevahelist suhtlust.

Kokkuvõtvalt lisab käesolev töö teadmisi inimese pre-ovulatoorse folliikuli miRNA-de tähtsuse kohta nii rakkude funktsioonide kui ka rakkudevahelises molekulaarse kommunikatsiooni mõistmisel. Rakkudevaheline molekulaarne kommunikatsioon munasarjas on ülitähtis naise viljakuse tagamiseks. Käesolev töö annab informatsiooni PCOS patsientide munasarja häiritud funktsioonide kohta ning võib luua uusi võimalusi PCOS haiguse raviks.

Appendix 1

Publication I

Rooda I, Hensen K, Kaselt B, Kasvandik S, Pook M, Kurg A, Salumets A, Velthut-Meikas A. Target prediction and validation of microRNAs expressed from FSHR and aromatase genes in human ovarian granulosa cells.
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Target prediction and validation of microRNAs expressed from FSHR and aromatase genes in human ovarian granulosa cells

Ilmatar Rooda[1,2], Kati Hensen[3], Birgitta Kaselt[1], Sergo Kasvandik[4], Martin Pook[3], Ants Kurg[3], Andres Salumets[2,5,6,7] & Agne Velthut-Meikas[1,2*]

MicroRNAs (miRNAs) are known post-transcriptional regulators of various biological processes including ovarian follicle development. We have previously identified miRNAs from human pre-ovulatory ovarian granulosa cells that are expressed from the intronic regions of two key genes in normal follicular development: FSH receptor (*FSHR*) and *CYP19A1*, the latter encoding the aromatase enzyme. The present study aims to identify the target genes regulated by these miRNAs: hsa-miR-548ba and hsa-miR-7973, respectively. The miRNAs of interest were transfected into KGN cell line and the gene expression changes were analyzed by Affymetrix microarray. Potential miRNA-regulated genes were further filtered by bioinformatic target prediction algorithms and validated for direct miRNA:mRNA binding by luciferase reporter assay. *LIFR*, *PTEN*, *NEO1* and *SP110* were confirmed as targets for hsa-miR-548ba. Hsa-miR-7973 target genes *ADAM19*, *PXDN* and *FMNL3* also passed all verification steps. Additionally, the expression pattern of the miRNAs was studied in human primary cumulus granulosa cell culture in relation to the expression of their host genes and FSH stimulation. Based on our findings we propose the involvement of hsa-miR-548ba in the regulation of follicle growth and activation via LIFR and PTEN. Hsa-miR-7973 may be implicated in the modulation of extracellular matrix and cell-cell interactions by regulating the expression of its identified targets.

Granulosa cell functions are essential in follicular development, maturation, and atresia. Granulosa cells support oocyte growth via continuous bidirectional communication to ensure oocyte quality and developmental competence. Due to the close communication cumulus (CGC) and mural granulosa cells (MGC) may reflect the characteristics of the oocyte and an understanding of the patterns of expression and functions of miRNAs in those cells may lead to a better understanding of follicle maturation and its dysfunction[1,2].

MicroRNAs (miRNAs) are short non-coding RNA molecules approximately 22 nucleotides in length[3]. MicroRNAs bind to their target mRNA 3′ untranslated region (3′UTR) at sites complementary to the miRNA 5′ seed region. MicroRNA biding to the target gene regulates gene expression by destabilizing the mRNA and/or inhibiting its translation[4]. In a few cases, an increase in gene expression has also been observed[5].

MicroRNAs have key role in the post-transcriptional regulation of various important biological processes, including cell proliferation, differentiation, apoptosis, and hormone biosynthesis and secretion[6]. Functional ovary requires precise coordination of follicle recruitment, selection and ovulation processes. Development of ovarian follicles is a complex process including oocyte maturation, granulosa cell proliferation and differentiation. MicroRNAs expressed in the ovary have regulative roles in ovarian follicle development and the expression of miRNAs in the ovary varies within the specific cell type and function[7]. The overall role of miRNAs in ovarian functions has been extensively studied in the mouse model as well as in humans. *Dicer1* conditional knock-out

[1]Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia. [2]Competence Centre on Health Technologies, Tartu, Estonia. [3]Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia. [4]Proteomics Core Facility, Institute of Technology, University of Tartu, Tartu, Estonia. [5]Institute of Clinical Medicine, Department of Obstetrics and Gynecology, University of Tartu, Tartu, Estonia. [6]Institute of Biomedicine and Translational Medicine, Department of Biomedicine, University of Tartu, Tartu, Estonia. [7]Department of Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland. *email: agne.velthut@taltech.ee

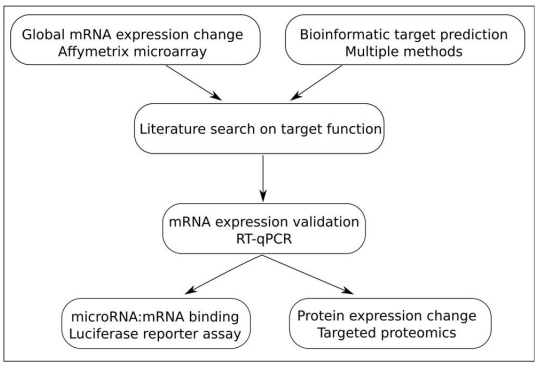

Figure 1. The rationale and methods used to identify and validate miRNA targets. Each arrow represents a filtering step, the conditions of which are specified in the text.

mice without the ability to process mature miRNAs in anti-Müllerian hormone expressing cells, including follicular granulosa cells, demonstrate abnormalities in ovulation, early embryonic development, and estrous cycles[8]. miRNA expression levels in CGC is altered in women diagnosed with polycystic ovary syndrome[9]. Additionally, there is a difference in the miRNA profile of CGC related to the meiotic maturation stage of the corresponding oocyte[10]. Therefore, granulosa cell miRNAs may serve as potential biological markers to increase the efficiency of assisted reproductive technologies by providing non-invasive means to assess oocyte quality and embryo survival potential[1].

miRNAs hsa-miR-548ba and hsa-miR-7973 were previously identified by deep sequencing of MGC and CGC populations isolated from women undergoing controlled ovarian stimulation and *in vitro* fertilization. Both miRNAs are of intronic origin: hsa-miR-548ba gene resides in the follicle stimulating hormone receptor (*FSHR*) gene and hsa-miR-7973 is located in the intron of the *CYP19A1* gene[11]. The regulatory mechanisms and target genes for those two miRNAs are currently not known.

Follicle stimulating hormone (FSH) activates time-related changes in granulosa cell gene expression by binding to FSHR promoting proliferation, differentiation, antrum formation, and oocyte maturation. Moreover, FSH stimulates aromatase expression and estrogens production[12,13]. Estrogens are produced by aromatization of androgens by aromatase enzyme encoded from *CYP19A1* gene[14]. Both FSHR and aromatase are crucial for follicle development and maturation[13].

The genomic locations of hsa-miR-548ba and hsa-miR-7973 in *FSHR* and aromatase genes, respectively, refers to potentially important regulatory roles of these miRNAs in follicle development and function. The primary aim of the current study was to identify the target genes of hsa-miR-548ba and hsa-miR-7973 in human granulosa cells by using granulosa KGN cell line as a model[15]. Secondly, the dependency of endogenous miRNA expression on their host genes and on FSH stimulation is investigated in primary human granulosa cells.

Results

Multiple methods and selection criteria were used to identify and narrow down the potential targets of hsa-miR-548ba and hsa-miR-7973. The methodological rationale for filtering the potential targets is depicted in Fig. 1.

Global gene expression changes upon transient expression of hsa-miR-548ba and hsa-miR-7973 in KGN cells. The first aim of the current study was to evaluate the effect of miRNA transfection on the global gene expression change in human granulosa cell line KGN. In non-transfected KGN cells the expression levels of hsa-miR-548ba and hsa-miR-7973 barely reached the detection limit (Supplementary Fig. 1). After optimization experiments (data not shown), the transfection of 12.5 nM miRNA mimic lead to considerably higher expression levels in comparison to primary granulosa cells (Supplementary Fig. 1). However, such level of over-expression did not influence cell viability or proliferation rate (Supplementary Fig. 2).

Genome-wide gene expression changes upon miRNA transfection were studied on Affymetrix GeneChip Human Gene 2.0 ST Array. The results demonstrated that upon hsa-miR-548ba transfection the expression level of 1,474 and upon hsa-miR-7973 the expression level of 1,552 genes changed with statistical significance (adjusted p-value < 0.01, Supplementary Table IIA,C). From those genes 1,015 were regulated by both miRNAs, 459 genes only by hsa-miR-548ba and 537 by hsa-miR-7973. Gene expression changes were calculated in comparison to the control samples transfected with miRNA cel-miR-39-3p that presumably has no target sequences in human cells.

Cluster analysis of microarray results expectedly revealed that cells transfected with different miRNA mimics formed separate clusters (Fig. 2). However, control samples expressing cel-miR-39-3p grouped separately from samples transfected with miRNAs hsa-miR-548ba and hsa-miR-7973. This is also confirmed by the overlapping number of commonly regulated genes by the human miRNAs.

Transfection of KGN cells with hsa-miR-548ba and hsa-miR-7973 leads to the regulation of several common as well as unique signaling pathways (Supplementary Table IIIA,B). Genes differentially expressed upon hsa-miR-548ba transfection are over-represented in syndecan interactions, glycosaminoglycan and fatty acid

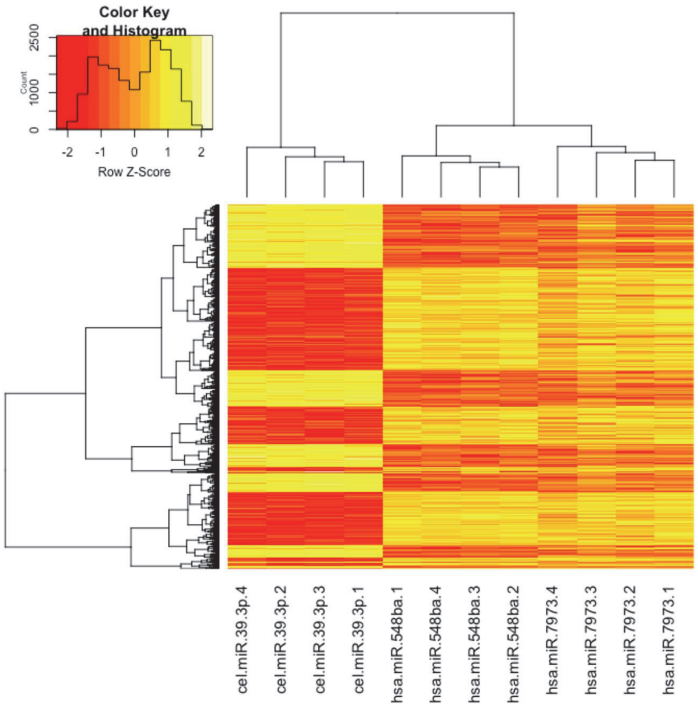

Figure 2. Cluster analysis of gene expression changes upon transfection of KGN cells with cel-miR-39p, hsa-miR-548ba or hsa-miR-7973 miRNA mimic. Gene expression changes were analyzed 72 h after transfection on Affymetrix microarray. Only statistically significant results are presented (adjusted p-value < 0.01, $n = 4$).

metabolism, unfolded protein response, G protein-coupled receptor (GPCR) and ephrin signaling among others (Supplementary Table IIIA). Expression of hsa-miR-7973 in KGN cells resulted in the regulation of genes involved in signaling by netrin-1, EGFR, PDGF, and TGF-beta receptor complex, as well as pathways related to the immune system (Supplementary Table IIIB). As several genes were regulated by both miRNAs, common targeted pathways include cholesterol biosynthesis and amino acid transport across cell membrane (Supplementary Table IIIA,B).

Bioinformatic target prediction. Microarray analysis results may partly demonstrate secondary effects on mRNA expression: gene expression changes triggered by primary miRNA targets. Therefore, to discriminate between primary and secondary target genes the differential expression results obtained from the microarray analysis method were compared to potential miRNA target mRNA-s predicted by bioinformatic algorithms. However, bioinformatic prediction may also provide false positive hits. In order to minimize such false positive findings, four target prediction programs were used, and a partial overlap of the results was used for further filtering of potential targets.

The overlap of statistically significant microarray results (adjusted p-value < 0.01) and bioinformatically predicted target genes are presented in Supplementary Table IIB and IID. Shortly, hsa-miR-548ba and hsa-miR-7973 potentially target the mRNA-s of 76 and 58 genes, respectively. Both miRNAs also share one common target mRNA of *TGFBR2* gene.

Validation of microarray results by RT-qPCR. Sixteen potential target genes from the overlapping list of microarray and bioinformatic target prediction results were validated by RT-qPCR: 8 potential targets of hsa-miR-548ba, 7 of hsa-miR-7973 and their common target *TGFBR2*. The list of validated genes was selected according to published data linking the molecular functions of these proteins to their potential importance in folliculogenesis (Table 1). As a result, *BCL2L11*, *LIFR*, *NEO1, PTEN* and *SP110* were statistically significantly down-regulated at mRNA level upon cell transfection with hsa-miR-548ba mimic ($p < 0.05$, Fig. 3a). From the list of potential hsa-miR-7973 targets the expression levels of *ADAM19*, *ATHL1*, *ATP6V1A, FMNL3* and *PXDN* were decreased with statistical significance ($p < 0.05$, Fig. 3b). The expression change of the common target gene *TGFBR2* was confirmed only in case of hsa-miR-7973 transient expression (Fig. 3b).

MicroRNA target binding validation by luciferase reporter assay. Potential miRNA targets that demonstrated significant gene expression change by microarray ($p < 0.01$) and RT-qPCR methods ($p < 0.05$)

Potential hsa-miR-548ba target genes			
Gene symbol	**Gene name**	**Cellular function**	**Ref**
BCL2L11	BCL2 Like 11	BCL-2 family activator of apoptosis.	33
GALNT7	Polypeptide N-Acetylgalactosaminyltransferase 7	Androgen-dependent GalNAc-transferase involved in mucin-type O-linked protein glycosylation.	61
INSIG1	Insulin Induced Gene 1	Regulates cholesterol synthesis.	62
LIFR	LIF Receptor Alpha	Growth initiation of human primordial follicles.	24
NEO1	Neogenin 1	Binds directly bone morphogenetic proteins (BMPs).	27
PTEN	Phosphatase And Tensin Homolog	Activation of primordial follicles from the dormant pool.	25
RARB	Retinoic Acid Receptor Beta	Nuclear receptor regulating the transcription of steroidogenic genes in the ovary.	63
SP110	SP110 Nuclear Body Protein	Nuclear hormone receptor coactivator, enhances retinoic acid receptor signal.	36
Potential hsa-miR-7973 target genes			
Gene symbol	**Gene name**	**Cellular function**	**Ref**
ADAM19	ADAM Metallopeptidase Domain 19	Cleaves extracellular matrix (ECM) proteins	38
ATHL1	Acid Trehalase-Like Protein 1	Cleaves ECM protein collagen.	64
ATP6V1A	ATPase H + Transporting V1 Subunit A	Involved in energy metabolism, expression correlates with human embryo quality.	65
FMNL3	Formin Like 3	Involved in cell-cell adhesion.	42
NPR3	Natriuretic Peptide Receptor 3	Maintains oocyte meiotic arrest.	66
PXDN	Peroxidasin	ECM protein with peroxidase activity.	39
STC1	Stanniocalcin 1	Regulates phosphate metabolism, potential luteinization inhibitor.	67
Potential hsa-miR-548ba and hsa-miR-7973 common target gene			
Gene symbol	**Gene name**	**Cellular function**	**Ref**
TGFBR2	Transforming Growth Factor Beta Receptor 2	Intercellular communication during ovarian follicle development.	68

Table 1. Potential target genes of hsa-miR-548ba and hsa-miR-7973 used in validation studies and their cellular functions.

were further validated for direct miRNA:mRNA binding by luciferase reporter assay. These included hsa-miR-548ba potential target genes: *BCL2L11*, *LIFR*, *NEO1*, *PTEN* and *SP110*, and hsa-miR-7973 target genes: *ADAM19*, *ATHL1*, *ATP6V1A*, *FMNL3* and *PXDN*. Although *TGFBR2* gene expression change was not confirmed by RT-qPCR in hsa-miR-548ba transfected cells, binding of its 3′UTR to both miRNAs under study was nevertheless assessed.

Potential target gene *PTEN* was tested with two versions of 3′UTR lengths: longer version obtained from the UCSC Genome browser and shorter sequence used by the miRDB bioinformatical prediction program, further noted as *PTEN* long and *PTEN* short, respectively. pmirGLO-3′UTR-PTEN vector and hsa-miR-21-5p were used as a positive control pair for miRNA:mRNA binding to confirm our luciferase assay reliability, as *PTEN* has been previously shown to be a target for hsa-miR-21-5p[16]. Upon direct binding of miRNA to the 3′UTR sequence of its target mRNA, reduction in the measured luciferase signal is expected. Such suppression of luciferase signal upon hsa-miR-21-5p binding to *PTEN* 3′UTR is demonstrated in Supplementary Fig. 3 for both long and short variants.

Luciferase assay results confirmed the direct binding of hsa-miR-548ba to *LIFR, NEO1*, and *SP110* 3′UTR sequences. Hsa-miR-548ba bound to its potential target *PTEN* 3′UTR sequence only when the longer version was used (Fig. 4a). From the tested potential targets of hsa-miR-7973 direct binding occurred on the 3′UTR of *ADAM19*, *FMNL3* and *PXDN* (Fig. 4b).

Effect of hsa-miR-548ba and hsa-miR-7973 transient expression on the endogenous protein levels of their target genes. The endogenous expression levels of target proteins were assessed at two time-points: 72 h (coinciding with the highest effect of miRNA transfection on target gene mRNA levels) and 96 h after transfection with miRNA mimics. As we are unable to account for effects triggered by long-term culture, secondary miRNA targets and the kinetics of each endogenous protein expression and stability, the difference in protein levels between the time-points and between cells transfected with miRNAs under study vs control miRNA cel-miR-39-3p were investigated. We observed different trends in endogenous protein expression levels for PTEN between cells transfected with has-miR-548ba and cel-miR-39-3p: while in former samples the expression level increased 4.34 times, in control cells the levels decreased 1.28 times from 72 h to 96 h (Supplementary Fig. 4A).

The level of PXDN, a potential target of hsa-miR-7973, decreased from 72 h to 96 h by 2-fold. In comparison, the level of PXDN was up-regulated 2 times during the observed time-frame in control samples. A trend for at least 2-fold change in protein expression between time-points and opposite direction of expression levels between cells transfected with hsa-miR-7973 and cel-miR-39-3p was also observed for TGFBR2. The expression of ATHL1 did not change in control experiment, while it increased 2-fold from 72 h to 96 h in cells transfected with hsa-miR-7973 (Supplementary Fig. 4B).

Protein expression remained below detection limit for BCL2L11, LIFR and ADAM19.

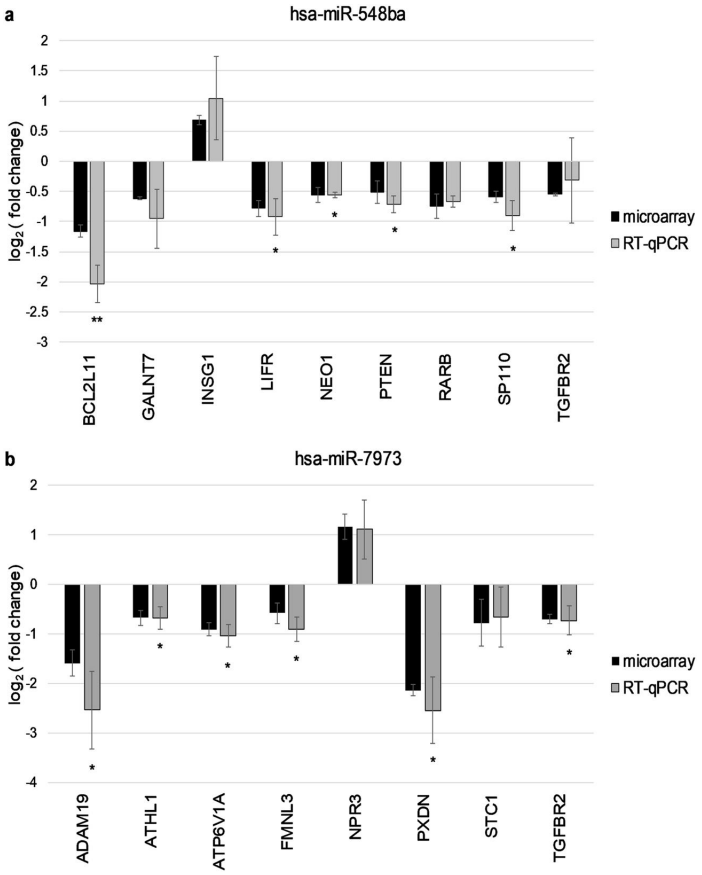

Figure 3. Validation of microarray results by RT-qPCR. (**a**) Potential target genes of hsa-miR-548ba and (**b**) Potential target genes of hsa-miR-7973. Gene expression change is calculated by comparing expression levels to samples transfected with control miRNA cel-miR-39-3p. Results are displayed as average fold change ±SD on $\log_2$ scale (*$p < 0.05$; **$p < 0.01$; Student t-test, $n = 3$). All presented microarray results were statistically significant (adjusted $p < 0.01$).

Influence of FSH stimulation on the expression of miRNAs and their host genes in primary granulosa cells. Hsa-miR-548ba and hsa-miR-7973 genes are located in the intronic regions of *FSHR* and *CYP19A1* genes, respectively. To test the correlation between miRNA and its host gene expression levels, long-term culture of primary human CGC and MGC was used. Cells were cultured in parallel with or without FSH stimulation (1 IU/ml) that is well known to increase the expression levels of both, FSHR and CYP19A1[13].

Hsa-miR-548ba was expressed well in correlation with its host gene *FSHR* both under stimulated and non-stimulated conditions similarly in both cell populations (Fig. 5a and Supplementary Fig. 5a). While the expression level of *CYP19A1* mRNA increased upon treatment of cells with FSH, such increase was not observed for the expression of hsa-miR-7973 in neither of the cell populations (Fig. 5b and Supplementary Fig. 5b). However, the expression of hsa-miR-7973 remained at a steady level up to 24 days in the presence of FSH in the culture medium in comparison to cells without FSH treatment. The latter result was statistically significant only in CGC (Fig. 5b).

Discussion

We present the first identification and validation results of hsa-miR-548ba and hsa-miR-7973 target genes.

The miRNAs of interest: hsa-miR-548ba and hsa-miR-7973, were both identified for the first time from human ovaries by deep sequencing approach of primary granulosa cells[11]. In the experiments of the current study we first used granulosa tumor-like KGN cell line as a granulosa cell model[15]. KGN cells express the functional FSH receptor and high levels of aromatase, encoded from miRNA hsa-miR-548ba and hsa-miR-7973 host genes *FSHR* and *CYP19A1*, respectively. KGN cell line has also been previously used for miRNA target identification and luciferase assay studies for direct miRNA:mRNA interactions[17].

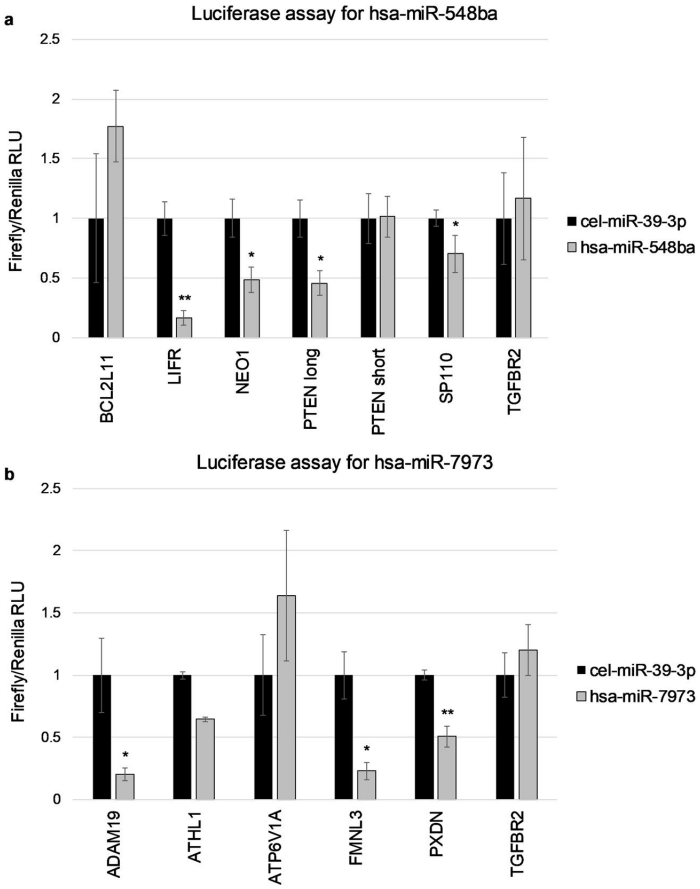

Figure 4. Validation of miRNA binding on the 3′UTR sequences of their potential target mRNAs by luciferase reporter assay. (**a**) hsa-miR-548ba potential target genes. (**b**) hsa-miR-7973 potential target genes. Results are shown as average normalized luciferase signal ± SEM, (*$p < 0.05$; **$p < 0.01$; Student one-tailed t-test, $n = 3$). RLU – relative light unit.

MicroRNAs are estimated to regulate 60% of all protein-coding genes and a single miRNA may regulate up to 400 different target mRNAs[18]. Our microarray results demonstrated approximately 1,500 differentially expressed genes upon transfection with either of the miRNAs under study, while 459 genes were regulated specifically by hsa-miR-548ba and 537 by hsa-miR-7973. Gene ontology analysis from hsa-miR-548ba regulated genes showed enrichment in fatty acid metabolism and ephrin signaling pathways and over-expression of hsa-miR-7973 showed enrichment in TGF beta receptor complex and immune system pathways. Commonly regulated genes by these two miRNAs targeted the cholesterol biosynthesis pathway. Cholesterol is a major component for steroidogenesis and sources of cholesterol for ovarian granulosa cells include plasma lipoproteins and de novo-synthesis[19]. Therefore, the regulation of cholesterol biosynthesis pathway by the miRNAs under study may influence steroid hormone production, contributing to the follicular developmental stage-dependent estradiol and progesterone production.

Although, miRNAs may regulate several hundreds of target genes, microarray results do not differentiate primary targets from secondary regulatory effects: i.e. the genes regulated by primary miRNA targets. Such secondary regulatory effects are common in over-expression conditions and large data-sets obtained by genome-wide methods[20]. The large number of commonly regulated genes by both miRNAs analyzed in the present study can be also explained by secondary effects on gene expression, as the over-expression of miRNAs upon transfection may lead to the saturation of RNA silencing complex (RISC) which results in altered gene expression regulation[21]. To distinguish primary miRNA targets from secondary ones, microarray results were compared to bioinformatically predicted target genes.

For direct miRNA:mRNA binding studies luciferase assay reporter vectors were cloned with potential target gene 3′UTR sequences. In this study full length 3′UTR sequences were used, except for *PTEN* in case of

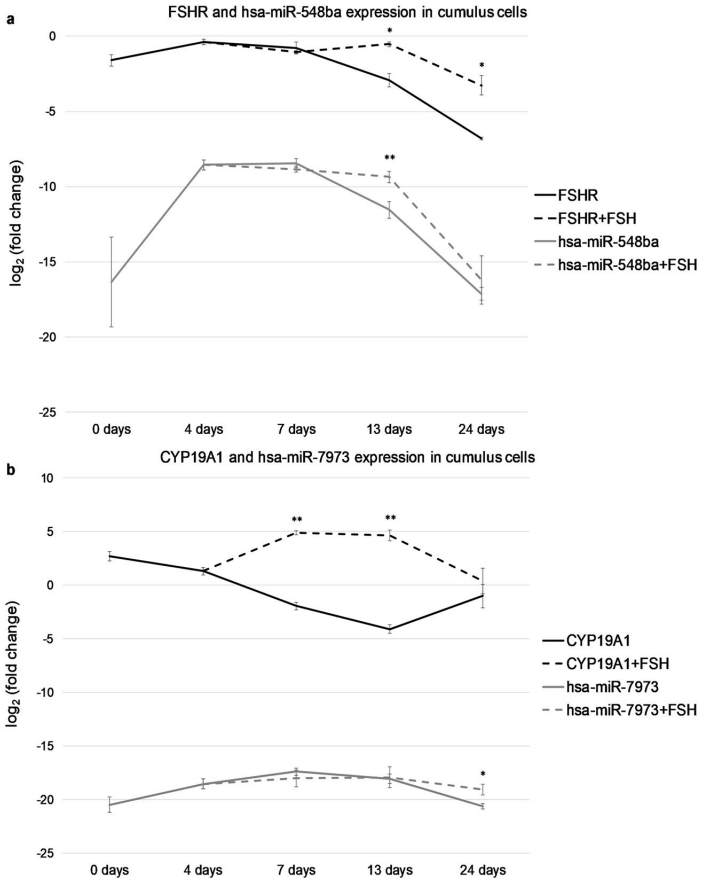

Figure 5. miRNA and host gene expression levels in primary human cumulus granulosa cells. mRNA and miRNA expression levels were normalized to SDHA or hsa-mir-132-3p, respectively. (**a**) hsa-miR-548ba and FSHR expression and (**b**) hsa-miR-7973 and CYP19A1 expression. Results are displayed as average fold change ± SD on $\log_2$ scale (*$p < 0.05$; **$p < 0.01$; Student t-test between cells exposed to 1 IU/ml FSH vs non-treated cells, n = 3).

which bioinformatic prediction algorithms do not use consistent 3′UTR sequence lengths and thus two different sequences were analysed. We chose to test full-length sequences for most of the target genes as we lacked the knowledge of functional miRNA target regions and the exact biding site identification within each potential target gene was not the goal of the current study. Additionally, testing a fragment of 3′UTR may easily lead to false results due to the different secondary structure from the full length sequence[22]. Direct binding assay confirmed *LIFR*, *PTEN*, *NEO1* and *SP110* as hsa-miR-548ba targets. For target gene *PTEN* direct binding occurred only with the longer 3′UTR sequence, although bioinformatic algorithms used in this work predicted target sequences into the shorter 3′UTR isoform. Such results can be explained by the differences in the secondary structure mentioned above. Moreover, bioinformatical programs might not predict all of the potential non-canonical miRNA target sequences.

The change in endogenous target protein expression levels was observed for PTEN (in case of transfection with hsa-miR-548ba), as well as for PXDN, TGFBR2 and ATHL1 (targets of hsa-miR-7973) in comparison to cells transfected with cel-miR-39-3p. As the regulation kinetics is expectedly different for each protein, the molecular mechanism behind the up-regulation of PTEN and downregulation of other targets cannot be explained based solely on our experiments. We demonstrate that the expression of the miRNAs under study influence the protein levels of their target genes, however, the changes in protein expression cannot be uncoupled from the effects of secondary targets and signaling pathways activated upon transient miRNA transfection.

The confirmed targets for hsa-miR-548ba LIFR, PTEN and NEO1 all play a role in the early stages of follicle development. Leukemia inhibitory factor (LIF), the ligand for LIFR, promotes primordial to primary follicle transition in the rat ovary[23]. In humans, the role of LIF and its receptor in the ovary is not well characterized, but LIF

and LIFR mRNA expression in human ovarian samples are consistent with the concept that LIF might be involved in growth initiation of human primordial follicles through its receptor[24]. The recruitment of primordial follicles is regulated by highly controlled mechanisms and the critical role is played by PI3K-Akt signaling pathway. This pathway is negatively regulated by PTEN and it has been shown that in mice lacking *PTEN* the entire primordial follicle pool becomes activated[25]. The levels of PI3K-Akt signaling pathway components in granulosa cells have a correlation with oocyte competence in bovine as decreased PTEN expression and increased expression of PTEN downregulating miRNAs correlate with the number of high-quality oocytes[26].

NEO1 is a receptor for repulsive guidance molecules, netrins, and is involved in bone morphogenetic protein (BMP) signaling pathways by directly binding BMPs[27]. Moreover, NEO1 activates RAC1-PI3K-AKT signaling pathway in human gastric cancer cells[28]. In mouse ovary, Rac1 is involved in primordial follicle formation by inducing nuclear transport of SMAD3[29]. As mentioned above, PI3K-AKT signaling pathway have crucial roles to balance follicle growth suppression, activation and progression also in humans[30] and BMPs have important role in follicle development including primordial germ cell development, oocyte-somatic cell interactions and modulating cumulus-oocyte complex (COC) formation and expansion[31].

Moreover, direct hsa-miR-548ba targets *PTEN*, *NEO1* and potential secondary target *BCL2L11* are also involved in regulating apoptosis[32–34]. In cattle, follicle atresia is regulated by LIF-STAT3 pathway which can be reversed with FSH administration[35]. In the current study, the transient expression of hsa-miR-548ba did not lead to changes in overall cellular viability during 72 h. Therefore, the role of hsa-miR-548ba in modulating apoptotic pathways in the human ovary needs to be investigated further.

The role of SP110 in the ovary is less clear. It has been suggested that SP110 may function as a nuclear hormone receptor transcriptional coactivator via binding to the retinoic acid receptor alpha (RARA) response element[36]. Retinoids are essential for steroid production and it has been demonstrated that bovine CGCs express active RARA[37].

The confirmed targets of hsa-miR-7973 according to our study were *ADAM19*, *PXDN* and *FMNL3*, all of which have been shown to be involved in extracellular matrix (ECM) modulation and cell-cell interactions[38,39]. ADAM19 is involved in ECM remodeling by cleaving ECM proteins, growth factors and cytokines like neuregulin[38]. Neuregulin 1 is expressed by granulosa cells and regulates the meiotic resumption time of the oocyte leading to proper oocyte developmental competence in cultured COCs[40].

Other two confirmed targets of hsa-miR-7973, PXDN and FMNL3, have not been studied in relation to folliculogenesis, but are involved in inter-cellular and cell-ECM contacts. PXDN is a peroxidase previously investigated in the context of ovarian cancer where the knockdown of this gene inhibits cellular proliferation, invasion and migration[39]. FMNL3 protein is involved in cytoskeletal organization, cell morphology, migration and cell-cell adhesion[41,42].

Genes demonstrating negative results in luciferase reporter assay can be considered as secondary targets of the miRNAs of interest, but one should consider that the experiments were performed with reporter vectors and in cell line conditions that may not fully reflect physiological conditions in primary cells. MiRNA target recognition may require multiple transacting factors available only in specific cellular contexts. Also the alternative polyadenylation of 3′UTRs in reporter vectors may increase the number of false-negative results[43]. In addition, many possible target genes were excluded in this study due to statistically non-significant results from microarray gene expression analysis and were thus not tested in the direct binding assay. Therefore, due to the stringent filtering approach used in the current study, the list of validated target genes for these two miRNAs of interest is not final.

Another goal of the study was to investigate, whether the expression of miRNAs coincides with the levels of their host genes. This may give indication on the biogenesis of the miRNAs under study, as intronic miRNAs may be generated alongside with the pre-mRNA intron splicing mechanism[44,45]. As KGN cells do not endogenously express detectable levels of hsa-miR-548ba and hsa-miR-7973, primary cell cultures of human stimulated cumulus and mural granulosa cells were used. We demonstrate that the miRNAs under study are stably expressed in the long-term culture of human primary granulosa cells for 24 days. It is clear from our results that the regulation of expression of the two miRNAs is achieved by different mechanisms. Hsa-miR-548ba is expressed in correlation with the mRNA of its host gene *FSHR*, both in presence or without FSH in culture medium. Such co-expression indicates the possibility that the miRNA is excised from the intron of *FSHR* pre-mRNA. However, there is no substantial co-expression of hsa-miR-7973 and its host gene *CYP19A1*. Hsa-miR-7973 is stably expressed over time, indicating a different mechanism of regulation: possibly an alternative promoter for the miRNA gene. However, additional experiments are necessary to draw firm conclusions about the biogenesis of these miRNAs.

Overall, we identified and confirmed several targets for hsa-miR-548ba and hsa-miR-7973 and showed that the endogenous expression of these miRNAs is maintained in long-term culture of primary granulosa cells. According to the previously known functions of their target genes we propose undescribed regulatory roles for these miRNAs in granulosa cell gene expression and in follicle development.

Materials and Methods

Cell culture of KGN cell line. Granulosa tumor-like cell line KGN was used as a model for cell line experiments[15]. Cells were grown in L-glutamine-containing Dulbecco's Modified Eagle's medium with 10% fetal bovine serum, 100U/ml penicillin and 0.1 mg/ml streptomycin on 60 × 15 mm culture plates at 37 °C and 5% CO_2. Cells were harvested with 0.05% trypsin and 0.02% EDTA (all solutions from Naxo, Tartu, Estonia).

Isolation of primary granulosa cells. The study was approved by the Ethics Committee of the University of Tartu and conducted according to the guidelines of the Declaration of Helsinki. An informed consent was obtained from all participants. For miRNA expression validation granulosa cells were collected from 8 women undergoing ovarian stimulation according to the GnRH antagonist protocol (described below), ovarian puncture (OPU) and oocyte intra-cytoplasmic sperm injection due to male-factor infertility. The general characteristics

of the study participants were as follows (mean ± SD): age 31.0 ± 6.2 years, BMI 22.9 ± 3.5, total FSH amount used 1528.0 ± 653.8 IU, the number of retrieved oocytes was 12.0 ± 9.1, of which 75.0 ± 22.2% were considered mature according to the visibility of the first polar body. For the long-term granulosa cell culture experiments samples were collected anonymously from consecutive *in vitro* fertilization patients and pooled for the reduction of inter-patient variability. Each pool consisted of granulosa cells from ≥ 10 patients.

Ovarian hormonal stimulation was conducted according to the GnRH antagonist (Cetrotide, Merck Serono, Geneva, Switzerland) protocol with the administration of recombinant FSH (Gonal-F, Merck Serono, or Puregon, Merck Sharp & Dohme Corp., Whitehouse Station, NJ, USA). All patients underwent OPU of follicles ≥ 15 mm in size after 36 h of hCG administration (Ovitrelle, Merck Serono).

MGCs were obtained from follicular fluid after OPU following the manual removal of cumulus-oocyte complexes and CGC aggregates devoid of the oocyte. The fluid from all follicles of a patient was pooled, centrifuged at 450 *g* for 10 minutes, followed by supernatant removal. The cells were separated on a 50% density gradient of PureSperm 100 (Nidacon; Mölndal, Sweden) in Universal IVF Medium (Origio; Jyllinge, Denmark), washed three times in Universal IVF Medium at 37 °C, depleted of CD45-positive leukocytes according to the manufacturer's suggested protocol (DynaMag and Dynabeads; Life Technologies, Carlsbad, CA, USA), lysed with QIAGEN miRNeasy Mini kit lysis buffer (QIAGEN, Hilden, Germany), and stored in liquid nitrogen.

CGC were collected 4 h after OPU during oocyte denudation lasting up to 5 min with type IV-S hyaluronidase extracted from bovine testes (Sigma-Aldrich, St-Louis, MO, USA) and diluted in Sperm Preparation Medium (Origio). The CGC from all oocytes irrespective of their maturity were pooled per patient and centrifuged at 450 g for 5 min and the supernatant was discarded. For direct analysis of miRNA content, the cells were lysed and stored as described above.

Cumulus granulosa cell culture. For primary cell culture experiments the collected cumulus cells were pooled (pools of 10–30 patients). Cells were counted and 500 000 cells were plated onto Matrigel-covered 6-well plates (Corning, New York, NY, USA) in DMEM/F12 growth medium (Lonza AG, Basel, Switzerland) with 20% KnockOut Serum Replacement (KSR) (Thermo Scientific, Waltham, MA, USA), 1% penicillin/streptomycin (Naxo, Tartu, Estonia) and Primocin (100 μg/ml) (InvivoGen, San Diego, CA, USA). IGF2 (50 ng/ml) (Bio-Techne, Minneapolis, MN, USA) and FGF2 (8 ng/ml) (Enantis, Brno-Bohunice, Czech Republic) were added to the growth medium. The effect of FSH (1 U/ml) (Gonal-F, Merck Serono) was tested starting from day 4, because it has been shown that GCs are not responsive to hormones right after collection[46]. Growth medium was changed daily. Cells were passaged using TrypLE (Thermo Scientific), half of the cells were re-plated and half were lysed using QIAzol lysis reagent (QIAGEN). All cell culture experiments were repeated three times.

Cell line transfection. For microRNA transfection Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) and miRCURY LNA miRNA mimics hsa-miR-548ba, hsa-miR-7973 and control miRNA with no mammalian homologue cel-miR-39-3p[47,48] were used according to manufacturer's protocol (Exiqon, Vedbaek, Denmark). MicroRNA mimics were transfected at previously optimized concentration of 12.5 nM and 72 h time-point after transfection was tested.

For miRNA and luciferase vector co-transfection Lipofectamine 2000 reagent (Invitrogen) and previously specified miRCURY LNA miRNA mimics were used according to manufacturer's protocol. MicroRNA final concentration in culture medium was 12.5 nM or 50 nM, while 100 ng of vector was transfected.

Cytotoxicity analysis. MicroRNA cytotoxicity analysis was performed using CytoTox-Glo Cytotoxicity Assay (Promega Corporation, Madison, WI, USA) according to the user manual. Five thousand cells were plated in each well of a white Greiner CELLSTAR 96-well plate (Sigma-Aldrich) and transfection was performed 24 h after plating. Non-transfected control samples were measured at 0, 24, 48, and 72 h. The cytotoxicity of cells transfected with either hsa-miR-548ba, hsa-miR-7973 or cel-miR-39-3p mimics was measured at 24, 48 and 72 h post-transfection. The above-mentioned assay uses a luminogenic peptide substrate to measure dead-cell protease activity, which is released from cells upon losing their membrane integrity. Luminescence was measured in two steps with Tecan GeniosPro luminometer (Tecan, Männedorf, Switzerland). Firstly, luminescence from dead cells and secondly luminescence from all cells after lysis was detected. Results were calculated as luminescence from all lysed cells minus luminescence from dead cells. All samples were measured in triplicates and the average value of three independent experiments was used in data analysis.

RNA extraction. Cells were lysed in QIAzol lysis reagent (QIAGEN). For total RNA extraction miRNeasy Mini Kit (QIAGEN) was used. In addition, small fraction RNA (≤ 200 nucleotides) extraction was performed separately using RNeasy Mini Elute Cleanup Kit (QIAGEN). Both total and small RNA extraction was performed according to the user manual. RNA concentrations were measured using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany).

The quality of all RNA samples analyzed on Affymetrix microarray was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany). All RNA samples were of high quality, with the RNA Integrity Number values between 9.2–9.8.

Affymetrix microarray. Affymetrix microarray was performed at the Array and Analysis Facility at Uppsala Biomedical Center, Sweden. From each sample 250 ng of total RNA were used to generate amplified and biotinylated sense-strand cDNA according to the GeneChip WT PLUS Reagent Kit User manual (Affymetrix, Santa Clara, CA). GeneChip Human Gene 2.0 ST Arrays were hybridized for 16 h in 45 °C incubator, rotated at 60 rpm. Arrays were washed and stained using the Fluidics Station 450 and scanned using GeneChip Scanner 3000 7 G. Affymetrix microarray data is available at Gene Expression Omnibus repository, accession number GSE122731.

Reverse transcription. For cDNA synthesis from mRNA SuperScript III First Strand Synthesis SuperMix (Invitrogen) was used according to user manual. 1 μg of cell line and 500 ng of primary granulosa cell RNA was used as input.

For microRNA detection cDNA was synthesized using miRCURY LNA™ Universal RT microRNA PCR Universal cDNA Synthesis Kit III (Exiqon). 200 ng of small fraction RNA was used as input.

Primer design. Primers used for RT-qPCR analysis were designed using NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Preferred primers spanned exon-exon junctions and amplified all splice isoforms of the given gene. Primer sequences are presented in Supplementary Table IA.

Primers for amplifying the 3′UTR sequences of potential miRNA target genes were designed in Benchling (https://benchling.com/). Primer binding specificity was tested using NCBI primer-BLAST (Supplementary Table IB).

For the amplification of miRNAs, commercially available primers were used (Exiqon).

RT-qPCR. The RT-qPCR analysis was carried out using SDS 2.3 software in 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California). For the detection of mRNA expression, Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) or Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) was used. cDNA from small RNA fraction was amplified by EXILENT SYBR Green Mastermix (Exiqon) according to the user manual. Each sample was run in triplicates on a 384-well plate up to 40 amplification cycles. The specificity of amplified PCR products was determined by melt curve analysis.

Luciferase reporter vector cloning and luciferase assay. The full length 3′UTR of hsa-miR-548ba potential target genes *BCL2L11*, *LIFR*, *NEO1*, *PTEN*, *RARB*, *SP110*, hsa-miR-7973 target genes *ADAM19*, *ATHL1*, *ATP6V1A*, *PXDN*, *FMNL3*, and the common target for both miRNAs *TGFBR2* were cloned into pmirGLO Dual-Luciferase vector (Promega) downstream of the Firefly luciferase gene. Target gene 3′UTR sequences were obtained from UCSC Genome Browser (https://genome.ucsc.edu/) with an exception of *FMNL3* where shorter isoform of 3′UTR was used. Bioinformatic prediction program TargetScan 7.1[49] uses *FMNL3* 3′UTR isoform with length 7,874 nt, while in UCSC Genome Browser the length is 9,316 nt. Moreover, for *PTEN* also a shorter version of 3′UTR isoform was cloned which is used by miRDB bioinformatical target prediction program[50]. Potential target gene 3′UTR sequences and predicted miRNA binding sites are available in Supplementary Material. Primers for amplifying full length 3′UTR from genomic KGN cell line DNA and restriction enzymes used for cloning are presented in Supplementary Table IB.

Validation of hsa-miR-548ba and hsa-miR-7973 direct binding to target gene 3′UTR was performed by using Dual-Glo Luciferase assay system (Promega) according to the user manual. Luciferase signal was measured 24 h after miRNA and vector co-transfection. The relative luciferase activities were determined by calculating the ratio of Firefly luciferase activities over Renilla luciferase activities. All experiments were repeated three times as triplicates on a white Greiner CELLSTAR 96-well plate (Sigma-Aldrich). Cel-miR-39-3p was used as a negative control.

Design of peptides for targeted proteomics. Synthetic peptides for targeted proteomics were designed based on the total proteome analysis of KGN cell lysate (described in Supplementary Materials). Two peptides per protein were selected. In addition, GAPDH was selected as an endogenous control. Peptides with the highest intensity in the library and without methionines and cysteines were preferred for the targeted analysis. The sequences of the synthetic peptides are presented in Supplementary Table IC.

Sample preparation for targeted proteomics analysis. KGN cells were transfected with hsa-miR-548ba, hsa-miR-7973 or cel-miR-39-3p mimics in triplicates as described above. After 72 h and 96 h post-transfection the cells were removed from the plate by trypsin, washed with PBS, and centrifuged at 1,700 g.

The pelleted cells were suspended in 10 volumes of 4% SDS, 100 mM Tris-HCl pH 7.5, 100 mM DTT lysis buffer. Samples were heated at 95 °C for 5 min, followed by probe sonication (Bandelin, Berlin, Germany) (60 × 1 s pulses, 50% intensity). Unlysed material were cleared with centrifugation at 14,000 g for 10 min, although, lysis was noted to be nearly complete. 30 μg of protein was precipitated with 2:1:3 (v/v/v) methanol:chloroform:water. Protein pellets were suspended in 25 μl of 7 M urea/ 2 M thiourea 100 mM ammonium bicarbonate (ABC) solution, followed by disulfide reduction and cysteine alkylation with 5 mM DTT and 10 mM chloroacetamide for 30 min each at room temperature. Proteins were predigested with 1:50 (enzyme to protein) Lys-C (Wako Chemicals, Neuss, Germany) for 4 h, diluted 5 times with 100 mM ABC and further digested with trypsin (Sigma Aldrich) overnight at room temperature. Peptides were desalted with in-house made C18 StageTips.

Targeted LC/MS/MS analysis. Peptides were injected to an Ultimate 3000 RSLCnano system (Dionex, Sunnyvale, CA, USA) using a 0.3 × 5 mm trap-column (5 μm C18 particles, Dionex) and an in-house packed (3 μm C18, Dr Maisch, Ammerbuch-Entringen, Germany) analytical 50 cm × 75 μm emitter-column (New Objective, Woburn, MA, USA). Peptides were eluted at 250 nl/min with an A to B 10–45% 60 min gradient (buffer A: 0.1% formic acid, buffer B: 80% acetonitrile + 0.1% formic acid) to a Q Exactive Plus (Thermo Fisher Scientific) MS/MS using a nano-electrospray source (positive mode, spray voltage of 2.6 kV). The MS was operated in a scheduled parallel reaction monitoring (PRM) mode by isolating and fragmenting only peptides from the selected proteins within ± 4 minute windows of their retention times. Retention time scheduling was calibrated using the indexed retention time (iRT) method[51]. MS/MS isolation window was 1.0 m/z and scans were performed at a resolution setting of 17,500. Ion target value of 2e5 and fill time of 60 ms were used. Normalized collision energy was set at 26.

Data analysis and statistics. *Affymetrix microarray data analysis.* Affymetrix microarray raw data was normalized in Expression Console, provided by Affymetrix (http://www.affymetrix.com), using the robust multi-array average (RMA) method. Gene annotations were drawn from the probeset ID-s of the HuGene-2_0-st chip. Differential gene expression analysis was carried out in the statistical computing language R (http://www.r-project.org) using packages available from the Bioconductor project (www.bioconductor.org)[52]. To detect differentially expressed genes between samples transfected with hsa-miR-548ba or hsa-miR-7973 and cel-miR-39-3p groups, an empirical Bayes moderated t-test was applied using the "limma" package[53]. The p-values were adjusted for multiple testing according to the method of Benjamini and Hochberg[54]. Statistical significance level was set at adjusted $p < 0.01$.

For cluster analysis of microarray data genes were ordered by the highest fold change expression difference upon hsa-miR-548ba transfection and Bioconductor package "heatmap" was used.

Gene ontology analysis. Analysis of over- and under-representation of Reactome pathways (version 58) was performed in the Panther classification system[55]. Genes differentially expressed (adjusted $p < 0.01$) on Affymetrix microarray upon transfecting KGN cells with either hsa-miR-548ba or hsa-miR-7973 mimics compared to the transfection with cel-miR-39-3p were used as input. Fisher's exact test with Benjamini and Hochberg false-discovery rate (FDR) correction was used to test the statistical significance of the results and over- or under-represented pathways obtaining $FDR < 0.05$ are reported.

MicroRNA target gene prediction. For miRNA bioinformatic target prediction four web based programs were used (DIANA microT v 3.0[56], microT CDS v 5.0[57], TargetScan 7.1[49] and miRDB[50]). Gene was considered as a potential miRNA target in case it was predicted by at least two programs out of four and its gene expression fold change according to microarray analysis was $\geq \log_2(|0.3|)$.

RT-qPCR. The gene-specific mRNA expression Ct values were normalized against *GAPDH* expression and miRNA expression levels were normalized for internal control hsa-miR-132-3p using the method of relative quantification by Pfaffl[58]. Several control small RNAs were tested for the stability of their expression levels and hsa-miR-132-3p was chosen due to the least variability in its expression between patients and granulosa cell populations (Supplementary Methods). In primary CGC culture experiments *SDHA* was used as an endogenous control for the normalization of mRNA levels. Experiments were run in technical triplicates three times. Results are shown as average ± SD (standard deviation). Statistical significance was calculated by two-tailed Student t-test in Microsoft Office Excel 2017. Statistical significance level was set at $p < 0.05$.

Luciferase assay. The relative luciferase activities were determined by calculating the ratio of Firefly luciferase relative light units (RLU) over Renilla luciferase RLU. All experiments were run independently three times as triplicates. Results are shown as average ± SE (standard error). Statistical significance was calculated by one-tailed Student t-test in Microsoft Office Excel 2017. Statistical significance level was set at $p < 0.05$.

LC/MS/MS data analysis. MS raw files were analysed with the Skyline software[59]. Spectral library was created from Mascot (Matrix Science, Woburn, MA, USA) database search results derived from measuring synthetic peptides (JPT Technologies, Berlin, Germany) with data-dependent LC/MS/MS mode. Only y-ions (starting from ion 4, y4 up to last ion −1) with charge states + 1 and + 2 were allowed. All extracted ion chromatogram (XIC) integrations were manually inspected for correct peak picking. Fragment XIC traces with strong interference and erroneously picked peaks (mass errors > ± 20 ppm, lack of fragment coelution) were removed.

For the comparison of protein expression levels between samples, peak areas referring to the same proteins were summed. Results were transferred to MaxQuant Perseus module[60], $\log_2$-transformed and filtered for at least 50% valid values in each group. Missing values were imputed by down-shifting and compressing the measured intensity distributions by 1.8 and to 0.3 standard deviation units, respectively, thereby simulating intensity measurements on the measurement threshold. All values were then normalized to GAPDH expression. Two comparisons per protein were made: (a) change in expression level between 96 h and 72 h after miRNA transfection, and (b) change in expression between cells transfected with either hsa-miR-548ba or hsa-miR-7973 and cel-miR-39-3p. Difference in the level of protein expression is reported, if the expression change between time-points is > 2-fold and if opposite direction of expression change is observed between the cells transfected with the miRNAs under study and the control miRNA.

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Author contributions

I.R., K.H., B.K., S.K., M.P. and A.V.-M. were involved in performing the experiments and data analysis. I.R., A.S., S.K. and A.V.-M. made substantial contribution to the concept and design of the study, wrote and revised the manuscript. A.K. contributed to the interpretation of the data and revision of the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.V.-M.

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Appendix 2

Publication II

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Hsa-mir-548 family expression in human reproductive tissues

Ilmatar Rooda[1,2*], Birgitta Kaselt[1], Maria Liivrand[1], Olli-Pekka Smolander[1], Andres Salumets[2,3,4,5] and Agne Velthut-Meikas[1]

Abstract

Background: Hsa-miR-548ba expressed in ovarian granulosa cells targets PTEN and LIFR, which are essential for ovarian follicle activation and growth. The expression pattern of hsa-miR-548ba correlates with its host gene follicle-stimulating hormone receptor (FSHR), and FSH has a positive influence on hsa-miR-548ba expression. However, hsa-miR-548ba is a member of a large hsa-mir-548 family with potentially overlapping targets. The current study aims to investigate the co-expression of hsa-mir-548 family members in FSHR-positive reproductive tissues and to explore the potential co-regulation of pathways.

Results: For the above-described analysis, small RNA sequencing data from public data repositories were used. Sequencing results revealed that hsa-miR-548ba was expressed at the highest level in the ovarian granulosa cells and uterine myometrial samples together with another twelve and one hsa-miR-548 family members, respectively. Pathway enrichment analysis of microRNA targets in the ovarian samples revealed the hsa-miR-548ba and hsa-miR-548b-5p co-regulation of RAB geranylgeranylation in mural granulosa cells. Moreover, other hsa-mir-548 family members co-regulate pathways essential for ovarian functions (PIP3 activates AKT signalling and signalling by ERBB4). In addition to hsa-miR-548ba, hsa-miR-548o-3p is expressed in the myometrium, which separately targets the peroxisome proliferator-activated receptor alpha (PPARA) pathway.

Conclusion: This study reveals that hsa-mir-548 family members are expressed in variable combinations in the reproductive tract, where they potentially fulfil different regulatory roles. The results provide a reference for further studies of the hsa-mir-548 family role in the reproductive tract.

Keywords: Hsa-mir-548 family, Hsa-miR-548ba, Granulosa cells, Myometrium, FSHR

Background

MicroRNAs (miRNAs) are a class of non-coding RNA molecules ~ 22 nucleotides in length with an important role in post-transcriptional gene expression regulation [1]. miRNAs target genes via the Watson-Crick complementarity principle. The seed sequences of miRNAs, the 2–7 nucleotides positioned in the 5′ region, play an important role in the precise targeting of mRNA [2, 3], while other regions in the miRNA sequence complement the target's specificity [4]. Overall, miRNAs play well-established roles in gene expression regulation in normal and pathological conditions [5]. Moreover, different tissues demonstrate variable miRNA expression patterns that determine tissue characteristics, differentiation, and functions [6].

miRNAs are categorized into families according to the mature miRNA sequence and/or structure of their pre-miRNAs [7]. Mir-548 family miRNAs originate from the mariner-derived element 1 (Made1) transposable elements [8]: primate-specific short miniature

* Correspondence: ilmatar.rooda@gmail.com
[1]Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
[2]Competence Centre on Health Technologies, Teaduspargi 13, 50411 Tartu, Estonia
Full list of author information is available at the end of the article

inverted-repeat transposable elements (MITEs) that form almost perfect palindromes. The secondary structure of Made1 RNA contains highly stable hairpin loops that are recognized by the miRNA-processing machinery [8]. Over the course of evolution, mir-548 family members have undergone several seed-shifting events, leading to changes in the seed sequences and hence the increased variability of their mRNA targets [9].

Human miRNA hsa-miR-548ba is a member of the mir-548 family and was originally described in granulosa cells of human pre-ovulatory follicles. The hsa-miR-548ba gene is located in the intronic region of the follicle-stimulating hormone receptor (FSHR) gene [10]. Hsa-miR-548ba target analysis has revealed PTEN and LIFR as its specific targets. Both of these genes play a well-established role in follicle activation and growth, indicating that hsa-miR-548ba may also have potential regulatory importance in follicle development [11].

Follicles are ovarian structures containing the oocyte and the supporting somatic cells: theca and granulosa cells, responsible for steroidogenesis and the metabolic support of the oocyte [12]. FSHR has important functions in follicle growth in the ovaries as well as sperm development in the testes [13]. By the time the follicle reaches the pre-ovulatory stage, granulosa cells have differentiated into cumulus and mural granulosa cell populations (CGC and MGC, respectively), and the follicle is filled with follicular fluid (FF) that physically separates these cell populations [12]. The main roles of CGC and MGC are providing essential metabolic support to the oocyte and steroid hormone production, respectively [12]. FSHR knock-out mice displayed disordered follicle growth and ovulation [14]. Similarly, point mutations in human FSHR result in arrested follicle development [15]. Therefore, disturbances in FSHR expression lead to female infertility [14, 15]. Analogously, Sertoli cells in the testes express FSHR, where FSH binding indirectly activates the proliferation of germ lineage cells. FSH also regulates the role of Sertoli cells as supporters of sperm cell development [16]. Male FSHR knock-out mice and humans with point mutations in the FSHR gene have decreased spermatogenesis rates and are subfertile [17].

In addition to the ovary and testis, FSHR expression is also detected in the following reproductive tissues: the endometrium [18] and myometrium [19] of the uterus, fallopian tube [20], and cervix [19]. The uterus is mainly composed of myometrial cells, the central roles of which are protecting the growing foetus and facilitating its delivery at the end of the pregnancy through muscular contractions [21, 22]. Myometrial smooth muscle cells express receptors for estrogen and progesterone important for myometrial cell growth and tissue activation during labour [21]. In addition, FSHR is present in the myometrium, where it may also participate in myometrial contractility [23]. The endometrium is a hormonally regulated inner lining of the uterus that is receptive for embryo implantation during only a short period of the menstrual cycle. During this period, the tissue develops specific functional and structural characteristics that allow the attachment of the embryo and its implantation [24, 25].

The aim of the current study is to understand the gene regulatory network between hsa-mir-548 family members that are co-expressed in a certain cell type or tissue. Distinguishing their unique and overlapping gene targets will allow a better interpretation of their importance in tissue function. Due to the importance of FSHR in folliculogenesis and the high level of expression of hsa-miR-548ba in granulosa cells, we aimed to investigate the expression of all hsa-mir-548 members in the context of reproductive tissues where FSHR expression has been detected. We start by providing an update to the status of the hsa-mir-548 family according to the latest miRBase version [26]. Finally, we provide a model of potential co-regulation of mRNA targets and pathways between hsa-miR-548ba and other hsa-mir-548 family members in human reproductive tissues.

The mir-548 family is primate-specific. Members of this family are found in *Homo sapiens*, *Pan troglodytes*, *Callithrix jacchus*, *Macaca mulatta*, *Pongo pygmaeus* and *Gorilla gorilla*. MiR-548ba has been reported uniquely in *Homo sapiens*; therefore, this study focuses only on the members detected in humans and excludes all other primates.

Results

According to the full version history of miRBase, the first members of the hsa-mir-548 family were added into v9. The number of members has since been increasing with almost all new releases of miRBase in correlation with the detection of new miRNA sequences due to the increasing availability of RNA sequencing data. MiRBase v22.1 contains 86 mature human mir-548 family sequences (Fig. 1A). Hsa-miR-548ba is a relatively new member of the family, added into v20.

Human mir-548 family distribution throughout the human genome

The human mir-548 family contains several multi-copy pre-miRNAs in the genome, and as a result, different miRNA precursor sequences give rise to the same mature sequences of hsa-miR-548. For example, hsa-miR-548f and hsa-miR-548h have 5 different pre-miRNAs in the human genome. There are in total 76 different hsa-mir-548 pre-miRNA sequences, located throughout the human genome (Fig. 1B). The highest enrichment is observed on chromosomes 6, 8, and X. Two human

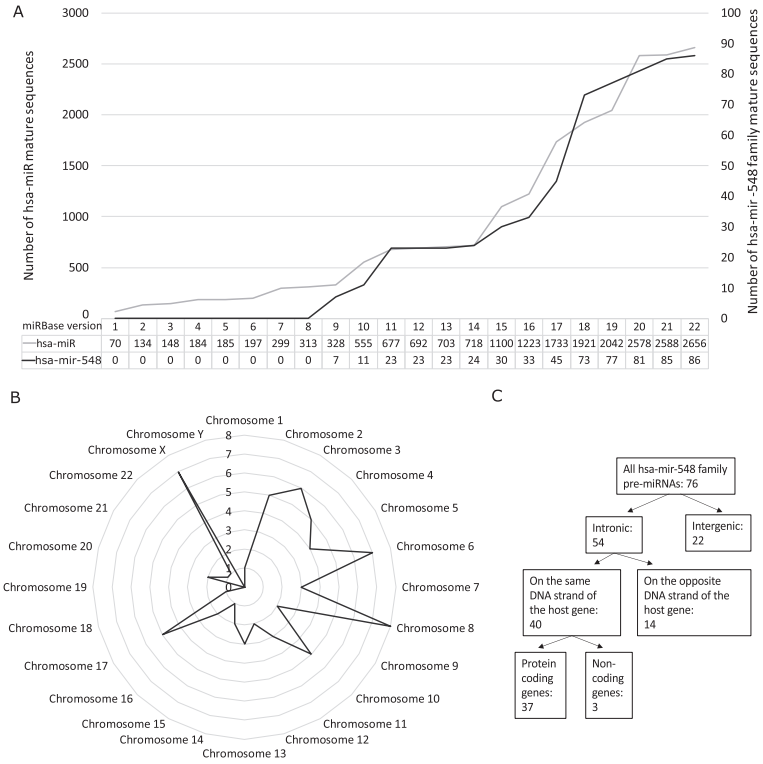

Fig. 1 Hsa-mir-548 family members in the miRBase database and in the human genome. (**A**) The number of all annotated human miRNA (grey line) and hsa-mir-548 family (black line) sequences in the full versions of the miRBase database. (**B** and **C**) Hsa-mir-548 family pre-miRNA sequences in the human genome: distribution by chromosome (**B**), distribution between the genomic loci (**C**)

chromosomes (19 and Y) lack hsa-mir-548 family sequences completely.

From the 76 hsa-mir-548 sequences, 54 are located in the intronic regions and 22 sequences are of intergenic origin (Fig. 1C). From 54 intronic miRNAs, 40 sequences were located on the same DNA strand as their host gene (Additional file 1 Supplementary Table S1). As 37 of these 40 are protein-coding genes, there is a potential co-transcription of the host gene and the corresponding intronic miRNA.

Sequence similarity analysis of hsa-mir-548 family members

Sequence similarity analyses were performed for both mature and pre-miRNA sequences. Shorter distances between mature sequences on the phylogenetic tree indicate higher conservation compared to pre-miRNA sequences (Additional file 2 Supplementary Fig. S1 and S2). The hsa-miR-548ba mature sequence displayed the shortest distances to the following miRNAs: hsa-miR-548 m, hsa-miR-548ag, hsa-miR-548d-5p, hsa-miR-548ay-5p, and hsa-miR-548ad-5p (Fig. 2C, Additional file 2 Supplementary Fig. S1). In addition, hsa-miR-548ag; hsa-miR-548ai and hsa-miR-570-5p share the critical seed sequence with hsa-miR-548ba (Fig. 2D), although the two latter miRNAs demonstrate dissimilarities in their 3'part and therefore reside more distantly in the phylogenetic tree (Additional file 2 Supplementary Fig. S1).

Moreover, a sequence similarity analysis was performed between hsa-mir-548 family members and Made1, the MITE elements giving rise to these miRNAs (Additional file 2 Supplementary Fig. S3 and S4, respectively). Hsa-miR-548-5p sequences demonstrate higher conservation and similarity to Made1 compared to hsa-miR-548-3p sequences. This result confirms a previous similar observation [9]. Hsa-miR-548ba belongs to the hsa-miR-548-5p sequences and is therefore a more conserved family member. Hence, it is highly probable that hsa-miR-548ba and other hsa-mir-548 family members co-expressed in a tissue regulate a set of the same mRNA targets.

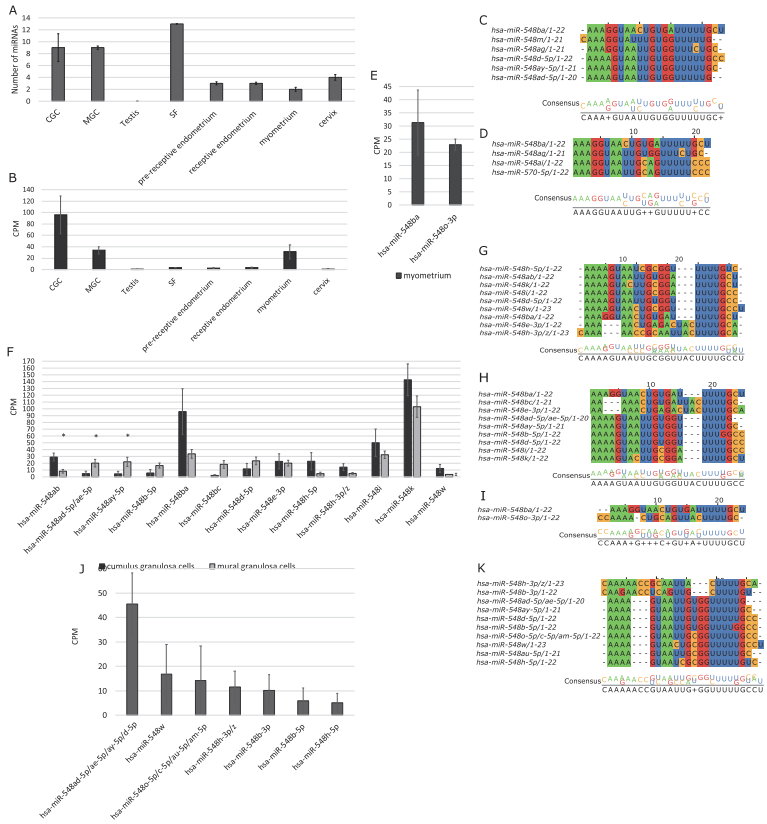

Fig. 2 The expression and sequence similarity of hsa-mir-548 family members in reproductive tissue samples. (**A**) The average number of hsa-mir-548 family members present in reproductive tissues with cut-off > 10 counts per million (CPM). (**B**) The expression levels of hsa-miR-548ba in reproductive tissues. The sequencing results are displayed as a mean of CPM ± SEM. CGC–cumulus granulosa cells ($n = 3$), MGC–mural granulosa cells (n = 3), testis ($n = 5$), SF–seminal fluid ($n = 1$), pre-receptive endometrium ($n = 12$), receptive endometrium (n = 12), myometrium (n = 3), and cervix ($n = 4$). (**C**) The closest hsa-mir-548 family members to hsa-miR-548ba according to sequence similarity. (**D**) Hsa-mir-548 family members which share a seed sequence with hsa-miR-548ba. The sequence length in nucleotides is noted after the slash. (**E**) miRNAs from the hsa-miR-548 family expressed in the myometrium. (**F**) miRNAs of the hsa-miR-548 family expressed in cumulus and mural granulosa cells. The alignment of hsa-miR-548 family sequences co-expressed with hsa-miR-548ba in the analysed tissues: (**G**) cumulus granulosa cells; (**H**) mural granulosa cells; (**I**) myometrium; (**J**) The expression of hsa-mir-548 family members in the cell-depleted follicular fluid of the ovarian follicle; (**K**) the alignment of extracellular miRNAs observed in follicular fluid. Expression levels are displayed as a mean of counts per million (CPM) ± SEM). *$p < 0.05$, Student's t-test

Hsa-mir-548 family expression in reproductive tissues

In order to quantify the expression levels of the hsa-miR-548 family members in human reproductive tissues, small RNA high-throughput sequencing results from male and female reproductive tissues were analysed (Table 1). Tissues were selected for analysis according to the availability of small RNA sequencing data and positivity for FSHR expression. From the male reproductive tissues, small RNA sequencing results were only available for the whole testis tissue homogenate and seminal fluid (SF). From the female reproductive tissues, data was available for MGC, CGC, and FF of the ovary, myometrium, and endometrium from the uterus and cervix.

The highest number of hsa-mir-548 family members was detected from the SF, CGC, and MGC samples (cut-off > 10 counts per million (CPM), Fig. 2A). From the testis samples, none of the hsa-mir-548 family members reached the set cut-off limit (Fig. 2A). The full lists of hsa-mir-548 family members expressed above > 10 CPM cut-off level are presented in Additional file 1 Supplementary Table S2.

Table 1 A description of data used in the hsa-mir-548 family analysis of reproductive tissues

Cellular samples	Tissue of origin	Cell type	Data repository	Accession number	Number of samples	Description
	Ovary	Granulosa	GEO	GSE46508	6	Two cell types of human granulosa cell samples were obtained from pre-ovulatory follicles: cumulus granulosa (CGC) and mural granulosa (MGC) collected from stimulated pre-ovulatory follicles [10].
	Uterus	Endometrial tissue	GEO	GSE108966	24	Human endometrial samples were collected from two time-points of the same menstrual cycle: early-secretory phase corresponding to pre-receptive endometrium and mid-secretory phase corresponding to receptive endometrium [27].
	Uterus	Myometrium tissue	GEO	GSE100338	3	[28]
	Uterus	Cervix tissue	GEO	GSE145372	4	[29]
	Testis	Testis tissue	GEO, ENCODE	ENCSR229WIW, ENCSR626GVP and GSE149084	5	Whole testis tissue sections [30].
	Reproductive track	Non-sperm cellular fraction of seminal fluid (SF)	GEO	GSE56686	1	Non-sperm cellular fraction of SF, which includes prostatic epithelial, urothelial and inflammatory cells [31].
Extracellular samples	**Tissue of origin**	**Cell type**	**Data repository**	**Accession number**	**Number of samples**	**Description**
	Ovary	Follicular fluid (FF)	GEO	GSE157037	8	Extracellular miRNAs were extracted from cell-depleted ovarian follicular fluid (FF) from stimulated pre-ovulatory follicles [32].

The highest expression levels of hsa-miR-548ba were observed in ovarian CGC and MGC samples (Fig. 2B). Outside of the ovary, hsa-miR-548ba expression was the highest in myometrial tissue compared to the other samples. Testicular, SF, endometrial, and cervical samples demonstrated expression levels with borderline detection values (Fig. 2B).

Hsa-mir-548 expression in granulosa cells and myometrium

To further study the potential and significance of the post-transcriptional co-regulation effect that hsa-miR-548ba may exhibit with its co-expressed family members, ovarian and myometrial samples were further analysed, as hsa-miR-548ba was only detected in these samples.

Sequencing results of granulosa cells revealed the expression of 13 different mature hsa-mir-548 family members (Fig. 2F). From those miRNAs, three (hsa-miR-548ab, hsa-miR-548ad-5p/ae-5p and hsa-miR-548ay) were differentially expressed between MGC and CGC samples ($p < 0.05$).

The miRNAs which share the same seed sequence with hsa-miR-548ba (Fig. 2D) are not co-expressed in granulosa cells. However, sequence alignment results reveal that a number of miRNAs detected in granulosa cells share the seed sequence with each other (Fig. 2G-H). Specifically, hsa-miR-548ab, hsa-miR-548d-5p, hsa-miR-548 h-5p, hsa-miR-548i, and hsa-miR-548w in CGC and hsa-miR-548ay, hsa-miR-548ae-5p, hsa-miR-548ad-5p, hsa-miR-548b-5p, hsa-miR-548d-5p and hsa-miR-548i in MGC contain the same seed sequences. Therefore, the co-regulation of common target genes by these miRNAs is possible and expected to occur in granulosa cells.

From miRNAs with the highest sequence similarity to hsa-miR-548ba, only hsa-miR-548ay-5p and hsa-miR-548ad-5p are present in MGC, and hsa-miR-548d-5p in both CGC and MGC are expressed above the cut-off > 10 CPM (Fig. 2F-H).

Compared to granulosa cells, the myometrium expresses only two hsa-mir-548 family members above the > 10 CPM cut-off: hsa-miR-548ba and hsa-miR-548o-3p (Fig. 2E). Hsa-miR-548o-3p is evolutionarily distant from hsa-miR-548ba, and these two miRNAs do not share a common seed sequence (Fig. 2I). In addition to the myometrium, hsa-miR-548o-3p is expressed in the endometrium, cervix, and SF samples (Additional file 1 Supplementary Table S2).

Extracellular hsa-mir-548 family miRNAs in the follicular fluid

miRNAs are known to be present in the extracellular space as a part of RNA-binding protein (RBP) complexes

or as loaded into extracellular vesicles (EV) [33]. However, the potential for the hsa-mir-548 family miRNAs to be secreted into extracellular spaces has not been studied. Due to the high expression levels of hsa-miR-548ba and several other members of the family in the ovarian follicular somatic cells, the extracellular profile was determined from the example of the ovarian cell-depleted FF [22], where seven family members were detected (50% of samples > 10 CPM cut-off, Fig. 2J). We observed that the miRNAs expressed at the highest levels in the cellular samples (hsa-miR-548k, hsa-miR-548ba and hsa-miR-548i) were not detected in FF. This suggests that those miRNAs are cell-specific and are not secreted into extracellular spaces. On the other hand, hsa-miR-548o-5p, hsa-miR-548c-5p, hsa-miR-548am-5p, and hsa-miR-548b-3p in FF are not expressed in granulosa cells above the determined cut-off level (Fig. 2J).

Specific motifs in the 3′ half of the miRNA sequence have the potential to determine whether miRNAs are secreted into the extracellular space or are retained in the cells: for example, GGAG and UGCA appear frequently in extracellular and cellular miRNAs, respectively [34]. In addition, the AGG motif may be involved in extracellular miRNA trafficking [35]. However, miRNAs present in FF samples do not contain GGAG nor AGG motifs (Fig. 2K). Cellular motif UGCA is present in hsa-miR-548 h-3p/z. The fact that those miRNAs are present in the extracellular space may be the result of non-specific secretion.

The signature of hsa-mir-548 family expression is characteristic for each female reproductive tissue

All ovarian follicle sample types form separate clusters according to their hsa-mir-548 family expression patterns (Fig. 3A-B). As expected, cellular and extracellular samples cluster separately. Moreover, two granulosa cell types form separate clusters according to their hsa-mir-548 expression patterns (Fig. 3B). MGC and FF samples display more similar expression patterns compared to CGC cells (Fig. 3A-B). This may indicate that MGC is the primary source of hsa-mir-548 members secreted into FF as MGC is the most abundant somatic cell type inside the pre-ovulatory follicle.

In addition to granulosa cells, hsa-miR-548ba exhibited high expression levels in the myometrial tissue. For clustering analysis, all available uterine tissue samples (endometrium, myometrium and cervix) were compared. The results exhibited a characteristic hsa-mir-548 family expression pattern for endometrium, myometrium, and cervical samples (Fig. 3C-D). Endometrial samples of pre-receptive and receptive stages clustered together (Fig. 3C-D), indicating that hsa-mir-548 family expression levels do not significantly change upon acquiring endometrial receptivity. Moreover, myometrial and cervical samples form closer clusters compared to endometrial samples (Fig. 3C).

Overall, the clustering analysis illustrates that it is possible to distinguish female reproductive tissues and cell types by the expression signature of the hsa-mir-548 family members. Therefore, this miRNA family possesses regulatory roles specific to cell type.

Pathways regulated by hsa-miR-548 members co-expressed in granulosa cells

Since multiple hsa-mir-548 family members are co-expressed with hsa-miR-548ba in the ovarian granulosa cells, we investigated their tissue-specific potential for regulating common signalling pathways with relevance to female fertility. Target genes were predicted for all miRNAs expressed above > 10 CPM cut-off level in CGC or MGC cells and the obtained lists were used as inputs for Reactome pathway enrichment analysis, the results of which are presented in Additional file 1 Supplementary Table S3. Target prediction results revealed that, despite the similarities between the sequences, miRNAs expressed in CGC or MGC target mostly individual genes with a small overlapping part (Fig. 4A and B, respectively).

Pathway enrichment analysis of targeted genes concluded that hsa-miR-548ba does not co-regulate common pathways in CGC with other cell-type-specific hsa-miR-548 family members. In MGC, hsa-miR-548ba revealed the co-regulation of “RAB geranylgeranylation” pathway with hsa-miR-548b-5p. From the other hsa-mir-548 family members hsa-miR-548d-5p and hsa-miR-548i co-regulate, “PIP3 activates the AKT signalling” pathway in both CGC and MGC. This pathway is additionally targeted by hsa-miR-548w and hsa-miR-548b-5p in CGC and MGC, respectively. Additionally, “PI5P, PP2A, and IER3 regulate PI3K/AKT signalling” pathway is commonly regulated by hsa-miR-548d-5p and hsa-miR-548b-5p in MGC (Additional file 1 Supplementary Table S3A and S3B).

In the context of ovarian function, the above-mentioned pathways “PIP3 activates AKT signalling” and “PI5P, PP2A and IER3 regulate PI3K/AKT signalling” have been previously studied [36–38]. In addition, the “Translocation of SLC2A4 (GLUT4) to the plasma membrane” targeted by hsa-mir-548ba in both CGC and MGC, as well as “Signalling by ERBB4” targeted by hsa-miR-548b-5p in MGC, demonstrate the importance of the corresponding miRNAs in ovarian functions [39, 40].

Pathways regulated by hsa-miR-548 members expressed in myometrium

Although the myometrial cells exhibited the expression of only two hsa-mir-548 family members (hsa-miR-

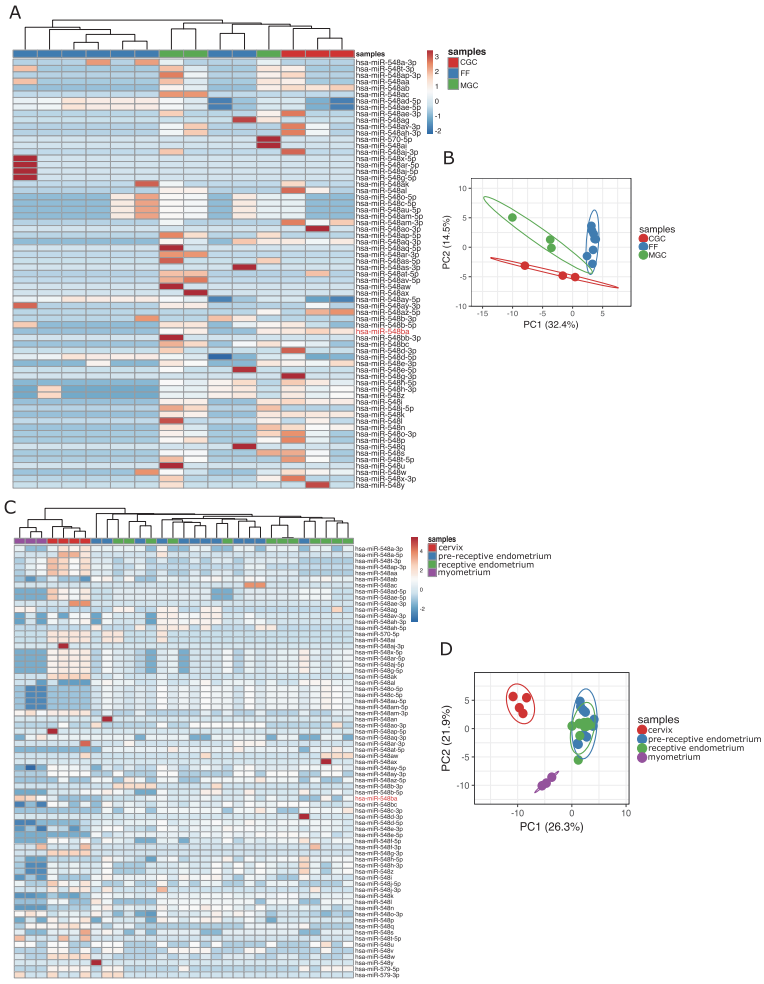

Fig. 3 Expression levels of hsa-mir-548 family miRNAs in the human ovarian follicle and uterine samples. (**A**) A heatmap of hsa-mir-548 family expression levels in individual ovarian samples; (**B**) a PCA plot of ovarian follicle cellular and extracellular samples according to the expression levels of hsa-mir-548 family miRNAs; (**C**) a heatmap of hsa-mir-548 family expression levels in individual uterine samples; (**D**) a PCA plot of uterine samples according to the expression levels of hsa-mir-548 family levels. FF–cell-depleted follicular fluid, CGC–cumulus granulosa cells, MGC–mural granulosa cells. The location of hsa-miR-548ba on the heatmap is highlighted in red. The heatmap colour scale displays ln(x + 1) transformed CPM values

548ba and hsa-miR-548o-3p), a commonly regulated pathway "Signalling by BRAF and RAF fusions" by these two miRNAs was detected.

In the context of myometrial functions, the following important regulatory pathways were targeted by hsa-miR-548o-3p: "PI5P, PP2A and IER3 regulate PI3K/AKT signalling" and "PPARA activates gene expression".

Discussion

Hsa-mir-548 is a primate-specific miRNA family derived from Made1 transposable element [8]. Made1 elements are MITEs with genomic locations either close to or within genes, where they may be involved in gene regulation [41]. Hsa-mir-548 family members are transcribed from most human chromosomes, while some other miRNA families exhibit chromosome-specific locations in the genome [7]. The distribution analysis of hsa-mir-548 family members in the human genome exhibited that the majority of pre-miRNA sequences (54/76) are located in the intronic regions of genes. This is in an accordance with the preferable genome locations of MITEs [41]. Moreover, chromosome Y, which contains the

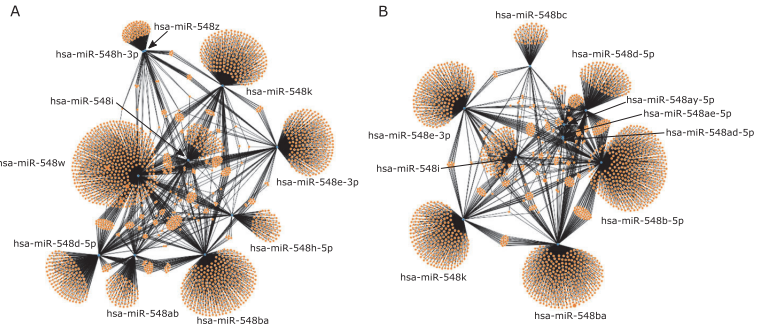

Fig. 4 Target prediction for hsa-mir-548 family miRNAs expressed in cumulus granulosa cells (**A**) and mural granulosa cells (**B**). Each orange node represents one target gene, and genes targeted by more than one miRNA are connected with an edge

smallest number of genes compared to other chromosomes [42], does not contain any hsa-mir-548 members. However, gene-rich chromosome 19 [43] likewise did not contain any hsa-mir-548 miRNA sequences. It is possible that not all hsa-mir-548 family members have been discovered. Since the first sequence of hsa-mir-548 was included to miRBase, new sequences have been added to almost every new miRBase release, correlating with the rapid development and reduced cost of high-throughput sequencing technologies. Moreover, using a bioinformatic approach, 34 additional precursor sequences of hsa-mir-548 have been discovered, indicating that this family could be larger [44]. However, their expression still needs experimental validation.

miRNAs are important gene expression regulators in both the male and female reproductive tissues and aberrant miRNA expression can lead to infertility [45, 46]. Hsa-miR-548ba expression analysis in reproductive samples revealed that this particular miRNA is expressed at the highest level in granulosa cells, where it was first discovered. It has been previously shown that the expression pattern of hsa-miR-548ba is similar to that of FSHR and FSH treatment upregulates hsa-miR-548ba expression levels in human granulosa cells [11]. Human ovarian granulosa cells [13], endometrium [47], myometrium [19], cervix [19] and testis Sertoli cells [13] all express FSHR. In addition to granulosa cells, hsa-miR-548ba exhibited high expression levels in the myometrial tissue. These results reveal the tissue-specific expression of hsa-miR-548ba that may be derived from a different miRNA expression regulation than that observed in the granulosa cells of its host gene, FSHR. However, differences in expression level may also be caused by technical errors. The datasets used for this study were obtained from data repositories and, therefore, RNA extraction and library preparation were not universal for all samples. This may be the cause of the lower expression of hsa-miR-548ba in the endometrium, cervix, and testis samples.

Overall, granulosa cells express 13 members of the hsa-mir-548 family. From those, miRNAs hsa-miR-548ab, hsa-miR-548ad-5p/ae-5p, and hsa-miR-548ay-5p were differentially expressed between MGC and CGC samples (p-value < 0.05). Although detected in a few other human sample types (hsa-miR-548ab expression is reported in human B-cells [48], hsa-miR-548ad-5p/ae-5p and hsa-miR-548ay-5p are present in synovial tissue samples [49], and hsa-miR-548ad-5p is present in blood plasma samples), the roles of these specific miRNAs have not been investigated. However, in our samples, hsa-miR-548ad-5p was detected in extracellular FF as one of the most abundant miRNAs. According to the comparisons in ovarian datasets, it can be deduced that hsa-miR-548ad-5p is secreted from MGC and may be involved in intercellular signalling in the follicle. Therefore, the investigated miRNA family potentially has unknown importance in follicular function.

miRNAs which share the same seed sequence with hsa-miR-548ba are not co-expressed in granulosa cells, which indicates that this miRNA potentially has an individual specific regulatory role in the ovary. However, despite this, some targets were shared between other co-expressed members of the hsa-mir-548 family with different seed sequences. It has been well established that one mRNA can be targeted by multiple miRNAs [50]. Moreover, target prediction algorithms like miRWalk use additional features to miRNA seed sequence for target prediction [51]. The hsa-mir-548 family has gone through several seed-shifting events, which has resulted in various seed sequences in the members [9]. Different seed sequence variants were also present in miRNAs expressed in ovarian and myometrial samples. Nevertheless, some hsa-mir-548 family miRNAs expressed in granulosa cells have common seed sequences and

consequently overlap with part of the predicted target genes; for example, hsa-miR-548b-5p, hsa-miR-548d-5p, and hsa-miR-548i expressed in MGCs.

The majority of enriched pathways were targeted by different individual miRNAs in the granulosa cells. All together there were three exceptions. The first exception was the "PIP3 activates AKT signaling" pathway, which is targeted by hsa-miR-548d-5p and hsa-miR-548i in CGC and MGC and additionally targeted by hsa-miR-548w and hsa-miR-548b-5p in CGC and MGC, respectively. This pathway is involved in regulating the balance between dormancy and activation of follicles, granulosa cell differentiation, and proliferation [38]. The second exception was the "PI5P, PP2A, and IER3 regulate PI3K/AKT signalling" pathway targeted by hsa-miR-548d-5p in CGC and MGC, and hsa-miR-548b-5p in MGC. IER3 is a part of a gonadotropin-EGR2-IER3 axis with a role in granulosa cell survival during follicle development [36]. Additionally, PP2A participates in the regulation of PKC-mediated inflammation in rat granulosa cells [37]. The last co-regulated pathway is "RAB geranylgeranylation" targeted by hsa-miR-548ba and hsa-miR-548b-5p in MGC. The depletion of the geranylgeranylation substrate geranylgeranyl diphosphate (GGPP) in mice oocytes inhibits Rab27a geranylgeranylation, which is required for Rab protein activation. Rab27a plays a possible role in oocyte protein secretion. Therefore, disturbances in this pathway impair oocyte-granulosa cell communication, which is necessary for normal follicle development [52].

Pathways targeted by individual hsa-mir-548 members have additional known roles in granulosa cells. For example, hsa-miR-548ba targets the "translocation of SLC2A4 (GLUT4) to the plasma membrane" pathway. GLUT4 is involved in glycose uptake and FSH stimulates this process in granulosa cells [39]. Granulosa cells of polycystic ovarian syndrome patients have a tendency to display abnormal glycose metabolism. Therefore, normal glycose metabolism is important for granulosa cell function [39]. Hsa-miR-548b-5p targets "Signalling by ERBB4" in MGC. ERBB4 plays a role in normal follicle development and disturbances in ERBB4 levels may lead to ovarian dysfunction [40]. To conclude, in addition to hsa-miR-548ba, other hsa-mir-548 family members regulate pathways important for granulosa cell functions.

Pathways "PIP3 activates AKT signalling" and "PI5P, PP2A and IER3 Regulate PI3K/AKT signalling" are targeted by miRNAs which share the seed sequences (hsa-miR-548d-5p, hsa-miR-548b-5p, hsa-miR-548i, and hsa-miR-548w). However, additional family members expressed in granulosa cells have the same seed sequence but do not target those pathways. Alignment results of miRNAs present in granulosa cells and alignment of the whole miRNA family demonstrated that, in addition to seed shifting events, nucleotide substitutions are present in miRNA sequences. These molecular events have changed potential targeting features [4] and have led to different target genes between miRNAs with the same seed sequence.

Myometrial samples express two hsa-mir-548 members: hsa-miR-548ba and hsa-miR-548o-3p. Both miRNAs regulate one common pathway: "Signalling by BRAF and RAF fusions". BRAF and RAF fusion is a result of chromosomal rearrangement events and is detected in distinct cancer types [53]. Therefore, in normal myometrial tissue, this pathway is not present. In addition, hsa-miR-548o-3p targets the "PI5P, PP2A, and IER3 regulate PI3K/AKT signalling" and "PPARA activates gene expression" pathways. From the first targeted pathway, PP2A regulates proteins involved in smooth muscle contraction [54]. In the second pathway, PPARA levels increase in the late pregnancy myometrium (gestation range 20–35 weeks) compared to nonpregnant women and decrease by the time of labour, suggesting that PPARA plays a role in maintaining pregnancy [55]. This indicates that the hsa-mir-548 family may have a regulatory role in myometrial gene expression regulation involved in contractile functions. Moreover, hsa-miR-548o-3p expression was not detected in ovarian samples but was present in endometrial and cervical samples, confirming its organ-specific expression.

miRNAs have been detected from all body fluids, including FF [32, 56], and may be involved in cell-to-cell communication [33]. miRNAs can be secreted from cells as a part of RBPs or packed into EVs [33]. Many possible sorting mechanisms are proposed for loading miRNAs into EVs: sequence characteristics, post-transcriptional modifications, subcellular location, and intracellular concentration [33]. In this study, FF which contains both RBPs and EVs, was searched for hsa-mir-548 family members. As a result, 7 miRNAs were detected in FF. Some of the miRNAs were only detected in FF and not in granulosa cells, for example, hsa-miR-548o-5p and hsa-miR-548c-5p. miRNAs expressed at the highest levels in cellular samples were not present in FF, indicating that the secretion mechanism is not based on the intercellular concentration of miRNA molecules. miRNAs present in extracellular samples were aligned and a possible export motif was searched for. Known miRNA secretion motifs GGAG [34] and AGG [35] are not present in the miRNA sequences of hsa-mir-548 family members present in FF. Nevertheless, hsa-mir-548 family members have been detected from other body fluids in addition to FF: hsa-miR-548b-5p, hsa-miR-548c-5p and hsa-miR-548i [57], and hsa-miR-548a-3p [58] in blood serum samples, hsa-miR-548b-3p in blood plasma, bronchial lavage and peritoneal fluid, and hsa-miR-548d-5p in amniotic fluid [59]. Some miRNAs infiltrate into

the FF from blood plasma [32], explaining the lack of their expression in the granulosa cells. Additionally, the oocyte has not been investigated as the source of miRNAs secreted into the FF due to the lack of such human data. Therefore, hsa-mir-548 family members are secreted into extracellular space by other cell types as well as ovarian granulosa cells. The mechanism by which hsa-mir-548 family members are selected for secretion remains unknown.

Conclusion

From all the analysed FSHR-positive samples, hsa-miR-548ba transcribed from the intronic region of FSHR gene can be detected in the ovarian granulosa cells and the myometrium. This suggests that the expression of hsa-miR-548ba and FSHR are differently co-regulated in other FSHR-positive tissues. In addition to hsa-miR-548ba, twelve and one other hsa-mir-548 family members are expressed in granulosa and myometrium samples, respectively. Moreover, hsa-mir-548 family members are detectable from the extracellular ovarian FF. miRNA target pathway enrichment analysis revealed that hsa-miR-548ba and hsa-miR-548b-5p co-regulate the RAB geranylgeranylation pathway in MGC. Disturbances in this pathway impair oocyte-granulosa cell communication. In addition to hsa-miR-548ba, other family members separately regulate essential pathways for granulosa cell function (PIP3 activates AKT signalling and signalling by ERBB4). This reveals that hsa-mir-548's family regulatory role in granulosa cells is wider than previously acknowledged. Moreover, hsa-miR-548o-3p expressed in myometrium targets the PPARA pathway which is associated with the maintenance of pregnancy. Furthermore, hsa-miR-548o-3p presents uterine-specific expression as it was detected only in myometrial, endometrial and cervical samples. Overall, hsa-mir-548 family members may play regulatory roles in ovarian follicle activation, development, granulosa cell differentiation, and proliferation. In the myometrium, the hsa-mir-548 family was predicted to regulate myometrial contractility and has a potential importance in the maintenance of pregnancy.

Methods

Hsa-mir-548 family members and sequences

The analysis of hsa-mir-548 family member curation was performed using the miRBase database [26]. Information about miRNA mature sequences was downloaded from all full miRBase versions with the exception of v22.1, which is the current release. Genomic locations of pre-miRNA sequences in the human genome were obtained from NCBI Gene [60] and Ensembl [61] databases.

Mature and pre-miRNA sequences were aligned in Jalview (v2.11.0) [62] using the Clustal Omega algorithm with standard settings [63]. Phylogenetic trees of mature and pre-miRNA sequences were constructed with the neighbour-joining method [64] from reads aligned with Clustal Omega in Jalview.

Hsa-mir-548 family expression in human reproductive tissues

All sequencing data used in the analyses were previously published and available in open data repositories (Table 1). From all available data, only samples from healthy control subjects were used. All miRNA raw FASTQ files were quality-filtered with Trimmomatic v0.39 [65] with the options of SLIDINGWINDOW:2:20. Adapter sequences were removed and reads below 17 nucleotides in length were discarded. The remaining filtered and trimmed reads were counted and mapped to the primary assembly of human genome GRCh38 and annotated miRNA sequences from miRBase v22.1 using miRDeep2 with standard settings [66]. All miRNA raw counts obtained from miRDeep2 results were normalized to counts per million (CPM) using the edgeR package v.3.28.1 [67]. miRNA results were filtered by expression levels, and the cut-off was set > 10 CPM for all cellular samples. The cut-off for extracellular samples was set to > 10 CPM in 50% of samples. Data visualization on heatmap and PCA plots was performed in ClustVis [68]. Statistical significance between CGC and MGC was calculated via a two-tailed Student's t-test. The statistical significance level was set at $p < 0.05$.

Target prediction and gene ontology analysis

Target genes were predicted for miRNAs with an expression cut-off level of > 10 CPM with miRWalk version 3 [51]. Obtained miRNA target lists were input for gene enrichment analysis with miRWalk pathway analysis tool, and a statistical significance threshold was set at Benjamini-Hochberg FDR < 0.1.

Abbreviations

CGC: cumulus granulosa cells; CPM: counts per million; EV: extracellular vesicles; FF: follicular fluid; FSHR: follicle-stimulating hormone receptor; Made: mariner-derived element 1; MGC: mural granulosa cells; MITEs: miniature inverted-repeat transposable elements; RBP: RNA-binding protein; SF: seminal fluid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12863-021-00997-w.

Additional file 1: Supplementary Table S1. Human hsa-mir-548 family pre-miRNA sequence locations in human genome. **Supplementary Table S2.** Hsa-mir-548 family miRNAs expressed in reproductive tissues. **Supplementary Table S3.** Reactome pathways of predicted miRNA targets

Additional file 2: Supplementary Fig. S1. Phylogenetic tree of mature sequences of hsa-mir-548 family members. **Supplementary Fig. S2.** Phylogenetic tree of pre-miRNA sequences of miR-548 family members.

Supplementary Fig. S3. Phylogenetic tree of Made1 and hsa-mir-548 family members. **Supplementary Fig. S4.** The alignment of Made1 and hsa-mir-548 family mature sequences

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Not applicable.

Authors' contributions
IR, ML, OPS, AS, and AVM contributed to the study design; IR, BK and AVM analyzed the data. All authors were involved in compiling the manuscript and approved the final version.

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Availability of data and materials
The datasets analysed during the current study are available in the Gene Expression Omnibus under accession numbers: GSE46508 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46508), GSE108966 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108966), GSE100338 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100338), GSE145372 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145372), GSE149084 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149084), GSE56686 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56686) and GSE157037 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157037) and ENCODE repository under accession numbers: ENCSR229WIW (https://www.encodeproject.org/experiments/ENCSR229WIW/) and ENCSR626GVP (https://www.encodeproject.org/experiments/ENCSR626GVP/).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author details
[1]Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia. [2]Competence Centre on Health Technologies, Teaduspargi 13, 50411 Tartu, Estonia. [3]Division of Obstetrics and Gynecology, Department of Clinical Science, Intervention and Technology (CLINTEC), Karolinska Institutet, 14186 Stockholm, Sweden. [4]Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tartu, L. Puusepa St. 8, 50406 Tartu, Estonia. [5]Institute of Genomics, University of Tartu, Riia 23b, 51010 Tartu, Estonia.

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Publisher's Note

Appendix 3

Publication III

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Cellular, Extracellular and Extracellular Vesicular miRNA Profiles of Pre-Ovulatory Follicles Indicate Signaling Disturbances in Polycystic Ovaries

Ilmatar Rooda [1,2,†], Mohammad Mehedi Hasan [3,†], Kristine Roos [1,4], Janeli Viil [3], Aneta Andronowska [5], Olli-Pekka Smolander [1], Ülle Jaakma [6], Andres Salumets [2,6,7,8], Alireza Fazeli [3,9] and Agne Velthut-Meikas [1,*]

1 Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia; ilmatar.rooda@gmail.com (I.R.); kristine.roos@gmail.com (K.R.); olli-pekka.smolander@taltech.ee (O.-P.S.)
2 Competence Centre on Health Technologies, Teaduspargi 13, 50411 Tartu, Estonia; andres.salumets@ccht.ee
3 Department of Pathophysiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 14B, 50411 Tartu, Estonia; mehedi.hasan@ut.ee (M.M.H.); janeli.viil@ut.ee (J.V.); alireza.fazeli@ut.ee (A.F.)
4 Nova Vita Clinic, A. H. Tammsaare tee 47, 11314 Tallinn, Estonia
5 Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima St. 10, 10-748 Olsztyn, Poland; a.andronowska@pan.olsztyn.pl
6 Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Fr. R. Kreutzwaldi 1, 51006 Tartu, Estonia; ylle.jaakma@emu.ee
7 Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tartu, L. Puusepa St. 8, 50406 Tartu, Estonia
8 Institute of Genomics, University of Tartu, Riia 23b, 51010 Tartu, Estonia
9 Academic Unit of Reproductive and Developmental Medicine, Department of Oncology and Metabolism, The Medical School, University of Sheffield, Sheffield S10 2SF, UK
* Correspondence: agne.velthut@taltech.ee
† These authors contributed equally to this work.

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Abstract: Cell-free RNAs have the potential to act as a means of gene expression regulation between cells and are therefore used as diagnostic markers describing the state of tissue environment. The origin and functions of such RNAs in human ovarian follicle, the environment of oocyte maturation, are unclear. The current study investigates the difference in the microRNA profiles of fertile women and polycystic ovary syndrome (PCOS) patients in three compartments from the same preovulatory follicle: mural granulosa cells (MGC), cell-free follicular fluid (FF), and extracellular vesicles (EV) of the FF by small RNA sequencing. In silico analysis was used for the prediction and over-representation of targeted pathways for the detected microRNAs. PCOS follicles were distinguished from normal tissue by the differential expression of 30 microRNAs in MGC and 10 microRNAs in FF (FDR < 0.1) that commonly regulate cytokine signaling pathways. The concentration of EV-s was higher in the FF of PCOS patients ($p = 0.04$) containing eight differentially expressed microRNAs ($p < 0.05$). In addition, we present the microRNA profiles of MGC, FF, and EV in the fertile follicle and demonstrate that microRNAs loaded into EVs target mRNAs of distinct signaling pathways in comparison to microRNAs in FF. To conclude, the three follicular compartments play distinct roles in the signaling disturbances associated with PCOS.

Keywords: extracellular vesicles; human ovarian follicle; granulosa cells; follicular fluid; polycystic ovary syndrome; PCOS; miRNA; intercellular communication

1. Introduction

Disturbances in normal ovarian physiology cause subfertility or infertility leading to prolonged effort or inability for a woman to conceive. Polycystic ovary syndrome (PCOS) is a common hormonal disturbance affecting up to 20% reproductive age women worldwide [1]. PCOS is a complex syndrome with reproductive, metabolic and psychological features and is characterized by hyperandrogenism, obesity, insulin resistance, polycystic ovarian morphology (PCOM) and/or anovulation [2,3]. The phenotype varies broadly depending on the genotype, ethnicity, and environmental factors [1]. Heterogeneity of PCOS is a challenge for diagnostics causing delayed detection and dissatisfaction with care [4]. The current knowledge regarding molecular mechanisms behind the dysfunction of PCOS is still incomplete.

The human ovarian follicle is a dynamic structure that supports oocyte maturation, ovulation, and steroid hormone synthesis. Granulosa, theca, and follicular immune cells are the somatic cell populations ensuring the flawless performance of the above-mentioned processes crucial for female fertility. By the pre-ovulatory stage, the follicle diameter expands above 20 mm and is filled with follicular fluid (FF) [5]. This fluid-filled environment enables long-distance cell communication between different cell populations via cell-secreted (lipo)proteins, ribo-protein complexes (RBPs), and extracellular vesicles (EVs) containing nucleic acids and proteins from the secreting cells [6]. The possible disorders in long-distance intercellular signaling in human polycystic ovaries have not been thoroughly investigated.

EVs are lipid bilayer-coated nanoparticles in varying size range [7]. Based on their size and mode of biogenesis EVs are classified into three major subtypes; exosomes (40–100 nm), microvesicles (100–500 nm), and apoptotic bodies (500 nm–2 um) [8]. The release of EVs and RBPs has been extensively studied and attributed to all cell types in the human body. Moreover, cell-free RNAs in RBPs and EVs have been detected in all investigated body fluids, including FF [9,10]. Small RNAs in EVs have caught more attention, however more than 90% of circulating miRNAs are present outside of EVs associated with AGO2, nucleophosmin 1, or high-density lipoprotein, among other proteins [6]. The secretion of RNA molecules via EVs is at least partly controlled by the releasing cells and the RNA content of EVs is cell specific [11]. However, AGO2-miRNA complexes may also be released non-specifically into extracellular space following cell death [6]. The RNA content of both EVs and RBPs can be taken up by recipient cells from the same or another cell population and potentially modulates signaling pathways in the recipient [12]. Examples of significance of such long-distance communication can be drawn from cancer studies and immunology [13], among other fields. In reproductive studies, fluorescently labelled EVs isolated from FF were taken up by ovarian granulosa cells in an equine in vitro model, suggesting that the exchange of RNA is potentially an important mean of communication also in the normal ovarian physiology [14]. Up to now, mainly miRNAs have been widely studied as the constituents of the follicular EVs due to their well-known molecular function. However, also other types of long and small RNAs have been described as the components of EVs and RBPs: mRNA, lncRNA, SRP RNA, circRNA, snRNA, snoRNA, vault RNA, Y RNA, piRNA, tRNA, and rRNA fragments [6]. The extracellular RNA content has been proposed as a diagnostic tool for disease states, as several cell populations have been demonstrated to change the repertoire of released cell-free RNAs upon external stimulus or disease [15,16].

The current study hypothesizes that there are differences in the cellular and extracellular miRNA expression levels between the ovarian follicles of healthy and PCOS patients indicating molecular signaling disturbances at preovulatory stage. We set out to investigate in a genome-wide manner the cellular and extracellular miRNA profile of three matched sample types collected from single follicles of healthy fertile women versus PCOS patients comprising of granulosa cells (MGC), cell-depleted FF and EVs purified from the FF. Such combined dataset for the human follicle is unique. The obtained information will provide new avenues for therapeutic approaches for PCOS patients, e.g., the development of new ovarian stimulation as well as in vitro oocyte maturation protocols for improving their infertility treatment outcomes.

2. Results

Intrafollicular communication differences between the fertile and PCOS ovaries were modelled by analyzing material from three distinct sources, each collected from the same follicle: small RNA from MGC, all cell-free small RNA populations from FF, and small RNA in EVs purified from the FF. The rationale of sample collection and compartmentalization is outlined in Figure 1.

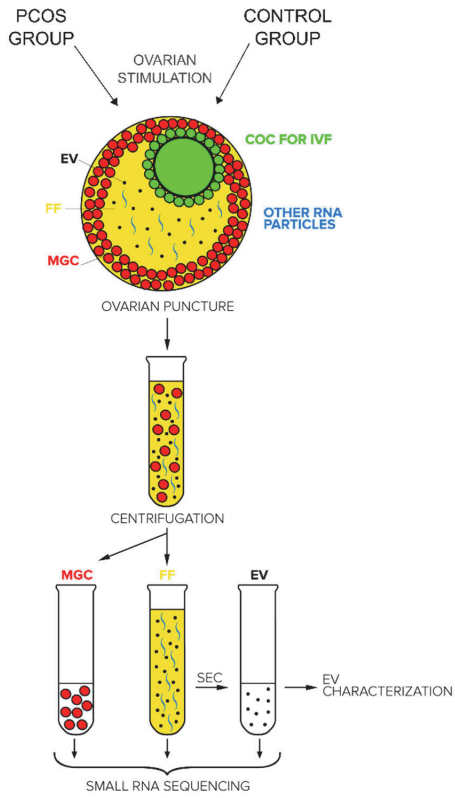

Figure 1. Schematic description of sample collection and processing from human pre-ovulatory follicles of polycystic ovary syndrome (PCOS) patients and fertile control group. COC—cumulus oocyte complex (green), FF—cell-free follicular fluid (yellow) containing extracellular vesicles (EV, black) and non-EV-bound RNA (blue), IVF—in vitro fertilization, MGC—mural granulosa cells (red).

EVs were characterized for their size, concentration, and surface markers. All three sample types underwent small RNA sequencing, and the results were validated by real-time quantitative PCR (RT-qPCR). The main characteristics of patients and the number of analyzed samples according to each method are presented in Table 1.

Table 1. General characteristics of study participants by used method.

		Age (Mean, Years)	SD (Years)	BMI (Mean, kg/m^2)	SD (kg/m^2)
Extracellular vesicle characterization:					
PCOS	n = 15	32.7	4.3	23.5	3.1
Oocyte donors	n = 15	25.8	3.2	22.3	2.8
p-value		<0.001		0.313	
Small RNA sequencing:					
PCOS	n = 7	34	4.8	22.7	2.6
Oocyte donors	n = 8	26.9	2.2	22.7	3.6
p-value		0.002		0.999	
miRNA expression validation (RT-qPCR):					
PCOS	n = 15	32.7	4.3	23.5	3.1
Control group	n = 16	33	4	23	3
p-value		0.626		0.831	

2.1. Characterization of Nanoparticles Isolated from Human Follicular Fluid as Extracellular Vesicles

FF-derived EVs from SEC fractions 6–9 (Supplementary Figure S1) were characterized by three independent methods: NTA, TEM, and Western blot (WB) analysis (Figure 2). According to the NTA size profile analysis (Figure 2A), most of the nanoparticles were under 200 nm in diameter with a large population range within 75–165 nm, which is a typical EV size range [17]. We observed a 4-nm difference between healthy women (mean 138.6± SEM 0.2 nm) and PCOS patients (142.7 ± 0.2 nm) FF-derived EVs ($p < 2.2 \times 10^{-16}$, Supplementary Figure S2). Secondly, the PCOS FF samples contain higher EV concentration compared to the control group (p = 0.04, Figure 2B).

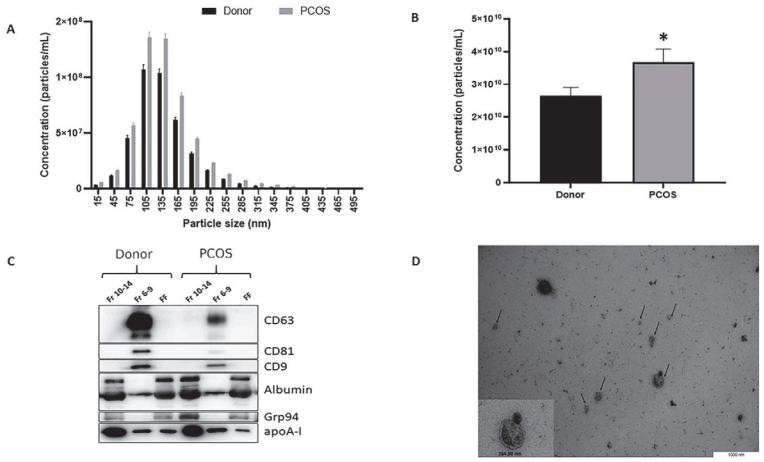

Figure 2. Characterization of extracellular vesicles (EVs) isolated from cell-free follicular fluid (FF). (**A**) Size profile of EVs in study groups (mean ± SEM). (**B**) Concentration of EVs in polycystic ovarian syndrome patients (PCOS) and control samples (mean ±SEM). (**C**) Positive signals of EV markers CD63, CD81, CD9 were detected from EV samples (Fr 6-9), while undetectable from protein fractions (Fr 10-14) and FF samples before EV isolation (FF). Albumin, Grp94, and apoA-I were used as markers of negative selection demonstrating diminished signal intensity in EV samples compared to the protein fraction and FF samples. (**D**) Transmission electron microscopy analysis of purified EVs, indicated by arrows. Data in (**A**,**B**) is demonstrated as mean ± SEM. * p = 0.04 Student's t-test.

Tetraspanins CD63, CD81, and CD9, considered as positive EV markers, were used to verify the presence and enrichment of extracellular vesicles in EV preparations. Based on WB analysis (Figure 2C), all tetraspanins were enriched in EVs, whereas the protein fractions of FF and non-purified

FF had undetectable levels of the studied EV markers. Endoplasmic reticulum protein Grp94, which is expected to be absent or under-represented in smaller EVs, notably exosomes [18], was indeed absent from EV samples, whereas protein fractions and non-purified FF were positive for Grp94. The purity of EVs and the efficiency of SEC was also tested by analyzing the presence of albumin and apolipoprotein A-I (apoA-I) as these proteins are often co-isolated with EVs. Strong signals were detected in FF samples, and although both of these proteins were also detectable in EV samples, the largest quantities were enriched in later fractions (Fr 10–14) corresponding to protein enrichment (Figure 2C). TEM analysis also confirmed the presence of EVs in the studied samples (Figure 2D).

2.2. Small RNA Profile of Granulosa Cells, Cell-Free Follicular Fluid and Extracellular Vesicles

Whole-genome small RNA sequencing was performed for all three sample types (MGC, FF and EV) to model intrafollicular signaling for eight oocyte donors and seven PCOS patients (Table 1). The sequencing depth and mapping efficiency for each sample type are represented in Supplementary Table S1. Analysis of sequencing read size distribution after adapter trimming demonstrated different patterns of small RNA sequence lengths between sample types (Supplementary Figure S3) referring to distinct variability in their content of small RNA populations. A prevalent size peak at 17–24 nt corresponding to the length of miRNAs appears in all samples. In addition, MGC and FF samples contain small RNAs of 28–36 nt length not detected in EV samples. Average sequence length is also shorter in EV samples compared to FF and MGC samples ($p < 0.005$, Supplementary Table S1). The current study focuses further on the miRNA content of each sample type.

2.3. miRNAs in Granulosa Cells, Cell-Free Follicular Fluid and Extracellular Vesicles

In total 1525 unique miRNAs were detected by at least one read: 658 miRNAs were observed in EVs, 1060 in FFs and 1381 in MGCs. All sample types share a large proportion of the most abundant miRNAs among the top 20 most represented sequences in every sample type (EV and FF share 15, FF and MGC share eight out of 20, Supplementary Table S2).

Clustering analysis revealed significant differences between sample types according to their miRNA content (Figure 3A,B). As expected, EV and FF samples cluster closer to each other in comparison to MGC samples (Figure 3A), as EV samples are a sub-compartment purified from the corresponding FF samples.

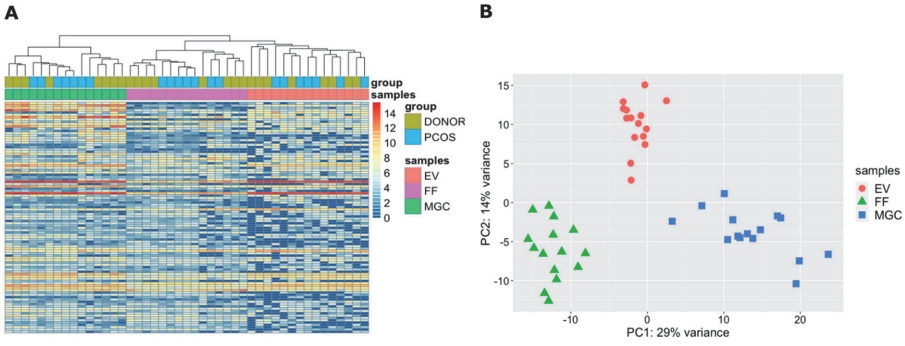

Figure 3. Cluster analysis of investigated samples and study groups. (**A**) Hierarchical clustering of top 100 most variable miRNAs across all samples. Results are depicted by DESeq2 normalized counts on $\log_2$ scale. (**B**) Principal component analysis based on expressed miRNAs per sample type. EV—extracellular vesicles, FF—cell-free follicular fluid, MGC—mural granulosa cells, PCOS—polycystic ovary syndrome.

2.4. Cellular and Extracellular miRNAs in the Healthy Ovarian Follicle

The analysis of samples from oocyte donors ($n = 8$) representing the healthy ovary revealed that in total 172 miRNAs are common to all three sample types (>5 reads observed in >50% of samples per

sample type). A set of 124 miRNAs were only present in MGC samples indicating that these are not secreted out of the cells (Figure 4A, Supplementary Table S3).

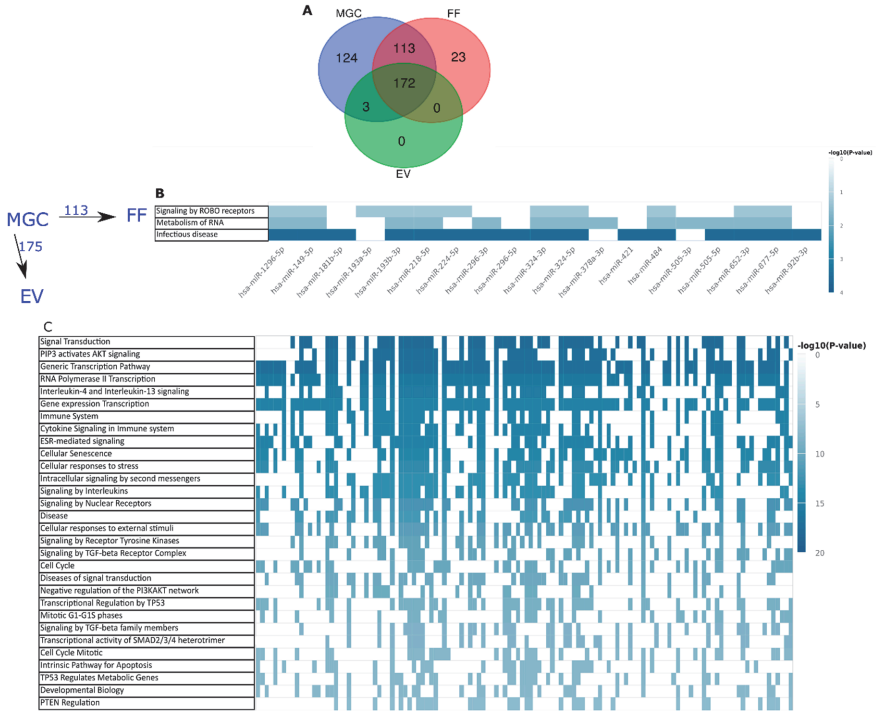

Figure 4. Cellular and extracellular miRNAs observed in ovarian follicles of fertile women. (**A**) Distribution of observed miRNAs (>5 reads in >50% of samples) between sample types. (**B**) Pathways over-represented by the targets of 113 miRNAs secreted by mural granulosa cells (MGC) into follicular fluid (FF) outside of extracellular vesicles (EV). (**C**) Top 30 pathways potentially regulated by 175 miRNAs secreted into FF in EVs. Each column in (**B**,**C**) corresponds to one miRNA regulating a pathway, if marked in blue.

EVs did not contain any unique miRNAs compared to FF or MGC. Three miRNAs (hsa-miR-374a-5p, hsa-miR-190a-5p and hsa-miR-196a-5p) were shared only between EV and MGC samples and not present in FF. We hypothesize that these miRNAs are specifically enriched into EVs. In the FF samples that contain RNA molecules present also in other forms these miRNAs remain below detection limit.

Moreover, MGC samples share 285 miRNAs with FF. Twenty-three miRNAs are exclusive to FF samples. Comparison to a recent study [19] analyzing female serum and plasma miRNAs extracted with the same protocol as in the current paper revealed that 10 of the miRNAs observed uniquely in FF potentially derive from plasma infiltrating into the follicle from perifollicular capillaries (Supplementary Table S3).

A list of 175 miRNAs were detected in EVs indicating that these miRNAs are secreted into the follicular space in vesicular form. At the same time, 113 miRNAs were common to MGC and FF only (Figure 4A), suggesting that these are secreted from cells in complexes other than EVs with characteristic surface markers. By comparing these two lists we were interested to test, if the secretion of miRNAs in EVs serves a different signaling purpose in comparison to other mechanisms of miRNA secretion. We investigated, if the miRNAs inside or outside of EVs have a potential to regulate overlapping signaling pathways. Indeed, only three pathways were commonly targeted by 11–16 FF miRNAs residing in FF outside of EVs (Figure 4B, Supplementary Table S4A). On the other hand, 436 pathways

were over-represented for miRNAs in EVs, the top 30 of the pathways were common to 30-94 miRNAs (Figure 4C, Supplementary Table S4B). The 23 miRNAs observed only in FF were omitted from the pathway over-representation analysis, as these are not probably secreted by MGC as mentioned above.

Significant enrichment depicting RNA secretion from cells to FF and the difference between miRNAs loaded into EVs in comparison to all cell-free miRNAs was analyzed by differential expression (DE) analysis in control group samples (FF vs. MGC and EV vs. FF, respectively, as depicted in Figure 5A).

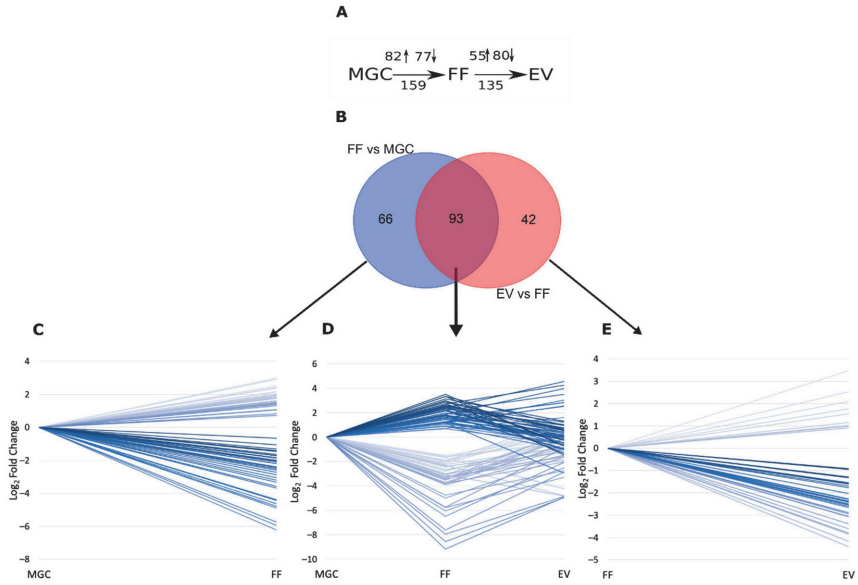

Figure 5. Comparison of cellular and cell-free miRNA expression levels. (**A**) Schematic representation of miRNA expression level comparisons performed between sample types and the number of differentially expressed (DE) results obtained. Arrows depict the number of upregulated (↑) or downregulated (↓) genes in each comparison. (**B**) Number of distinct and shared DE miRNAs between comparisons. (**C**) DE results unique to FF vs. MGC comparisons (**D**) DE results common for FF vs. MGC and EV vs. FF comparisons. (**E**) DE results unique to EV vs. FF comparisons. Each line in panels (**C**–**E**) depicts the statistically significant (FDR < 0.05) expression level difference between sample types of one miRNA. EV—extracellular vesicles, FF—cell-free follicular fluid, MGC – mural granulosa cells.

Comparison between FF and MGC samples, resulted in 159 differentially expressed miRNAs (FDR < 0.05, Figure 5A, Supplementary Figure S4A, Supplementary Table S5), while the DE analysis of EV and FF samples demonstrated the statistically significant expression of 135 miRNAs (FDR < 0.05, Figure 5A, Supplementary Figure S4B, Supplementary Table S6). From all differentially expressed miRNAs 93 were common to both comparisons: FF vs. MGC and EV vs. FF (Figure 5B). Figure 5D illustrates the expression level changes of those 93 miRNAs throughout MGC, FF and EV samples. Figure 5C,E illustrates miRNA expression changes of 66 and 42 miRNAs differentially expressed between FF vs. MGC or EV vs. FF, respectively. Significant miRNA expression changes between the analyzed compartments indicate potentially different secretion mechanisms: miRNAs with constantly increasing levels from MGC to FF and EV are more probably packed specifically into EVs. In contrast, miRNAs with the highest abundance in FF have a higher probability to be secreted into extracellular space in other macromolecular complexes.

2.5. Differences in miRNA Expression between PCOS Patients and Oocyte Donors

A comparison of samples between patient groups revealed that 30 and 10 miRNAs in MGC and FF, respectively, were differentially expressed between PCOS and oocyte donor patients (FDR <0.1, Figure 6A,B, Supplementary Table S7A,B). Due to a higher variation of miRNA expression levels across patients in EV samples (Figure 3A), no miRNAs reached the same FDR cut-off level. However, seven miRNAs were differentially expressed in EV samples between the two groups without considering the FDR (p-value < 0.05, Figure 6C, Supplementary Table S7C). Hsa-miR-200c-3p was the only commonly up-regulated miRNA in the extracellular samples FF and EV. All other DE miRNAs between the patient groups were unique to each sample type.

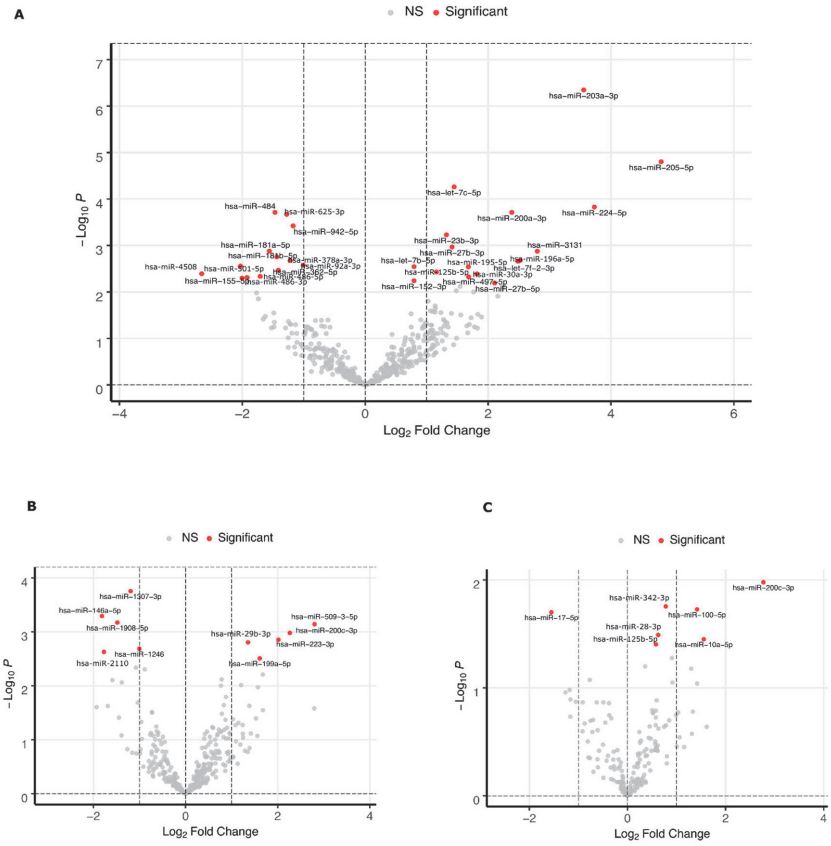

Figure 6. Differentially expressed miRNAs between oocyte donors and PCOS patients in mural granulosa cell samples (**A**), cell-free follicular fluid samples (**B**) and extracellular vesicle samples **(C)**. Statistical significance cut-off is FDR < 0.1 (A and B) or p < 0.05 (**C**).

Validation of the RNA sequencing results was performed by RT-qPCR for miRNAs with the highest fold change and that have been previously related to ovarian functions (Table 2). Since oocyte donors are generally young women the average age difference between the two study groups in RNA sequencing experiment was statistically significant (p-value = 0.002, Table 1). As there is previous evidence that the expression of some miRNAs can be affected by age [20,21], a validation cohort of age-matching women undergoing IVF due to male-factor infertility was added to the oocyte donor samples used for RNA sequencing. The average age difference between the PCOS and RT-qPCR validation control group was therefore not statistically significantly (p-value = 0.626, Table 1).

From five of the validated miRNAs in MGC samples the expression change direction was confirmed with statistical significance for hsa-let-7c-5p, hsa-miR-196a-5p and hsa-miR-203-3p. In FF samples four of the five validated miRNAs presented same directional change, out of which two (miRNAs hsa-miR-509-3-5p and has-miR-200c-3p) were also statistically significant. All three validated miRNAs from EVs displayed the same directional change without reaching the statistically significant level (Supplementary Figure S5).

Table 2. Differentially expressed miRNAs between PCOS patients and oocyte donor samples selected for RT-qPCR validation.

A) MGC PCOS vs. donors:		
miRNA	**Log_2 Fold Change**	**miRNA role in ovary**
hsa-miR-205-5p	4.82	Expression is upregulated in MI oocytes upon IGF-1 treatment [22]. Upregulated in ovarian cancer (OC) cells compared to control group and is associated with poor survival rates. Proposed miRNA targets are SMAD4 and PTEN [23].
hsa-miR-203a-3p	3.56	Expression levels are higher in granulosa cells of young women with normal ovarian reserve compared to young women with diminished ovarian reserve [24].
hsa-miR-196a-5p	2.49	Detectable in bovine granulosa cells at day 3 but not at day 7 of the estrous cycle [25].
hsa-let-7c-5p	1.45	Expressed higher in human CGC compared to MGC cells. miRNA expression is decreased in granulosa cells of early and progressive atretic follicles and in case of premature ovarian failure syndrome (measured from plasma) [26].
hsa-miR-181a-5p	−1.56	Expressed higher in human CGC compared to MGC [27]. In mouse granulosa cells miR-181a-5p targets ACVR2A (Activin Receptor IIA) and inhibits granulosa cell proliferation [28]. In oxidative stress conditions miRNA expression is upregulated in mouse granulosa cells and mediates granulosa cell apoptosis [29].
B) FF PCOS vs. donors:		
miRNA	**Log_2 Fold Change**	**miRNA role in ovary**
hsa-miR-509-3-5p	2.80	Expression is higher in FF of PCOS patients compared to controls [30].
hsa-miR-200c-3p	2.26	Expression is higher in granulosa cells [31] as well as in FF samples [32] of PCOS patients compared to control group.
hsa-miR-223-3p	2.02	EVs obtained from FF show expression of hsa-miR-223-3p [33]. miRNA expression is decreased in cumulus cells of PCOS patients [34].
hsa-miR-1908-5p	−1.48	Low expression predicts poor prognosis for ovarian cancer [35].
hsa-miR-146a-5p	−1.81	Expression is higher in human MGC samples compared to CGC [36].
C) EV PCOS vs. donors:		
miRNA	**Log_2 Fold Change**	**miRNA role in ovary**
hsa-miR-200c-3p	2.77	Expression is higher in granulosa cells samples [31] as well as in FF [32] obtained from PCOS patients compared to control group.
hsa-miR-100-5p	1.42	Associated with cell proliferation regulation [37]. Downregulated in young women with diminished ovarian reserve compared to normal ovarian reserve [37].
hsa-miR-17-5p	−1.55	Expression is downregulated in granulosa cells and FF of PCOS women compared to controls [38]. miRNA expression is detected in EVs obtained from FF [33].

2.6. Distinctive Functions Are Dysregulated in Each Analyzed Follicular Compartment of PCOS Patients

As one miRNA may target several genes and one gene may be targeted by several miRNAs, we subsequently aimed to detect any common pathways changed in PCOS patients by the differentially expressed miRNAs. Reactome pathway over-representation was performed for all differentially expressed miRNA-s according to RNA sequencing in each sample type. Lists demonstrating higher and lower miRNA expression levels in PCOS group compared to controls were analyzed separately.

miRNAs that are significantly upregulated in the PCOS group regulate in total 20 pathways in MGC, 25 pathways in FF and 13 pathways in EV (Figure 7, Supplementary Table S8). Transcription regulation and cell cycle pathways constitute the majority of the over-represented terms in MGC.

In addition, miRNAs regulating signaling by estrogen receptors (ESR) and nuclear receptors in general were more abundant in the MGC of PCOS patients.

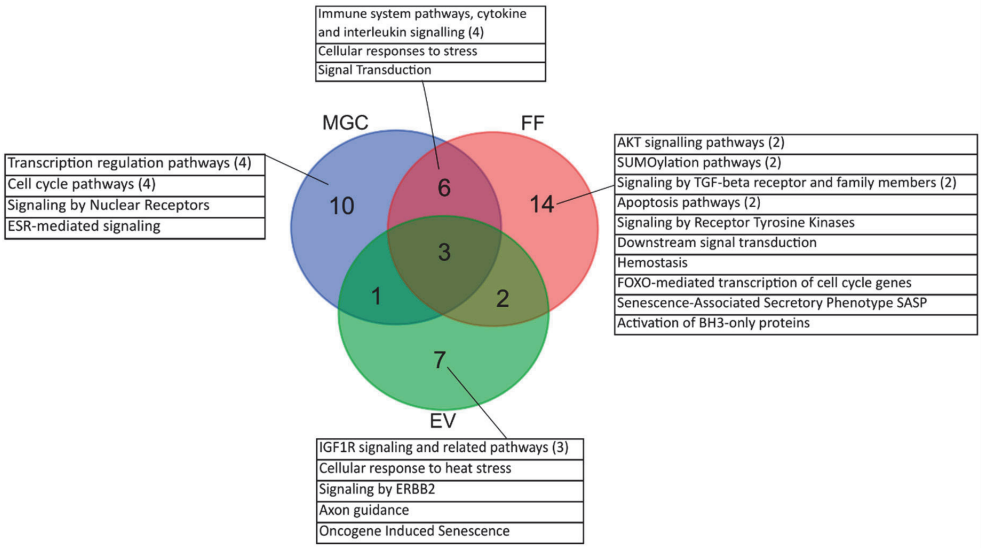

Figure 7. Number of Reactome pathways over-represented for miRNAs that are more abundantly expressed in PCOS patients compared to the fertile control group in each sample type. Numbers in brackets refer to combined pathways with similar outcome. EV—extracellular vesicles, FF—cell-free follicular fluid, MGC—mural granulosa cells.

Over-represented miRNAs in FF regulate several signal transduction pathways: AKT, TGF-beta, as well as pathways related to apoptosis and protein modification by SUMOylation appeared as the most frequent terms. The most common terms for miRNA-s that were significantly more abundant in the EVs of PCOS women were related to IGF1R signaling pathways.

In addition, miRNAs up-regulated in the MGC and FF samples of PCOS women target common immune system related pathways that are not apparent predicted targets for miRNAs in the EVs.

miRNAs that were less abundant in the MGC of PCOS patients are involved in pathway "Cellular responses to external stimuli". No over-representation of pathways was achieved for miRNAs with low abundance in FF and EV in the PCOS group (Supplementary Table S8).

2.7. Potential Novel miRNA as Marker for Follicular EVs

RNA sequencing data can be useful for predicting novel, yet unannotated miRNAs. After filtering candidate novel miRNA sequences suggested by miRDeep2 algorithm, we propose one potential new miRNA (mature sequence: CCUGGGCAUGGGACUGG, predicted stem-loop sequence in Figure 8A) that was expressed in 12 different patients and in all three sample types (EV, FF and MGC). It was most frequently detected in EV samples (in nine EV samples, four FF, and five MGC samples) demonstrating significantly higher expression levels compared to FF and MGC samples (Figure 8B). Moreover, validation with RT-qPCR demonstrated significantly higher levels of this sequence in EVs compared to FF samples (Figure 8B). The expression levels of the novel miRNA did not differ between PCOS and donor group in any of the sample types (data not shown). Five previously annotated miRNAs share a similar seed sequence (nucleotides 2–8 from 5′ end) with the potential novel miRNA (Figure 8C). miRDB predicted 1430 potential targets for the novel miRNA that were further enriched into 82 terms in the ontology domain of biological processes (FDR < 0.05, Supplementary Table S9). These were further reduced by semantic similarity analysis to three

largest categories: regulation of cell communication, cell junction organization, and nervous system development (Supplementary Figure S6).

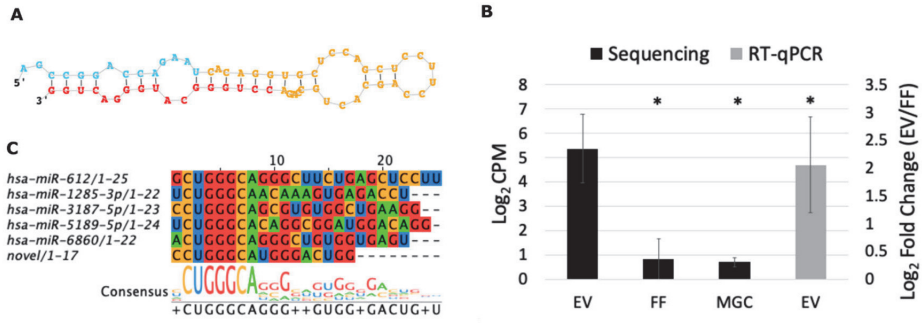

Figure 8. Novel miRNA detected by small RNA sequencing of single follicle components. (**A**) Predicted stem-loop sequence by miRDeep2. (**B**) Novel miRNA expression levels in the extracellular vesicles of follicular fluid (EV), cell-free follicular fluid (FF) and in granulosa cells (MGC) according to RNA sequencing displayed as a mean of count per million (CPM) ±SEM on $\log_2$ scale (n = 15). RT-qPCR validation result is displayed as fold change ±SEM on $\log_2$ scale (n = 15). (**C**) Novel miRNA aligned to previously annotated miRNAs with similar seed sequence. * $p < 0.05$, Student's t-test.

3. Discussion

A key aspect of cellular and organismal homeostasis in the higher mammals is intercellular communication, where cells are required to communicate with each other in order to maintain the vital functions of the body. Some of the important mediators of this cell-cell communication are cell-free RBPs and nanoparticles, including EVs that contain molecules from a plethora of RNA biotypes [6]. All body fluids, including the follicular fluid, are rich sources of cell-free nucleic acids and EVs [39]. EVs are heterogeneous, and their subtypes; exosome and microvesicles share a similar size range, which imposes a challenge in their efficient isolation, purification, and separation [40]. There are several different methods used for EVs isolation, and every method has its own limitations towards the purity of EVs from other RNA-containing particles. According to the Minimal Information for Studies of Extracellular Vesicles 2018 guidelines (MISEV) [39], purification methods should be chosen based on the downstream application of EVs. In the current study the MISEV-approved SEC method that gives the flexibility to separate the EVs according to their size ranges was used to acquire pure and functional EVs with reasonable recovery rates [41–43].

Several studies suggest that EVs are involved in intercellular communication in both normal physiology and pathological condition [44]. A recent study presented the higher blood plasma concentration of platelet-derived microparticles in PCOS patients as compared to healthy women [45]. Our study additionally detected significantly more EVs in the FF of PCOS women compared to oocyte donors with normal ovarian morphology.

We have, for the first time, analyzed matched cellular and extracellular small RNA profiles from individual pre-ovulatory follicles by whole-genome small RNA sequencing. In addition, the extracellular small RNAs were analyzed in two fractions: small RNAs loaded into EVs and total cell-free small RNAs present in FF. While separate RNA sequencing studies of MGC [27], FF [9] and EV [9] samples from the human follicle are available, combining the data from the three compartments of the follicle as a closed biological system is unique to the current study. The functionality of extracellular RNAs can roughly be divided into three: known function, predicted function and unknown function [46]. MiRNAs belong into the known function group and therefore we continued to investigate the miRNA profiles of the samples. We observed that the miRNA profile clearly distinguishes the three sample types indicating that the miRNAs are released in EV- and non-EV-mediated mechanisms serve distinct functional purposes. To support our results, it has been

demonstrated that the cell-free and EV-associated miRNAs also have different profiles in matching plasma samples [47,48].

There are many possible sorting mechanisms proposed for loading miRNAs into EVs: sequence characteristics, post-transcriptional modifications, subcellular location and intracellular concentration have all been shown to pose an effect to the segregation of miRNAs into EVs [6]. The heterogeneity of EVs (exosomes, microvesicles or apoptotic bodies) additionally plays a role in the nucleic acid content [49]. An even larger proportion of extracellular small RNAs is secreted outside of EVs in composition of other macromolecular complexes, e.g., non-vesicular RBPs. The mechanisms by which proteins interact with miRNAs before secretion remain unclear and may depend on the particular protein in the complex, but the export of miRNAs via these pathways has been shown to be an energy-dependent process [6].

According to our study, 172 miRNAs were present in all sample types, indicating that those miRNAs are non-selectively secreted from cells via both methods (EVs and/or non-vesicular RBP pathway). Moreover, 113 miRNAs were detected only in cells or FF, meaning those miRNAs are likely secreted out of the cells in RBP, while 175 miRNAs were observed also in the EVs. The over-representation analysis of the EV and FF miRNA targets indicate that miRNAs loaded into EVs carry specific molecular signals. It is considered that cargo segregation into EVs is not a random event: for example the human bone marrow- and adipose-mesenchymal stem cell-derived exosomes contain distinctive small RNA molecules linked to their differentiation status [50]. Moreover, studies in the equine and bovine models have demonstrated that follicular fluid EVs were taken up by granulosa cells in vitro and this process affected the expression of genes involved in follicle development [14,51]. Furthermore, some miRNAs in follicular EVs may also regulate oocyte growth, as alterations in their expression were observed between follicles with different oocyte maturation stages [52]. All these studies lead to a conclusion that EVs in follicular fluid most likely have important regulatory roles.

The miRNAs in EV samples potentially target the following Reactome terms: "ESR-mediated signaling", "Signaling by Nuclear Receptors", and "PTEN Regulation", among others. Estrogen receptors belong to the nuclear receptor family and together with other members, like the androgen receptors, are involved in follicle development and ovulation [53,54]. PTEN participates in follicle activation and growth, higher PTEN levels being associated with poor oocyte competence [55,56]. To summarize, miRNA profile analysis of samples from fertile women indicates specific miRNA segregation into vesicles with various targeted pathways downstream. At the same time, the selection of miRNAs for secretion by non-EV mediated pathway tends to be a relatively random event in the follicle.

One may argue whether all the miRNAs detected in extracellular space participate in cell-to-cell signaling and what the relevant concentration of EV-, or protein-bound miRNAs would be to have a physiologically significant function. Chevillet et al. have calculated that EVs contain less than 1 copy of miRNAs per EV [57]. In sequencing experiments the small RNA cargo is analyzed in bulk, therefore there is no information whether all individual EVs are equally loaded with miRNA molecules or if there is a certain distribution of cargo into different EVs. A specific segregation of miRNA molecules may significantly raise the copy number of individual miRNAs per EV as it is proposed with low-occupancy/high-miRNA concentration distribution model indicating that rare EVs in population contain many copies of a certain miRNA [57].

As the main goal of this study, we were interested, whether the ovarian miRNA expression, secretion, or segregation into EVs are compromised by PCOS. We observed that each studied follicular compartment is affected differently by PCOS, since the miRNA profiles are dissimilar in MGC, FF and EV also in the fertile ovary. Therefore, separate pathways are affected by PCOS in MGC and in the potential recipient cells for the extracellular miRNA in the FF and EV.

Several miRNAs have been shown to be differentially expressed in the granulosa cells and/or FF between fertile and PCOS women [9,30,58,59]. In the current study, the highest number of differences in miRNA expression were detected from cellular material. At single follicle level the EV samples

diverged the least between the two groups, although some differences could be detected without multiple testing adjustment. Fewer differences in EV may be caused by the more complex processing of EV samples before RNA extraction compared to MGC and FF that may affect the results.

We identified several miRNAs differentially expressed between the patient groups which have not been previously associated with PCOS but are involved in the regulation of gene expression in follicles or in other ovary-related disorders. For example, hsa-miR-224-5p [60], which was up-regulated in the MGC of PCOS women in our study, downregulates SMAD4, which is involved in the regulation of apoptosis of granulosa cells [61]. It has been shown that hsa-miR-203a-3p, hsa-miR-195-5p, hsa-miR-486-3p, and hsa-miR-484 levels are altered in the granulosa cells of women with diminished ovarian reserve [24]. Interestingly, all the four mentioned miRNAs are expressed in the MGC of our PCOS group according to the same pattern as in normal ovarian reserve (NOR) patients: hsa-miR-203-3p and hsa-miR-195-5p are both more abundant in NOR and our PCOS group, while hsa-miR-486-3p and hsa-miR-484, are less abundant in NOR as well as in our PCOS samples. PCOS women have been shown to have a slower age-related decline in antral follicle count (AFC) compared to non-PCOS patients [62] and AFC is considered to be a reliable marker for ovarian reserve evaluation [63]. Slower decline in AFC in PCOS women may also explain our miRNA expression results.

The expression differences of hsa-miR-486-5p [64] in MGC have been previously associated with PCOS compared to women undergoing IVF due to male factor infertility with same directional expression as in our study. Moreover, hsa-miR-200a-3p [34] and hsa-miR-30a-3p [32] have been previously linked to PCOS, but in other follicular compartments. In these studies, hsa-miR-200a-3p in cumulus granulosa cells and hsa-miR-30a-3p in FF were less abundant in PCOS women which is opposite to our results. Differential expression of hsa-miR-509-3-5p [30] and hsa-miR-200c-3p [30,31] in FF has been previously associated with PCOS with mutual expression direction to our results. Hsa-miR-1307-3p [65] and hsa-miR-223-3p [34] are also altered in cumulus granulosa cells of PCOS patients, but with an opposite direction to our result. These comparisons demonstrate the dependence of miRNA expression disturbances depending on the cellular environment. By the pre-ovulatory follicular stage, when the samples have been obtained, cumulus granulosa cells have differentiated from MGC and have significant dissimilarities in gene expression and post-transcriptional regulation patterns [27,66]. From EV samples hsa-miR-200c-3p [30,31], hsa-miR-17-5p [38] have been previously shown to be altered in PCOS women in line with the results of our study.

Our results clearly demonstrate that the effects of miRNA expression differences brought upon PCOS lead to different molecular outcomes depending on the investigated sample type. For example, cytokine-mediated signaling was affected in the cellular compartment and by non-EV-mediated RNA secretion, while EV-mediated signaling potentially affects the IGF1R pathways in PCOS patients. Those results emphasize the importance of studying the follicle as a system to better understand inter-cellular signaling and possible molecular disturbances in the PCOS ovary.

In conclusion, the current study proposes novel miRNAs and their regulated signaling pathways, underlying the infertility of patients with PCOS.

4. Materials and Methods

4.1. Ethics Statement

The study was approved by the Research Ethics Committee of the University of Tartu, Estonia on January 21st, 2019 with the approval number 289/M-8. Written informed consent was obtained from all participants.

4.2. Patients and Sample Collection

FF and MGCs were collected from women undergoing ovarian stimulation and oocyte pick-up by ovarian puncture (OPU). Ovarian hormonal stimulation was conducted according to the gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide, Merck Serono, Darmstadt, Germany)

protocol with the administration of recombinant follicle-stimulating hormone (Gonal-F, Merck Serono, or Puregon, Merck Sharp & Dohme Corp., Whitehouse Station, NJ, USA). All patients underwent OPU if at least two follicles were ≥18 mm in size 36 h after human chorionic gonadotropin administration (Ovitrelle, Merck Serono).

Samples were collected from two groups of women: PCOS patients (n = 15) and fertile women (IVF patients from couples with male factor infertility (n = 16) and oocyte donors (n = 15)). General characteristics of study participants are presented in Table 1. The PCOS group was formed according to the Rotterdam Consensus [67] with PCOM observed by ultrasound being the primary criterion for recruitment. Control group consisted of women with regular menstrual cycles, without any infertility diagnosis nor PCOM. All recruited women were <40 years of age.

FF containing all cellular material was collected from the first aspirated follicle visibly clear of blood contamination. The sample was first centrifuged 10 min at 300 g to remove whole cells. The supernatant was subsequently centrifuged 10 min at 2000× g to remove cell debris. The final cell-free FF was stored at −80 °C until further analysis. The remaining cell pellets of MGCs from the first centrifugation were lysed using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) and stored at −80 °C until RNA extraction.

4.3. Isolation of Extracellular Vesicles from Follicular Fluid Samples

Five hundred μL of each FF sample was concentrated to 150 μL using 10 kDa Amicon® Ultra centrifugal filter units (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Ireland). Commercially available size exclusion chromatography (SEC) column (qEVsingle/70 nm by Izon Sciences, UK) was used for the isolation of EVs. The column was prewashed with 10 mL filtered (0.2 μm Minisart® syringe filters) Dulbecco's phosphate-buffered saline (DPBS, Sigma® Life Science, UK) and 150μl of the concentrated sample was added to the top of the column filter. After the sample had passed down, DPBS was added immediately on the top of the column filter and a total of 20 fractions, 200 μL each, were collected separately. The concentration of nanoparticles (NP) of each fraction was measured on ZetaView® nanoparticle tracking analyzer (NTA, PMX 120 by Particle Metrix GmbH, Inning am Ammersee, Germany). The protein concentration of each fraction was determined with the Quick Start™ Bradford Protein Assay (Bio-Rad, California, USA) according to the manufacturer's protocol. Based on these analyses, fraction 6-9 (800 μL) showed the presence of the highest number of particles and the least protein contamination Supplementary Figure S1). Fractions 6–9 were pooled together, concentrated with 10 kDa Amicon® Ultra 2 centrifugal filter units, and used for downstream experiments.

4.4. Nanoparticle Tracking Analysis

The size profile and concentration of NPs/EVs in the samples were carried out using the ZetaView® nanoparticle tracking analyzer. A standard of 100 nm particles (Applied Microspheres BV, Leusden, the Netherlands) was used for instrument calibration. During the analysis, standard manufacturer's procedure was followed for NPs/EVs size distribution and concentration measurement. The size profile and concentration of NPs/EVs were measured using the scatter mode under the following settings: sensitivity 85, shutter speed 70, frame rate 30 frames per second, and the number of cycles 3. All samples were measured in triplicates. In order to minimize the inter-sample contamination, the measurement cell of the instrument was washed thoroughly using Milli-Q® water, and the cell was filled with DPBS before the injection of the next sample.

4.5. Western Blot Analysis

Fractions 6–9 (contain follicular fluid EVs) and fractions 10–14 (contain FF proteins) were obtained by SEC as described earlier. Respective fractions were pooled and concentrated with 10 kDa Amicon® Ultra-15 centrifugal filter to 300 μL. To precipitate the proteins, 100 μL of water, 400 μL of methanol (Sigma-Aldrich, Schnelldorf, Germany), and 100μl of chloroform (Lach-Ner, Neratovice, Czech Republic) were added to the concentrated sample and centrifuged for 5 min at

14,000× *g*. The top layer was removed, and proteins in the interphase were washed with 400 µL of methanol. After centrifugation, the pellet was dried, resuspended in 0.25% SDS, and the protein concentration was measured with Bradford assay. Proteins from 50µl of FF were precipitated using the same protocol. For each sample, 10 µg of protein was mixed with either non-reducing Laemmli buffer or reducing Laemmli buffer, heated for 5 min at 95 °C and separated by 12% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Thermo Scientific, Rockford, IL, USA), and the membranes were incubated in blocking buffer (5% nonfat dry milk in PBS-Tween 0.05%) for 1 h at room temperature (RT). Subsequently, the membranes were incubated with the following primary antibodies overnight at 4 °C: mouse anti-human CD63 antibody (556019, 1:1000, BD Biosciences, San Jose, CA, USA), mouse anti-CD9 antibody (sc-59140, 1:250, Santa Cruz Biotechnology Inc., Dallas, TX, USA), mouse anti-apoA-I antibody (sc-376818, 1:1000, Santa Cruz Biotechnology Inc.), mouse anti-human CD81 antibody (555675, 1:1000, BD Biosciences), rabbit anti-Grp94 antibody (ADI-SPA-851-D, 1:1000, Enzo Life Sciences, Farmingdale, NY, USA) and rabbit anti-albumin antibody (16475-1-AP, 1:10 000, Proteintech, Chicago, IL, USA). Membranes were washed with PBS-Tween 0.05% and then incubated with either HRP-conjugated goat anti-rabbit IgG secondary antibody (G21234, 1:20 000, Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) or goat anti-mouse IgG secondary antibody (G21040, 1:20 000, Invitrogen, Thermo Fisher Scientific) for 1 h at RT. After washing the membranes with PBS-Tween 0.05% and incubating in ECL Select Western Blotting Detection Reagent solution (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), the protein bands were visualized using ImageQuant RT ECL Imager (GE Healthcare).

4.6. Transmission Electron Microscopy

Fractions 6-9 (800 µL) of isolated EVs on SEC were pooled and subsequently concentrated to 150µl using Amicon® Ultra 2 centrifugal filter units (10 kDa) (Merck Millipore Ltd.). A previously described method [39] was followed for *transmission electron microscopy* (TEM) analysis. A droplet from the purified EV samples was deposited on Formvar-carbon-coated 200 mesh copper grids (Agar Scientific, Essex, UK) and allowed to absorb for 20 min. The sample was fixed on a grid in 2% paraformaldehyde (Sigma-Aldrich) and 1% glutaraldehyde (Polysciences, Warrington, PA, USA), contrasted in uranyl oxalate (a mixture of 4% uranyl acetate (Polysciences) and 0.15 M oxalic acid (Sigma-Aldrich)) and embedded in a mixture of methylcellulose (Sigma-Aldrich) and uranyl acetate (Polysciences). Samples were observed with a JEM 1400 transmission electron microscope (JEOL Ltd. Tokyo, Japan) at 80 kV, and digital images were acquired with a numeric camera (Morada TEM CCD camera, Olympus, Germany).

4.7. RNA Extraction

miRNA extraction from isolated EVs was performed using miRNeasy Micro kit (QIAGEN) according to the user manual with the exception of 5 µg of glycogen (Thermo Scientific) added to chloroform.

Starting amount of miRNA extraction from FF was 500 µL. Extraction was performed with miRNeasy Micro kit (QIAGEN) with some modifications to the user manual [68]. Shortly, 500 µL of FF was transferred into a 15 mL tube and 5x volumes of QIAzol Lysis Reagent (QIAGEN) was added. After incubation 500 µL chloroform and 5 µg of glycogen (Thermo Scientific) were added to the tube. Following steps of RNA exactions were performed according to the miRNeasy Micro kit (QIAGEN) user manual.

Total RNA from cells was extracted with miRNeasy Mini kit (QIAGEN). In addition, small fraction RNA (≤200 nucleotides) was separated by RNeasy Mini Elute Cleanup Kit (QIAGEN). Both total and small RNA extraction were performed according to the user manual.

The quality and concentration of cellular RNA samples was evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

4.8. Small RNA Library Preparation and Sequencing

Small RNA libraries were prepared with QIAseq miRNA Library Kit (QIAGEN) according to the manufacturer's protocol. Starting amount of RNA in library preparation was 10 ng from cellular small RNA fraction and 5 µL of RNA from EV and FF samples. Final libraries were separated and excised from 5% TBE gels (Bio-Rad Laboratories) after staining with 1X SYBR Gold stain (Thermo Fisher Scientific). Gel pieces containing the miRNA libraries were crushed with pellet pestles (Fisher Scientific). 300 µL RNase free water (Thermo Fisher Scientific) was added to the gel debris and rotated for 2 h at RT to elute miRNA libraries. Eluate and gel debris were transferred to the Spin X centrifuge tube filter (Merck, Darmstadt, Germany) and centrifuged 2 min at 16,000 g. Thereafter 2 µL glycogen (Thermo Scientific), 30 µL 3 M NaOAc (Thermo Fisher Scientific), 1 µL 0.1x Pellet Paint (Merck) and 975 µL of cold 100% ethanol (Naxo, Tartu, Estonia) were added to the eluate, and centrifuged for 20 min at 20,000× *g* at 4 °C. Pellet was washed with 500 µL of 70% ethanol and centrifuged for 2 min at 20,000× g. The final libraries were resuspended in 7 µL of resuspension buffer (PerkinElmer, Massachusetts, USA). The size of libraries was estimated with Agilent DNA High Sensitivity chips on the Agilent 2100 Bioanalyzer system (Agilent Technologies). Library concentrations were measured using Qubit High Sensitivity Assay kit (Thermo Fisher Scientific) before pooling in equimolar amounts. Single-end sequencing of 75 bp length was performed on NextSeq 500 platform with NextSeq 500/550 High Output Kit v2.5 (Illumina, San Diego, CA, USA).

4.9. cDNA Synthesis and RT-qPCR

For the validation of miRNA expression levels cDNA was synthesized using miRCURY LNA RT Kit (QIAGEN) from 30 ng of cellular small RNA fraction or 5 µL of extracted small RNA fraction from FF and EV samples.

The RT-qPCR analysis was carried out on LightCycler 480 instrument (Roche, Basel, Switzerland). For the detection of miRNA expression miRCURY LNA SYBR Green (QIAGEN) was used according to the user manual. The specificity of amplified PCR products was determined by melt curve analysis. miRCURY LNA miRNA PCR Assay primers were used in all reactions (QIAGEN).

4.10. Data Analysis and Statistics

4.10.1. miRNA Sequencing Analysis

Raw FASTQ files were quality-filtered with Trimmomatic v 0.39 [69] with the options of SLIDINGWINDOW:2:20. Adapter sequences (3'adapter AACTGTAGGCACCATCAAT and 5´ adapter GTTCAGAGTTCTACAGTCCGACGATC) were removed and reads below 17 nucleotides in length were discarded and the remaining filtered and trimmed reads were counted and mapped to the primary assembly of human genome GRCh38 using miRDeep2 with standard settings [70].

Count tables from individual samples were merged using edgeR package v.3.28.1 [71] and formed count matrix was used as input for DESeq2 v.1.26.0 [72] in R version 3.6.3 [73] for differential gene expression analysis between groups with standard options. miRNAs expressed at low level were removed from analysis: cut-off was set at ≥5 raw reads in 50% of samples. For visualization purposes, variance stabilizing transformation of data was performed with option blind = FALSE.

The statistical significance cut-off for differentially expressed miRNAs in DESeq2 analysis was set at false discovery rate (FDR) <0.05 in case of comparing three tissue types in oocyte donor samples. Cut-off for statistical significance was set at FDR <0.1 when comparing patient groups.

4.10.2. RT-qPCR Data Analysis

miRNA expression levels in cellular fraction were normalized for U6 snRNA and hsa-miR-132-3p. Endogenous control for FF and EV samples was hsa-miR-16-5p. All normalizations were performed according to the ΔΔCt method of relative quantification [74]. Statistical significance was calculated

by two-tailed Student's t-test in Microsoft Office Excel 2017. Statistical significance level was set at $p < 0.05$.

4.10.3. miRNA Target Prediction, Gene Ontology and Over-Representation Analysis

Novel miRNA targets were predicted with miRDB [75] custom prediction tool. Obtained miRNA targets list was an input for gene enrichment analysis with g:Profiler [76], using g:GOst functional profiling tool where significance threshold was set at FDR <0.05. Results were visualized with REVIGO [77].

For annotated miRNAs the lists of statistically significant differentially expressed miRNA lists were used as input to miRNA Enrichment Analysis and Annotation Tool (miEAA), that performs miRNA target prediction and over-representation analysis of gene ontology terms simultaneously by combining linked external databases [78]. Over-representation analysis was performed for Reactome Pathways via miRPathDB [79] for each up- and down-regulated miRNA list separately. Background list was created from all detected miRNAs in our small RNA sequencing dataset. Pathways targeted by >50% of miRNAs (minimum 3) in each list with Benjamini-Hochberg FDR < 0.05 are reported.

Similarly, miEAA was used to analyze Reactome Pathway over-representation for all miRNAs observed exclusively in FF or in EV samples.

4.10.4. Novel miRNA Candidate Filtering

Predicted novel miRNAs were filtered with a cut-off of miRDeep2 score >1. Remaining potential novel miRNA candidates were aligned against human transcriptome with NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), discarded if the sequences overlapped with a coding region of an annotated gene, demonstrated high similarity to other know miRNAs, were detected only in one sample or with the average occurrence in positive samples <10 raw counts. miRNA sequences with similar seed region to the potential novel miRNA were obtained from miRBase v22.1 and visualized in Jalview 2.11.1.0 [80].

4.10.5. EV Size Profile and Concentration

To test if the sample means of the EV size profiles are non-normally distributed 1000 samples of 1000 EVs were drawn from the NTA data of PCOS and donors' group, their means calculated and tested using Shapiro–Wilk test. Student's t-test was then used to test if the difference between the size profile means is statistically significant. The behavior of p-values with smaller sample size was further analyzed by drawing 1000 random samples of size 100, 1000, 2000, and 5000 EVs each and plotting the p-value histograms (Supplementary Figure S2). The difference in EV concentrations was tested using two-tailed Student's t-test and a p-value <0.05 was considered statistically significant.

4.10.6. Data Availability

The datasets generated for this study can be found in the Gene Expression Omnibus repository (GSE157037).

5. Conclusions

The current study proposes novel signaling pathways underlying the infertility of patients with PCOS. We demonstrate that the follicular environment is affected by the PCOS differently depending on the studied compartment, i.e., MGC, EV, and FF, indicating potential changes in intercellular communication in the ovaries of these patients. We predict that alterations in cellular miRNA expression levels lead to changes in estrogen receptor signaling and the dysregulation of transcription and apoptosis. EV-mediated miRNA signalization potentially affects IGF1R pathways in the recipient cells.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/24/9550/s1.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

AFC	Antral follicle count
CGC	Cumulus granulosa cells
COC	Cumulus oocyte complex
DE	Differential expression
ESR	Estrogen receptors
EV	Extracellular vesicles
FDR	False discovery rate
FF	Follicular fluid
MGC	Mural granulosa cells
NOR	Normal ovarian reserve
NP	Nanoparticles
NTA	Nanoparticle tracking analyzer
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovary syndrome
RBP	Ribo-protein complexes
SEC	Size exclusion chromatography
TEM	Transmission electron microscopy
WB	Western blot

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Curriculum vitae

Personal data

Name:	Ilmatar Rooda
Date of birth:	30.12.1991
Place of birth:	Tallinn
Citizenship:	Estonia

Contact data

E mail:	ilmatar.rooda@gmail.com

Education

2016–2021	Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology, Chemistry and Gene Technology, PhD
2014–2016	Tallinn University of Technology, Faculty of Science, Department of Gene Technology, Gene Technology, MSc
2011–2014	Tallinn University of Technology, Faculty of Science, Department of Gene Technology, Gene Technology, BSc
2008–2011	Tallinn 32. High School

Language competence

Estonian	Native
English	Fluent
Russian	Basic
French	Basic

Professional employment

2016–…	The Competence Centre on Health Technologies, Specialist (0.5)

Scholarships & awards

2021	Dora Plus T1.2 mobility scholarship for visiting the Karolinska Institute, Sweden (September-October).

Courses and conferences

July 2020	Conference “36th virtual annual meeting of ESHRE”; poster presentation “Whole-genome microRNA expression profiles from single pre-ovulatory follicles of oocyte donors and polycystic ovarian syndrome (PCOS) patients”
May 2019	Summer School “Basics to Work With Extracellular Vesicles”, Tartu, Estonia
October 2019	Course “RNA-seq workshop for beginners: from sequences to visualization using Galaxy and R”, Tartu, Estonia
January 2019	Course “Software carpentry”, Tartu, Estonia
October 2018	Course “Comparative aspects of reproduction and its failure in humans and animals”, Tartu, Estonia
August 2018	Conference “Frontiers in Delivery of Therapeutics”, Tartu, Estonia
September 2018	Course “Developmental Biology Minisymposium“, Tallinn, Estonia
September 2018	Conference “Women's and Fetal Health and Wellbeing”, Tartu, Estonia
September 2018	Conference “The management of infertility”, Tartu, Estonia
June 2018	Conference “The 18th International Gene Forum”, Tartu, Estonia

May 2018	Workshop “Workshop on Reproductive Medicine”, Helsinki, Finland; presentation “Target identification of microRNAs expressed from follicle stimulating hormone receptor (FSHR) and aromatase genes”
June 2017	Conference “The 17th International Gene Forum”, Tartu, Estonia

Supervised dissertations

Birgitta Kaselt, Master’s Degree, 2020, (sup) Agne Velthut-Meikas; Ilmatar Rooda, Description and role of hsa-mir-548 family microRNAs in the human endometrium, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology

Birgitta Kaselt, Bachelor’s Degree, 2018, (sup) Agne Velthut-Meikas; Ilmatar Rooda, Target identification of microRNAs expressed from follicle stimulating hormone receptor (FSHR) and aromatase genes, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology

Publications

Kasvandik S, Saarma M, Kaart T, **Rooda I**, Velthut-Meikas A, Ehrenberg A, Gemzell K, Lalitkumar PG, Salumets A, Peters M.
Uterine Fluid Proteins for Minimally Invasive Assessment of Endometrial Receptivity. J Clin Endocrinol Metab. 2020 Jan 1;105(1):dgz019. doi: 10.1210/clinem/dgz019. PMID: 31512719.

Rooda I, Hensen K, Kaselt B, Kasvandik S, Pook M, Kurg A, Salumets A, Velthut-Meikas A.
Target prediction and validation of microRNAs expressed from FSHR and aromatase genes in human ovarian granulosa cells.
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Rooda I*, Hasan MM*, Roos K, Viil J, Andronowska A, Smolander OP, Jaakma Ü, Salumets A, Fazeli A, Velthut-Meikas A.
Cellular, Extracellular and Extracellular Vesicular miRNA Profiles of Pre-Ovulatory Follicles Indicate Signaling Disturbances in Polycystic Ovaries.
Int J Mol Sci. 2020 Dec 15;21(24):9550. doi: 10.3390/ijms21249550. PMID: 33333986; PMCID: PMC7765449.

Rooda I, Kaselt B, Liivrand M, Smolander O-P, Salumets A, Velthut-Meikas A.
Hsa-mir-548 family expression in human reproductive tissues. BMC Genom Data. 2021 Oct 8;22(1):40. doi: 10.1186/s12863-021-00997-w. PMID: 34625017; PMCID: PMC8501715.

* Equal contribution

Elulookirjeldus

Isikuandmed

Nimi:	Ilmatar Rooda
Sünniaeg:	30.12.1991
Sünnikoht:	Tallinn
Kodakondsus:	Eesti

Kontaktandmed

E post:	ilmatar.rooda@gmail.com

Hariduskäik

2016–2021	Tallinna Tehnikaülikool, Loodusteaduskod, Keemia ja biotehnoloogia instituut, Keemia ja geenitehnoloogia (geenitehnoloogia), PhD
2014–2016	Tallinna Tehnikaulikool, Loodusteaduskond, Geenitehnolooogia instituut, geenitehnoloogia, MSc
2011–2014	Tallinna Tehnikaulikool, Loodusteaduskond, Geenitehnolooogia instituut, geenitehnoloogia, BSc
2008–2011	Tallinna 32. keskkool

Keelteoskus

Eesti keel	Emakeel
Inglise keel	Kõrgtase
Vene keel	Algtase
Prantsuse keel	Algtase

Teenistuskäik

2016-...	Tervisetehnoloogiate Arenduskeskus AS, Tartu, spetsialist (0.5)

Stipendiumid ja tunnustused

2021	Dora Plus T1.2 õpirände stipendium Karolinska Instituudi külastuseks Rootsis (september-oktoober).

Kursused ja konverentsid

Juuli 2020	Osalemine konverentsil “36th virtual annual meeting of ESHRE”; posterettekanne “Whole-genome microRNA expression profiles from single pre-ovulatory follicles of oocyte donors and polycystic ovarian syndrome (PCOS) patients”
Mai 2019	Osalemine suvekoolis “Basics to Work With Extracellular Vesicles”, Tartu, Eesti
Oktoober 2019	Osalemine kursusel “RNA-seq workshop for beginners: from sequences to visualization using Galaxy and R”, Tartu, Eesti
Jaanuar 2019	Osalemine kursusel “Software carpentry”, Tartu, Eesti
Oktoober 2018	Osalemine kursusel “Comparative aspects of reproduction and its failure in humans and animals”, Tartu, Eesti
August 2018	Osalemine konverentsil “Frontiers in Delivery of Therapeutics”, Tartu, Eesti
September 2018	Osalemine kursusel „Developmental Biology Minisymposium”, Tallinn, Eesti
September 2018	Osalemine konverentsil “Women's and Fetal Health and Wellbeing”, Tartu, Eesti

September 2018	Osalemine konverentsil "The management of infertility". Tartu, Eesti
Juuni 2018	Osalemine konverentsil "The 18th International Gene Forum", Tartu, Eesti
Mai 2018	Osalemine töötoas "Workshop on Reproductive Medicine", Helsingi, Soome; ettekanne "Target identification of microRNAs expressed from follicle stimulating hormone receptor (FSHR) and aromatase genes"
Juuni 2017	Osalemine konverentsil "The 17th International Gene Forum", Tartu, Eesti

Juhendatud väitekirjad

Birgitta Kaselt, magistrikraad, 2020, (juh) Agne Velthut Meikas; Ilmatar Rooda, Hsa-mir-548 perekonna mikroRNAde kirjeldus ja roll inimese endomeetriumis, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

Birgitta Kaselt, bakalaureusekraad, 2018, (juh) Agne Velthut-Meikas; Ilmatar Rooda, Folliikuleid stimuleeriva hormooni retseptori (FSHR) ja aromataasi geeni intronitest ekspresseeruvate mikroRNAde sihtmärkide tuvastamine, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

Publikatsioonid

Kasvandik S, Saarma M, Kaart T, **Rooda I**, Velthut-Meikas A, Ehrenberg A, Gemzell K, Lalitkumar PG, Salumets A, Peters M.
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* Võrdne panus

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