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# Ovarian Follicle as the Environment of Oocyte Maturation: The Role of Granulosa Cells and Follicular Fluid at Pre-Ovulatory Development

AGNE VELTHUT-MEIKAS

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TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Centre for Biology of Integrated Systems

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Supervisors: **Professor Andres Salumets, PhD**
Institute of Bio- and Translational Medicine, University of Tartu, Estonia; Women's Clinic of Tartu University, Estonia

**Professor Madis Metsis, PhD**
Institute of Mathematics and Natural Sciences, Tallinn University, Estonia

**Senior Researcher Anu Aaspõllu, PhD**
Centre for Biology of Integrated Systems, Tallinn University of Technology, Estonia

Opponents: **Professor Ursula Eichenlaub-Ritter, PhD**
Faculty of Biology, Institute of Gene Technology/Microbiology, University of Bielefeld, Germany

**Professor Toivo Maimets, PhD**
Institute of Molecular and Cell Biology, University of 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 the Tallinn University of Technology has not been submitted for any other academic degree.

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# Munasarja folliikul kui munaraku küpsemise keskkond: granuloosrakkude ja follikulaarvedeliku roll ovulatsioonieelses arengus

AGNE VELTHUT-MEIKAS

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KIRJASTUS

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*These authors contributed equally to this work.

**II**

**Velthut-Meikas A**, Simm J, Tuuri T, Tapanainen JS, Metsis M, Salumets A. Research resource: small RNA-seq of human granulosa cells reveals miRNAs in FSHR and aromatase genes. Mol Endocrinol. 2013 Jul;27(7):1128-41. doi:10.1210/me.2013-1058. Epub 2013 May 9. PubMed PMID: 23660593.

**III**

Sarapik A, **Velthut A**, Haller-Kikkatalo K, Faure GC, Béné MC, de Carvalho Bittencourt M, Massin F, Uibo R, Salumets A. Follicular proinflammatory cytokines and chemokines as markers of IVF success. Clin Dev Immunol. 2012;2012:606459. doi: 10.1155/2012/606459. Epub 2011 Oct 5. PubMed PMID: 22007253; PubMed Central PMCID: PMC3189459.

# INTRODUCTION

Delicate communication between the germline and ovarian somatic cells is the basis for all processes in ovarian physiology: the formation of follicles in the foetus, follicular dynamics throughout the menstrual cycles starting at puberty, as well as follicular atresia occurring during human development and reproductive lifetime.

Post-pubertal stages of folliculogenesis encompass the meiotic maturation of the oocyte and its successful ovulation. These processes are accomplished by pituitary gonadotrophin stimulation that reach the ovary via blood flow, but also by intricate local molecular signalling between the oocyte and the surrounding somatic cells: theca and granulosa cells. Disturbances at either the systemic or local levels of molecular interaction may lead to severe consequences regarding the fertility of the woman: anovulation, incomplete oocyte maturation, premature ovarian failure etc.

The molecular mechanisms of oocyte maturation and folliculogenesis have been revealed to a degree, which allows medical manipulation of these processes. A large proportion of infertile couples can now be aided by controlled ovarian stimulation, collection of oocytes via ovarian puncture, *in vitro* fertilization (IVF) and the transfer of *in vitro* grown embryos to the uterus. In Europe, depending on the country, 1-3% of children are already born aided by the assisted reproductive technologies (ART).

Although the first IVF baby was born already in 1978, making the history of ART rather long, the average success rate of each stimulation and IVF cycle remains at approximately 30%. Increasingly more attention is being turned to women, for whom conventional ovarian stimulation is contradictory. Protocols for *in vitro* maturation of oocytes and follicles are extensively being sought for, but the success rate using such techniques is even lower. All this creates substantial emotional as well as economic burden for the infertile couples.

In order to increase the success rate of ovarian stimulation and improve *in vitro* maturation techniques, it is of utmost importance to understand the molecular mechanisms underlying *in vivo* folliculogenesis. Furthermore, regarding the individual nature of each patient, the knowledge of how the general parameters of female physiology influence the outcome of follicle development is far from complete today.

During oocyte collection at ovarian puncture, the follicular fluid and granulosa cells become available for research without creating further discomfort for the patient. These components are valuable, as they constitute a major part of the follicular environment of oocyte maturation. With the available whole genome-wide methods, it has become within reach to gain vast amount of information from individual samples, bringing the solutions to the described challenges closer at hand.

The present thesis concentrates on identifying the gene expression and regulation characteristics of two granulosa cell populations: the mural and cumulus granulosa cells, with distinct functions in folliculogenesis. Secondly, the follicle-specific roles of cytokines and apoptosis markers as means of communication between the granulosa cell populations are under focus. Finally, follicular fluid levels of these markers are correlated with the etiology of female infertility and the outcome of ovarian stimulation and IVF.

# ABBREVIATIONS

AGO – argonaute protein
AMH – anti-Müllerian hormone
BMP – bone morphogenetic protein
CD44(v6) – CD44 antigen containing variable exon 6
CGC – cumulus granulosa cells
COC – cumulus-oocyte complex
COS – controlled ovarian (hyper)stimulation
CYP19A1 – cytochrome P450, family 19, subfamily A, polypeptide 1; aromatase
FAS – Fas cell surface death receptor
FASLG – FAS ligand
FF – follicular fluid
FSH – follicle stimulating hormone
rFSH – recombinant FSH
FSHR – FSH receptor
G-CSF – granulocyte colony-stimulating factor
GDF9 – growth differentiation factor 9
GnRH – gonadotrophin-releasing hormone
hCG – human chorionic gonadotrophin
IFN – interferon
IL – interleukin
IVF – *in vitro* fertilization
LH – luteinizing hormone
LHCGR – luteinizing hormone/chorionic gonadotrophin receptor
MCP-1 – monocyte chemotactic protein 1
M-CSF – macrophage colony-stimulating factor
MGC – mural granulosa cells
MIP – macrophage inflammatory protein
miRNA – microRNA
mRNA – messenger RNA
OHSS – ovarian hyperstimulation syndrome
PCOS – polycystic ovary syndrome
Poly(A) RNA – polyadenylated RNA
RANTES – regulated on activation, normal T cell expressed and secreted
RNA-seq – RNA sequencing by next generation high-throughput sequencing methods
sFAS – soluble FAS
SLC – solute carrier family
TFI – tubal factor infertility
TGF-β – transforming growth factor beta
TNF-α – tumour necrosis factor alpha
VEGF – vascular endothelial growth factor

# REVIEW OF THE LITERATURE

## 1. Overview of human folliculogenesis

The first follicular structures, primordial follicles, are formed by breaking down the syncytium of mitotically proliferating oogonia and recruiting a layer of flattened pre-granulosa cells to surround each oocyte starting its first meiotic division. There are three signalling pathways considered to be responsible for this process: Notch pathway, transforming growth factor beta (TGF-β) pathway and neurotrophic factors nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NTF4) (reviewed in Pepling, 2012). Oogonia that fail to switch to meiotic cell cycle and are not recruited into primordial follicles are eliminated by apoptosis (Fulton et al., 2005). The primordial follicles thereafter remain quiescent with the oocyte arrested in the prophase of the first meiotic division. Only after puberty follicle development and oocyte maturation continue periodically as depicted in Figure 1 (reviewed in Tripathi et al., 2010).

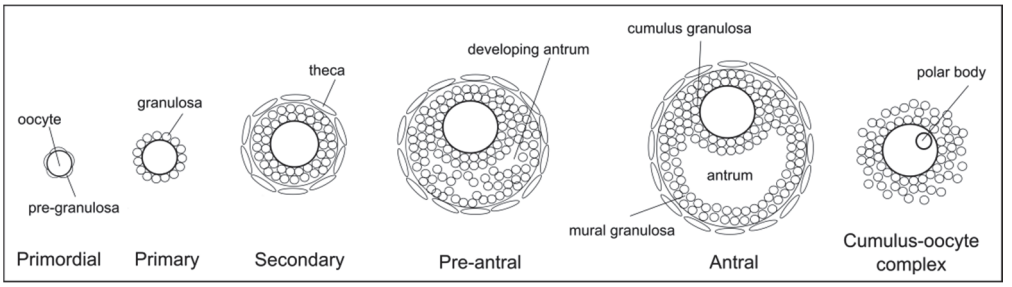


**Figure 1.** Simplified schematic representation of post-pubertal follicle development from primordial to antral stages. Cumulus-oocyte complex is extruded from the follicle after ovulation.

Subsets of primordial follicles are activated to develop into primary stage continuously throughout female reproductive life; this process is not dependent on the periodical nature of the menstrual cycle. Some signalling pathways have been proposed to be of higher importance in maintaining the balance between the resting and activated follicles: KIT-PI3K-AKT pathway and basic fibroblast growth factor (bFGF) expression in the oocyte, LIF-JAK-STAT pathway in the granulosa cells and TGF-β family protein signalling networks are involved in follicle activation, while anti-Müllerian hormone (AMH) in granulosa cells and tuberous sclerosis 1 (TSC1), phosphatase and tensin homolog (PTEN), and cyclin-dependent kinase inhibitor p27 in the oocyte are responsible for maintaining the resting follicle pool (reviewed in Oktem et al., 2010, Sobinoff et al., 2013). Disturbance in this balance leads to the recruitment of all follicles at once and subsequently to premature ovarian failure, a phenomenon that has been especially well demonstrated in case of the PTEN/phosphatidylinositol-3-kinase (PI3K)/AKT pathway in the oocyte (Reddy et al., 2008). The best notable morphological change during primordial to primary follicle transition is that of the granulosa cells that acquire a cuboidal shape. Precursor theca cells are recruited to the follicle via the expression of KIT ligand

(KITLG) by the granulosa cells. The theca cells in return produce keratinocyte growth factor (KGF) that further stimulates follicle activation (Skinner, 2005).

Thereafter, extensive mitotic proliferation of granulosa cells coincides with the enlargement of the oocyte. Follicles containing multiple layers of granulosa cells are referred to as secondary or pre-antral. Follicle growth at this stage is not gonadotrophin dependent, but may be influenced by gonadotrophins, as granulosa cells at this stage express the follicle stimulating hormone receptor (FSHR) on their plasma membrane in mouse, sheep, as well as human (reviewed in Binelli et al., 2010, Findlay et al., 1999). The ovary-specific proteins responsible for follicle maturation at this stage again include the TGF-β family members: bone morphogenetic protein 15 (BMP15) and activin A have been shown to promote granulosa cell proliferation, while inhibin B and AMH counter-balance this process. Oocyte-derived growth differentiation factor 9 (GDF9) is responsible for granulosa cell proliferation and survival (reviewed in Sanchez et al., 2012).

At the same time with active granulosa cell proliferation the peri-follicular theca cell layer becomes vascularised. Serum infiltrates into the follicle due to an osmotic gradient caused by the synthesis of hyaluronan and chondroitin sulphate proteoglycans by granulosa cells. Both, passive liquid transport due to the lack of tight junctions between granulosa cells and active transport via aquaporins contribute to this process (Clarke et al., 2006, Rodgers et al., 2010).

Expansion and liquid infiltration lead to the formation of a fluid-filled cavity (or antrum) inside the follicle resulting in the division of granulosa cells into two sub-populations: cumulus granulosa cells (CGC) that remain close to the oocyte forming the cumulus-oocyte complex (COC), and mural granulosa cells (MGC) that are separated from COC by the cavity and remain lining the basal membrane from the inside of the follicle. Follicle growth from pre-antral to antral stage is dependent on gonadotrophin secretion from the pituitary gland: FSH is necessary for antrum formation (Dierich et al., 1998), while both FSH and luteinizing hormone (LH) are required for antral follicle expansion (Burns et al., 2001, Zhang et al., 2001). FSH also induces the expression and activation of aromatase (CYP19A1), the key enzyme in estradiol-17β synthesis, marking the beginning of steroidogenesis in the follicle (Burns et al., 2001, Danilovich et al., 2000). As previously, ovarian-specific inter-cellular signalling modulates the effect of gonadotrophins. More specifically, granulosa cells grown without the oocyte in 3D culture do not form an antrum. GDF9 and BMP15 are two oocyte-secreted proteins partly responsible for the process (reviewed in Binelli et al., 2010). In addition, insulin-like growth factor 1 (IGF1) expressed by somatic cells enhance the expression of FSHR (Zhou et al., 1997), while BMP4 and BMP7 signalling from theca cells modulate FSH-induced steroidogenesis in granulosa cells (Shimasaki et al., 1999). Activin-inhibin pathways in granulosa cells are necessary for antrum formation and growth (reviewed in Sanchez et al., 2012).

It is at the gonadotrophin-dependent stage of folliculogenesis that the dominant follicle selection occurs in mono-ovulatory species leading to the ovulation of a single oocyte. This process is accomplished by intricate communication between the growing follicles and the pituitary gland via gonadotrophins, steroid hormones and proteins secreted by follicular somatic cells.

In short, estradiol synthesized by the granulosa cells as well as follicular inhibins are the inhibitors of pituitary FSH production (Findlay et al., 1990, Zeleznik et al., 1985). Therefore, competition for capturing the required amount of FSH for further growth commences between the antral follicles. The follicle with granulosa cells able to express the highest number of FSH receptors becomes dominant, while other follicles undergo atresia due to FSH starvation (reviewed in Mihm et al., 2008).

It is noteworthy that by pre-ovulatory stage the human follicle has expanded to over 20 mm in diameter, compared to only about 30 μm at primary stage (Gougeon et al., 1987, Griffin et al., 2006). This considerable distance between CGC and MGC results in significant differentiation between the properties and functions of these two somatic cell populations. According to various experimental proofs, the rate of differentiation is positively correlated with the distance from the oocyte, suggesting that signalling molecules secreted by the oocyte are affecting mainly the most adjacent layers of granulosa cells, *ie* CGC (Diaz et al., 2007b, Diaz et al., 2007a, Hussein et al., 2005).

Communication between CGC and the oocyte takes place via paracrine signalling (reviewed in Gilchrist et al., 2008), as well as through physical connections (gap junctions, adherens junctions and transzonal projections) (Anderson et al., 1976, Motta et al., 1994). The latter are used for the transport of ions, amino acids, pyruvate, nucleotides, and possibly ATP and glucose from CGC into the oocyte (Collado-Fernandez et al., 2012). It is clear that the resumption of meiosis in the oocyte is triggered by the closure of gap junctions with CGC, which leads to the drop of cGMP and cAMP concentrations and subsequently the re-activation of meiotic cell cycle (Norris et al., 2009). Several studies in other biological systems (cardiac myocytes, various immortalized cell-lines and human embryonic stem cells) have shown that bidirectional communication between cells via gap junctions may also involve RNA molecules that can alter gene expression in the adjacent cell (Kizana et al., 2009, Valiunas et al., 2005, Wolvetang et al., 2007). It has however not yet been shown, if such communication exists between the oocyte and CGC.

The oocyte-secreted factors TGF-β1, GDF9, BMP15, and activin A that were mentioned in regard with previous stages of folliculogenesis are also involved in inhibiting CGC luteinization, enhancing CGC proliferation and cumulus expansion before ovulation via paracrine signalling (Elvin et al., 1999, Vanderhyden et al., 2003, Yoshino et al., 2006). The latter process involves the expression of such CGC transcripts as hyaluronan synthase 2 (HAS2), tumor necrosis factor alpha-induced protein 6 (TNFAIP6), prostaglandin-endoperoxide synthase 2 (PTGS2), and pentraxin 3 (PTX3), all necessary for the restructuring of the hyaluronan-rich extracellular matrix between CGC (reviewed in Gilchrist et al., 2008). Reduction in cumulus expansion is strongly associated with oocyte incomplete meiosis resumption in *in vitro* fertilization (IVF) patients (Testart et al., 1983) as well as in the bovine model (Aardema et al., 2013).

MGC remain in the proximity of theca cells and the capillaries that transport hormones and other bioactive molecules to and from the follicle. The theca-MGC tandem acquires the activity to synthesize steroid hormones that influence FSH and LH release from the pituitary as well as the granulosa cell response to

gonadotrophins (Hillier et al., 1994, Liew et al., 2010, Pincus et al., 1958). In addition, MGC of the pre-ovulatory follicle express LHCGR earlier than CGC (Eppig et al., 1997, Maman et al., 2012) and are therefore responsible for responding to the LH surge that sets off several processes necessary for ovulation: tissue remodelling for COC expulsion and MGC luteinization required for progesterone production among many others (reviewed in Russell et al., 2007). The signal from LH surge is mediated to the COC from MGC by EGF-like factors amphiregulin, epiregulin and beta-cellulin, constituting additional signals for COC maturation processes (Park et al., 2004).

After ovulation has taken place, MGC and theca cells form *corpus luteum* with the main function of progesterone synthesis to prepare the endometrium for potential embryo implantation (reviewed in Stocco et al., 2007). CGC also has several roles once outside the follicle: it is clear that interaction between the extracellular matrix of expanded COC and epithelial lining of the oviductal infundibulum is necessary for COC transport towards the uterus (experimentally proven in hamster model in Lam et al., 2000). In addition, there is evidence that CGC performs as a filter for sperm cells before they reach the *zona pellucida* of the oocyte (Hong et al., 2004, Jin et al., 2011, Van Soom et al., 2002).

## 2. Infertility

Infertility refers to a couple's inability to conceive after 12 months of unprotected regular intercourse and according to this criterion affects approximately 9-15% of couples worldwide (Boivin et al., 2007, ESHRE, 2014). The diagnosis of infertility affects both the male and female partner more or less equally, however in approximately 10-20% cases the etiology of infertility remains unknown (ESHRE, 2014). Male factor infertility is diagnosed according to sperm parameters: the motility, morphology and concentration of spermatozoa in ejaculate (Guzick et al., 2001). The most frequent etiologies of female infertility are divided as follows (according to Molinaro et al., 2009):

a. Endometriosis caused by the flourishing of endometrium outside of the uterine cavity. The nature and severity of endometriosis varies significantly depending on the location and size of endometriotic foci (reviewed in Adamson, 2013). The connection between endometriosis and infertility is extensively studied, but still not clear, as only up to 50% of endometriosis patients are infertile (Bulletti et al., 2010). Several pathologies leading to infertility have been proposed: from pelvic distortion to inflammation, imbalance in local hormonal profile leading to disrupted ovulation, failure in oocyte capture, inefficient uterotubal sperm transport and reduced embryo implantation rate (reviewed in ASRM, 2012). All this information refers to the fact that endometriosis patients are probably a very heterogenic group in regards of molecular or physical etiologies of infertility.
b. Tubal factor infertility (TFI) caused by obstructed or removed oviducts after ectopic pregnancy, salpingitis, adhesions or tubal polyps (Kodaman et al., 2004).

c. Polycystic ovary syndrome (PCOS) characterized by oligo- or anovulation, hyperandrogenism and/or polycystic ovaries (The Rotterdam PCOS Consensus Group, 2004).
d. Diminished ovarian reserve diagnosed in case of women reaching menopause below the age of 40 (reviewed in Cox et al., 2014, Molinaro et al., 2009)
e. Uterine conditions: fibroids, adenomyosis, polyps, intrauterine adhesions that potentially interfere with embryo implantation or the development of pregnancy (reviewed in Coughlan et al., 2014).

## 3. Assisted reproductive technologies

### *3.1. Controlled ovarian stimulation*

Several infertility treatment strategies involve pharmacological or surgical solutions. However, during the last 35 years the development of assisted reproductive technologies (ART) has been substantial. The first child was born from an *in vitro* fertilized oocyte in 1977 (Steptoe et al., 1978) followed by 5 million others estimated by 2009 (Ferraretti et al., 2013). Today, depending on country, approximately 1-3% of children are born via the use of ART (Ferraretti et al., 2013, Sunderam et al., 2013).

The knowledge of gonadotrophin production and signalling mechanisms has paved a way to the development of controlled ovarian stimulation (COS) protocols used for increasing the success rate of IVF procedures. The universal aim of COS is to abolish dominant follicle selection by providing sufficient levels of FSH allowing multiple follicle maturation. The use of recombinant FSH (rFSH) produced by genetically engineered cell-lines is preferred, due to its higher efficiency and purity compared to the alternative preparation purified from the urine of postmenopausal women (Palagiano et al., 2004). Endogenous gonadotrophin secretion is down-regulated by gonadotrophin-releasing hormone (GnRH) antagonists or agonists and ovulation is triggered most frequently by human chorionic gonadotrophin (hCG) administration (reviewed in Santos et al., 2010), which binds to LH receptors, but is more stable in bloodstream than LH (McFarland et al., 1989, Yen et al., 1968). Alternative stimulation protocols have been developed and used depending on the etiology of infertility as well as patient response to exogenous gonadotrophins (extensively reviewed, *eg* in Hillier, 2013, Humaidan, 2012).

An important question is, whether COS could alternate the follicular milieu and hence the developmental potential of the oocyte. However, studies to answer this question are difficult to perform in human *in vivo*. De los Santos *et al* performed a study measuring follicular hormone production, gene expression levels of CGC and evaluating oocyte developmental competence in women undergoing either natural or stimulated cycles (de los Santos et al., 2012). GnRH agonist long protocol with rFSH stimulation and recombinant chorionic gonadotrophin (rCG) ovulation triggering were used in COS procedure, while only rCG triggering was used in the natural cycle group. Although significant differences were found between groups

regarding individual steroid hormones in the follicular fluid (FF), the estradiol-testosterone ratio remained unaltered. Also, LH level was significantly lower in the stimulated group due to GnRH agonist exposure. Eighteen transcripts showed statistically significant expression between groups. As a main result, no differences in oocyte meiotic maturation, fertilization rate or subsequent embryo developmental potential were observed. Hence the authors concluded that the observed differences in CGC and FF do not probably contribute to IVF outcome.

### *3.2.In vitro fertilization*

Oocytes are collected via transvaginal follicle puncture at about 36-38 hours after hCG administration (Dellenbach et al., 1985). The IVF procedure may involve conventional fertilization on a dish or in case of severe male infertility, intracytoplasmic sperm injection (ICSI) is used (Palermo et al., 1992). For performing ICSI, CGC layer is removed by hyaluronidase treatment and the maturation state of the oocyte is inspected. In case of conventional IVF the COC is not mechanically manipulated prior fertilization. If fertilization was successful, further embryo development is monitored under the microscope. Single or multiple embryos with the highest morphological quality are transferred to the uterus at 4-8-cell or blastocyst stage. Implantation and early pregnancy is supported by the exogenous administration of progesterone, compensating for the lack of natural *corpus luteum* development (common practice in Estonian IVF clinics, personal communication with Dr Elle Talving and Dr Peeter Karits from Nova Vita Clinic).

Despite the long history and large number of IVF cycles performed, the success rate of conventional COS-IVF or -ICSI in Europe has remained at around 29% per ovarian puncture or 32% per embryo transfer (Ferraretti et al., 2013). The rate may be higher in countries where multiple embryo transfers are more popular, for example in the USA the pregnancy rate per transfer was 46.1% and live-birth delivery rate 37.6% (Sunderam et al., 2013). As a downside, multiple embryo transfer results in higher number of multiple pregnancies that may cause complications both for the mother and the foetuses (reviewed in Norwitz et al., 2005). Currently the direction in IVF practice is steadily moving towards single embryo transfer in order to minimize these risks (Chambers et al., 2013, Ferraretti et al., 2013). But to aim for maintaining and increasing the IVF success rate with single embryo transfer, good oocyte or embryo selection criteria are needed.

### *3.3.In vitro maturation*

There are several occasions, when standard COS is not successful or even not possible to perform:

a. Women with high risk of potentially lethal ovarian hyperstimulation syndrome (OHSS) (Nastri et al., 2010). This group often involves PCOS patients, who produce large number of immature oocytes and elevated peak estradiol levels after conventional COS that are both risk factors for developing OHSS (Swanton et al., 2010, Tummon et al., 2005). The alternative option to COS for this group is to collect the oocyte from

immature follicles or the single dominant follicle produced during a natural or mildly stimulated cycle (reviewed in Chian et al., 2013).

b. Cancer patients undergoing chemotherapy or irradiation that severely damage the ovarian follicles, often leaving these women infertile. Ovarian tissue can be cryopreserved prior to cancer treatment, re-implanted into the ovary and conventional COS performed to retrieve healthy oocytes after treatment (reviewed in Chian et al., 2013). However, gonadotrophin administration during COS elevates serum estradiol levels and is therefore not advisable for patients with estrogen receptor positive cancer history (Balkenende et al., 2013). In such cases, maturing the follicles isolated from cortical strips *in vitro* is necessary. Another option is to cryopreserve immature oocytes collected before gonadotoxic therapy and further *in vitro* maturation (IVM), as described for group a.

It is clear that various situations may require different IVM protocols, depending on the time of oocyte or follicle collection and hence the developmental stage. It is common knowledge that preserving inter-cellular connections and 3-dimensional structure during follicle IVM is necessary for normal oocyte maturation. However, depending on the follicular stage, this may appear difficult. Follicles isolated at early stages expand substantially in diameter, necessitating the studies of artificial matrixes that could support such growth, while preserving the follicular structure (Krotz et al., 2010, Tagler et al., 2013). Since the hypothalamus-pituitary-ovarian axis is disrupted in IVM, it is of utmost importance to know the micro- and macromolecules that are necessary to add to cultured follicles or COC-s at various stages for full maturation of the oocyte.

IVM of immature oocytes obtained from human antral follicles is being routinely used in infertility clinics. However, the results are suboptimal compared to standard COS and IVF: implantation rates of embryos generated by fertilizing IVM oocytes are lower by approximately 50% (reviewed in Coticchio et al., 2012). Full *in vitro* growth and maturation protocols from primordial follicles have so far provided viable offspring only in mice (Eppig et al., 1996, O'Brien et al., 2003). COC IVM has led to live births also in cow (Hirao et al., 2004). Unfortunately, in primate models the most successful IVM cases of primary or secondary follicles have halted in zygote or early cleavage stages (Xu et al., 2013, Xu et al., 2011a, Xu et al., 2010). All these results indicate that our current knowledge of folliculogenesis is not sufficient enough to obtain clinically useful IVM protocols.

## 4. Antral follicle markers in IVF

Clinical and basic scientific studies regarding human biological material may be conducted under strict ethical guidelines (WMA, 2013, Rickham, 1964). There are two options to retrieve human follicular material. Firstly, ovarian cortical strips can be maintained in culture, enabling the investigation of folliculogenesis *in vitro*, as described above. But as mentioned, the tissue culture conditions are suboptimal and do not yet mimic the natural environment. Therefore, information gathered from such experiments, although very valuable, is not always applicable to *in vivo* systems. Second option is to gather material from ovarian biopsies or ovarian

puncture procedures during an IVF cycle. Such biological material is derived from its natural environment, providing information on *in vivo* biological processes. However, it describes only the tissue stage it was obtained from and no invasive time-lapse *in vivo* experiments in human are possible due to ethical reasons.

FF containing the COC and MGC is aspirated during ovarian puncture and all these intrafollicular components, except for the oocyte used for fertilization, are available for further study (Figure 2). Gene expression studies are most frequently performed from granulosa cells, while more recent interest in cell-free miRNAs measured from FF has arisen (Diez-Fraile et al., 2014, Roth et al., 2014, Sang et al., 2013). In addition, FF is a source of a large variety of soluble proteins, steroid hormones and metabolites secreted by the intrafollicular cells or infiltrated from plasma (Rodgers et al., 2010). From oocyte polar body biopsy the chromosomal consistency of the oocyte after the first or second meiotic division can be extrapolated (Montag et al., 2013).

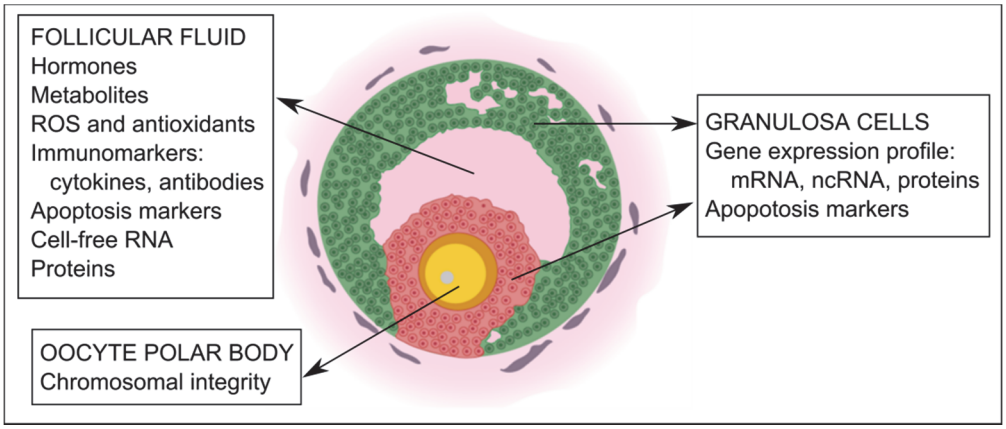


**Figure 2.** Follicular material available for analysis after ovarian puncture. The most frequently studied oocyte quality or infertility markers are presented. ncRNA – non-coding RNA, ROS – reactive oxygen species.

The purpose of studies on human intrafollicular material can be divided into three broad categories of high importance:

a. Descriptive studies using high-throughput screening technologies to identify novel intrafollicular components: gene expression profile of COC (Assou et al., 2006, Hernandez-Gonzalez et al., 2006), metabolome (Pinero-Sagredo et al., 2010), or full proteome of FF (Ambekar et al., 2013, Angelucci et al., 2006) are only a few examples.
b. Studies correlating intrafollicular molecular components to the physiological parameters, lifestyle or other background information regarding the patient provide new knowledge on their possible influence on folliculogenesis. To mention only a few examples, the effect of age (Adriaenssens et al., 2010, Manau et al., 2000, Pinero-Sagredo et al., 2010) and body-mass index (Robker et al., 2009, Wu et al., 2007), nutritional (Boxmeer et al., 2008, Ozkaya et al., 2010, Ozkaya et al., 2011) and smoking habits (reviewed in

Dechanet et al., 2011), etiology of infertility (Buyuk et al., 2008, Haouzi et al., 2012, Velthut et al., 2013), and hormone stimulation protocols (Assou et al., 2013, Brannian et al., 2010, de los Santos et al., 2012) on follicular environment and hence fertility have been unravelled. Ovarian response to gonadotrophins during COS has also been correlated to intrafollicular molecular markers (*eg* Adriaenssens et al., 2010, Velthut et al., 2013). Especially of high importance in this regard is finding the molecular markers that would distinguish between IVF patients with an elevated risk for developing the potentially lethal OHSS. The proposed markers in FF for OHSS cases are elevated interleukin 6 (IL-6) (Geva et al., 1997), decreased vascular endothelial growth factor (VEGF) (Gao et al., 2011, Pellicer et al., 1999), as well as inhibin A and inhibin B (Moos et al., 2009), when compared to women with an average response to COS and no prevalence of OHSS.

c. Studies aiming to find biomarkers that would predict the outcome of an IVF procedure. Several research groups have sought to find non-invasive molecular markers to predict the maturity and developmental potential of the oocyte (reviewed in Fragouli et al., 2014, Revelli et al., 2009). Finding such markers of high predictive value would enable a huge step towards improving IVF outcome by using single embryo transfer. These studies therefore have a potential clinically applicable value. The largest number of publications in this field describe the search for biomarkers in the highly accessible granulosa cell transcriptome, as genome-wide screening methods for nucleic acids are well developed and give quantitative as well as qualitative results (Uyar et al., 2013). The most frequently appearing marker for embryo morphological quality is the mRNA expression level of gremlin 1 (GREM1) in CGC (Adriaenssens et al., 2010, Assou et al., 2013, Cillo et al., 2007, McKenzie et al., 2004). However, studies accounting for inter- and intra-patient gene expression differences (Feuerstein et al., 2012, Hamel et al., 2010), the collection of samples at multiple centres (Iager et al., 2013), patient and stimulation characteristics (Wathlet et al., 2013, Wathlet et al., 2012) do not propose GREM1 as a marker for IVF success. To the contrary, all these studies propose different markers or algorithms for predicting oocyte developmental potential and/or pregnancy outcome. The described results indicate that further large-scale multicentre studies are necessary and successful biomarkers, instead of being single molecules, might involve complicated multi-factorial algorithms.

### *4.1.Role of cytokines in the antral follicle*

Various cytokines play a crucial role in folliculogenesis. Perhaps the best-studied cytokines regarding several processes of folliculogenesis are the TGF-β family members involved in oocyte-somatic cell communication as described above. In addition, several cytokines originally described in the immune cells have both, immune-system-related as well as alternative roles in the ovarian follicle (reviewed in Field et al., 2014). It is noteworthy that the concentration of several cytokines is

higher in the pre-ovulatory FF as compared to peripheral blood (Asimakopoulos et al., 2006). By the pre-ovulatory follicular stage several leukocytes invade the theca layer, but do not penetrate MGC before the follicular basal membrane has ruptured during ovulation, as seen in both, humans and rats (Brannstrom et al., 1994, Oakley et al., 2010). It has therefore been proposed that most cell types in and around the follicle produce cytokines that then either infiltrate through the basal membrane (from theca cells and immune cells residing between them) or are directly secreted into the FF by the granulosa cells or the oocyte (Field et al., 2014).

During antral growth the follicle experiences hypoxic conditions and several mechanisms for peri-follicular neo-angiogenesis have been described. It has been shown that FF IL-1β induces VEGF expression by rat granulosa cells (Levitas et al., 2000). VEGF in turn induces the expression of chemokine IL-8, another pro-angiogenic factor in granulosa and theca cells (Murayama et al., 2010) also involved in neutrophil attraction (Baggiolini et al., 1992). In addition, IL-8 affects steroidogenesis by decreasing estradiol and increasing progesterone production in bovine pre-ovulatory follicle (Shimizu et al., 2012). During follicle growth tumour necrosis factor alpha (TNF-α) expressed by the non-immune cells of the follicle is involved in granulosa cell proliferation (Son et al., 2004).

Ovulation has been compared to inflammatory processes similar to injury and wound healing (first comparison by Espey, 1980). It involves tissue remodelling by several proteinases before follicle rupture, and rebuilding the ovarian epithelial layer afterwards. Therefore it is not surprising that several cytokines secreted by follicular cells act as chemoattractants for inflammatory leukocytes (reviewed in Field et al., 2014). As an example, TNF-α as well as IL-1α both induce granulosa and theca expression of monocyte chemotactic protein 1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) (Kawano et al., 2004) that are chemoattractants for peri-follicular macrophages (Dahm-Kahler et al., 2009, Nishimura et al., 1995). Macrophages secrete RANTES (regulated on activation, normal T cell expressed and secreted) required for the recruitment of T-cells, eosinophils and mast cells (Alam et al., 1993, Schall et al., 1990). The recruited leukocytes then participate in tissue remodelling and clearing of apoptotic follicular cells as well as modulate *corpus luteum* development and degeneration (reviewed in Pate et al., 2010).

Cytokines have additional functions in ovulatory processes that are independent of leukocytes. TNF-α is involved in the upregulation of collagenase bioactivity in the FF of ewes (Johnson et al., 1999) as well as collagenase expression by human ovarian surface epithelial cells (Yang et al., 2004), hence the remodelling of the basal membranes of the follicle and the ovarian surface epithelium prior to follicle rupture. IL-1β inhibits MGC proliferation and drives this cell population towards differentiation (Karakji et al., 1995). IL-1β also modulates granulosa cell prostaglandin E production (Hurwitz et al., 1995) that determines the site of follicle rupture at the apical basal membrane (Gaytan et al., 2002). IL-6 expressed by mouse MGC and COC upon hCG stimulation promotes CGC expansion and germinal vesicle breakdown of the oocyte (Liu et al., 2009).

### *4.2.Apoptosis in the pre-ovulatory follicle*

During human natural folliculogenesis a cohort of primordial follicles is recruited for further development. Usually only a single dominant follicle survives up to ovulation and the subordinate follicles undergo atresia making apoptosis a natural process during folliculogenesis. However, markers of apoptosis may also be connected with the outcome of IVF procedure. The apoptosis rate of CGC was observed to be higher if the corresponding oocyte had not successfully completed the first meiotic division during COS (Host et al., 2000). In addition, CGC around oocytes that were successfully fertilized *in vitro* had the lowest apoptosis rate (Host et al., 2000). The amount of apoptotic CGC at OPU has been correlated with woman's age, providing an explanation to the lower number of matured follicles and reduced embryo quality in older patients (Lee et al., 2001). The health of the oocyte is considered as one of the determinants of whether the follicle survives or not: it has been shown that several oocyte-secreted factors create a pro-survival gradient on the adjacent cumulus cells. IL-7, BMP-6 and BMP-15 are three of such well-studied examples, where the ligand is secreted by the oocyte and receptors being expressed on CGC (Cheng et al., 2011, Hussein et al., 2005).

Apoptosis rate in MGC has also been negatively correlated with the developmental potential of the corresponding oocyte (Oosterhuis et al., 1998, Suh et al., 2002). However, there are also studies that strongly question this association (eg Jancar et al., 2007). Higher percentage of apoptotic MGC may also be one of the possible underlying causes of unexplained infertility (Idil et al., 2004).

Several signalling pathways are involved in maintaining the balance between survival and apoptosis in all cell types in the follicle. Some of the above-mentioned cytokines have been described to have additional roles in retaining this balance. TNF-α is one of those cytokines with various and sometimes opposing roles in follicle biology, depending on the signalling partners and receptors that convey the signal. Reduced oocyte apoptosis was observed in TNF-α knockout mice (Cui et al., 2011). An apoptosis pathway is suggested to be triggered by this cytokine in mouse granulosa cells together with excessive amounts of NGF (Garcia-Rudaz et al., 2011) and signalling through receptor TNFR1 rather than TNFR2 (Tartaglia et al., 1991). However, TNF-α expressed by theca cells plays a different role prior to ovulation. It has been demonstrated in ewes that plasmine cleaves the transmembrane TNF-α from theca cell membrane that thereafter induces apoptosis in local ovarian surface epithelium cells, thus aiding in dissolution of tissues necessary for follicle rupture (Murdoch et al., 1999).

FAS ligand (FASLG) and fas cell surface death receptor (FAS) are members of the TNF and TNFR superfamily, respectively (Itoh et al., 1991, Suda et al., 1993). FAS-FASLG system is well studied for their role in triggering apoptosis in many tissues, including the ovarian follicle (Watanabe-Fukunaga et al., 1992). The FAS receptor is expressed by all cell types in human follicle, but the expression level depends on follicle stage and may not be evenly distributed between the different layers of CGC, MGC or theca cells. FASLG, on the other hand, is predominantly expressed by the oocyte at primordial and primary stage and by somatic cells in atretic follicles (Cataldo et al., 2000, Jose de los Santos et al., 2000). In addition, a soluble FAS receptor isoform (sFAS) is present in the FF (Jose de los Santos et al.,

2000). This isoform is generated via alternative splicing that eliminates the transmembrane region of the receptor. As a result, the soluble form binds FASLG without transferring the death signal inside of the cell, thus acting as an anti-apoptotic agent (Cheng et al., 1994).

IL-6 is a cytokine that exerts its pro-survival effect in porcine follicles through its soluble receptor sIL6R and trans-membrane IL-6 signal transducer (IL6ST) (Maeda et al., 2007a). It was shown that the expression levels of all these three components diminished in atretic follicles compared to healthy ones, in contrast to the trans-membrane IL6R (Maeda et al., 2007a, Maeda et al., 2007b). The role of the latter in follicle atresia is yet not known.

CD44 is a highly glycosylated transmembrane cell surface protein with at least 42 different alternative splicing isoforms in human, according to the Ensembl database (http://www.ensembl.org). In the ovarian follicle, CD44 standard isoform (CD44s) is well known as the receptor for hyaluronic acid, the main component of extracellular matrix between the cells of COC (Culty et al., 1990, Underhill et al., 1987). It has been shown that the anti-apoptotic effect of hyaluronic acid on CGC is mediated by CD44 (Kaneko et al., 2000, Tunjung et al., 2009). However, follicular atresia in the pig model is associated with elevated expression of CD44 on peri-follicular macrophages, probably indicating the increased invasive and migratory properties of these cells (Miyake et al., 2006). CD44 is also expressed in a sub-population of MGC, but at a lower level compared to CGC (Ohta et al., 1999). In addition, CD44s as well as the variable splice isoforms (CD44v) may be cleaved from the cell surface by proteases and become soluble (reviewed in Nagano et al., 2004). Such soluble CD44 molecules have been detected in the FF (Ohta et al., 2001). The role of soluble CD44 protein isoforms is not known, but there is an increase in their concentration in the FF upon luteinization, described by high positive correlation with progesterone and hCG and negative correlation with estradiol levels (Ohta et al., 2001).

## 5. The role of microRNAs in folliculogenesis

### *5.1.Biogenesis of microRNAs*

MicroRNAs (miRNAs) are a class of short RNA molecules (on average 21-22 nucleotides long) that have an important role in post-transcriptional gene regulation in most eukaryotes. Biogenesis of miRNAs involves transcription, cleavage steps by endoribonucleases, RNA degradation and loading of the intermediate products to various protein complexes that carry out these processes. According to the canonical pathway, miRNAs are transcribed from miRNA genes by RNA polymerase II, the obtained product, named primary miRNA (pri-miRNA), is 5' capped and 3' polyadenylated as are mRNA strands (Cai et al., 2004, Lee et al., 2004). The secondary structures of pri-miRNAs are recognized by the DROSHA/DGCR8 (DiGeorge syndrome critical region 8) complex, the RNase III component of which cleaves the pri-miRNA into a short hairpin RNA, referred to as precursor miRNA (pre-miRNA) (Lee et al., 2003). The short hairpin structure is recognized by another type III RNase DICER in a multi-protein complex that cleaves the pre-miRNA

molecule, producing a double-stranded, imperfectly paired RNA duplex (Hutvagner et al., 2001). The duplex is then loaded into miRNA induced silencing complex (miRISC), where argonaut (AGO) protein has a central role (reviewed in Stroynowska-Czerwinska et al., 2014). One of the RNA strands (the mature miRNA strand) is preserved and the antisense strand (passenger strand or star strand) is degraded (Schwarz et al., 2003). The canonical pathway of miRNA biogenesis is presented in Figure 3.

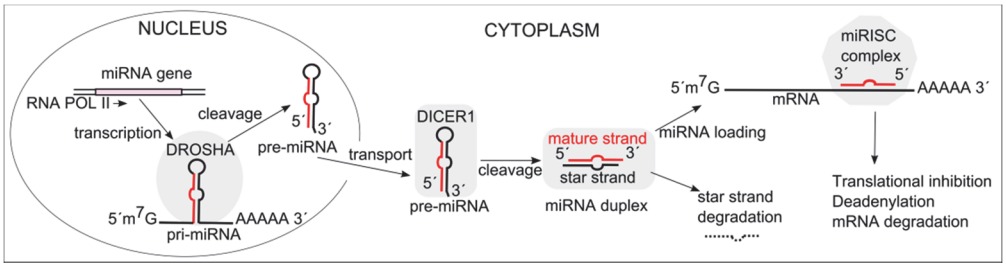


**Figure 3.** Simplified scheme of the canonical miRNA processing pathway. DROSHA and DICER1 endoribonucleases act in protein complexes and pre-miRNA transport is performed by exportin 5 (not shown). In addition, several non-canonical pathways have been proposed as indicated in text.

During recent years several non-canonical pathways of miRNA biogenesis have been described. The perturbations from the canonical pathway may include every possible step: the miRNA gene may be transcribed by RNA polymerase III (Borchert et al., 2006), the cleavage steps by either DROSHA/DGCR8 (Ruby et al., 2007) or DICER (Cheloufi et al., 2010, Cifuentes et al., 2010) may be skipped. A subclass of miRNAs, the mirtrons, is generated by splicing mechanisms from intronic regions (Berezikov et al., 2007, Okamura et al., 2007). These introns are sufficiently short to form the pre-miRNA molecule without the need of cleavage by DROSHA/DGRC8 complex.

A class of miRNAs residing in the introns of coding genes, but generated independently of the splicing machinery has also been described. The exact components necessary for the biogenesis of the so-called “simtrons” have not been fully defined. It seems that the intronic region itself contains all necessary sequences for triggering miRNA biogenesis as the flanking exonic regions are not necessary for this process (Havens et al., 2012).

However, miRNAs from longer introns need canonical biogenesis pathways and DROSHA processing to cleave the unnecessary portions of pri-miRNAs. In such cases the processes of pre-mRNA splicing by supraspliceosomes and pri-miRNA cleavage by the microprocessor complex temporally overlap and significantly influence the efficiency of one another (Agranat-Tamir et al., 2014). Adjacent or overlapping pri-miRNA sequence and mRNA splice cite may activate such interplay between splicing and miRNA processing mechanisms that lead to the generation of alternative mRNA molecules and completely abolish miRNA expression (Agranat-Tamir et al., 2014, Mattioli et al., 2013, Melamed et al., 2013). Therefore, the machinery of miRNA generation may also modulate gene expression

co-transcriptionally. In addition, it has been shown that approximately 35% of intronic miRNAs are generated according to the canonical pathway, but independently of their host genes or the host gene promoters (Monteys et al., 2010).

Computational predictions have proposed that miRNAs can also be generated by virtually any hairpin-forming RNA molecules: short hairpin RNAs (shRNAs), small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) etc (Castellano et al., 2013). However, the proportion of miRNAs generated by non-canonical pathways seems to be modest and their nature evolutionarily less conserved (reviewed in Westholm et al., 2011).

### *5.2.Function of microRNAs*

The sequence of the single-stranded mature miRNA bound to AGO in miRISC is matched to a target mRNA strand (Ameres et al., 2007). Targeting does not require perfect complementarity between miRNA and mRNA. The majority of miRNA binding regions appear in the 3' UTR of the mRNA strands, however coding sequences and 5' UTR may also be targeted (reviewed in Stroynowska-Czerwinska et al., 2014). There are two main outcomes of miRNA-mRNA duplex formation that may be dependent on each other: the mRNA molecule may become degraded via deadenylation (Wu et al., 2006) or the translation of that mRNA is inhibited (Humphreys et al., 2005). Either of the two mechanisms leads to the reduction in the concentration of the final protein product. Only a few cases in which miRNA action has led to an increase in translation efficiency have been observed (Vasudevan et al., 2007).

It has to be mentioned that the ability to define miRNA target regions on mRNA strands by current methods and bioinformatic algorithms is far from flawless. Due to imperfect complementarity between miRNA/mRNA duplexes one single miRNA may target different mRNAs and several miRNAs are able to target the same mRNA (Lewis et al., 2003). It has been shown that the number and location of miRNA target sites on a particular mRNA strand are in correlation with the level of mRNA degradation and/or translational inhibition (Saetrom et al., 2007). However, these dynamical aspects are not yet well understood. It is also difficult to distinguish between direct and indirect miRNA targets from high-throughput gene expression studies. Therefore, all reports involving miRNA target predictions in biological systems must be interpreted with great caution.

Several studies have proven that in addition to intercellular functions, miRNAs are also secreted from the cells in extracellular vesicles (microvesicles, exosomes, apoptotic bodies), high- and low-density lipoproteins or RNA-binding proteins like AGO2 (reviewed in Kosaka et al., 2013). Due to their stability in such complexes, miRNAs have been detected in nearly all biofluids (Weber et al., 2010), including ovarian FF (Sang et al., 2013). Hence, cell-free miRNAs have gained potential diagnostic importance in several fields of biomedicine, especially in the studies of cardiovascular diseases and cancer (Creemers et al., 2012, Kosaka et al., 2010).

An even more novel field involves verifying the purpose of cell-free miRNAs. There are two main hypotheses: the secreted miRNA complexes may be

cellular by-products or in fact targeted means of paracrine and endocrine communication (Valadi et al., 2007). Depending on circumstances, both hypotheses may be correct. As proofs for the latter, there are studies showing that packaging of miRNAs into secreted vesicles in the cells is not random (Guduric-Fuchs et al., 2012), and the up-take of such cell-free vesicles via endocytosis has been demonstrated for various cell types, including granulosa cells (da Silveira et al., 2012, Sohel et al., 2013).

### *5.3.MicroRNAs in ovarian biology and folliculogenesis: studies in mammalian models and cell-lines*

Since the DICER1 mouse knockout has an embryonic lethal phenotype (Bernstein et al., 2003), conditional knockout models have been used to study the importance of miRNAs in reproductive tissues. Two methods have been used thus far in mice: deleting the gene in AMHR2 positive cells in the reproductive tract (Hong et al., 2008, Lei et al., 2010, Nagaraja et al., 2008) or by creating lines with hypomorphic DICER expression (Otsuka et al., 2008). It appeared that the morphology and function of the oviducts and uterus were more severely hampered in conditional knockout mice compared to that of the ovaries (Hong et al., 2008, Nagaraja et al., 2008). Although mice without DICER1 could produce fertilizable oocytes, reduced follicular recruitment, maturation and ovulation rates as well as increased follicular atresia and diminished progesterone production by *corpora lutea* were observed (Hong et al., 2008, Lei et al., 2010, Nagaraja et al., 2008, Otsuka et al., 2008). No knockout models deleting DROSHA/DGCR8 complex or its components has been created for studies in reproduction to our knowledge.

According to functional studies, miRNAs appear to be involved in the fine-tuning of virtually all processes in folliculogenesis. miR-145 plays an important role in maintaining the primordial follicle pool in mouse and the proposed mode of action is via targeting TGF-β receptor 2 mRNA (Yang et al., 2013). In functional studies using adult granulosa cells, this miRNA suppressed their proliferation by targeting activin receptor IB (Yan et al., 2012). Further follicular development into primary, secondary and antral stages is influenced by miR-143, miR-125b, let-7a, let-7b, let-7c, and miR-21, the expression of miR-143, let-7a, and miR-15b being down-regulated by FSH stimulation (Yao et al., 2009). The expression of miR-181A is decreased upon follicular development from primary to pre-antral and antral stages, which coincides with the up-regulation of its target activin receptor IIA (Zhang et al., 2013).

Some of the best-studied biochemical pathways in growing follicles are related to steroidogenesis. A few publications have addressed the role of miRNAs in the process. miR-133b was shown to target FOXL2 mRNA in the human granulosa cell-line KGN and cultured mouse primary granulosa cells. It is a transcription factor responsible for repressing the expression of steroidogenic acute regulatory protein (STAR) and aromatase genes (Kuo et al., 2012). As a result of miR-133b over-expression, FOXL2 expression was down-regulated and estradiol secretion was expectedly increased (Dai et al., 2013). miR-378 targets aromatase mRNA in porcine granulosa cells in culture, leading to decreased estradiol production. *In vivo*

the expression of this miRNA was inversely correlated with that of aromatase gene with significantly higher levels in small follicles (Xu et al., 2011b). In mouse, miR-383 production was activated by steroidogenic factor 1 leading to enhanced estradiol release from granulosa cells by suppressing the c-Myc pathway (Yin et al., 2012).

Insufficient steroidogenesis is correlated with apoptotic processes in the follicle and one link between the two processes was revealed in a mouse *in vitro* study showing that androgens inhibit apoptosis by increasing miR-125b, which further targets mRNAs of such pro-apoptotic genes as Bcl-2 homologous antagonist/killer (BAK), Bcl-2-associated X protein (BAX), tumour suppressor protein p53 and Bcl-2 modifying factor (BMF) (Sen et al., 2014). Other anti-apoptotic miRNAs expressed in mouse granulosa cells are miR-21 (Carletti et al., 2010) and miR-224, the latter targeting the signal transduction protein SMAD4 mRNA (Yao et al., 2010). High expression level of miR-26b was shown to target *ataxia telangiectasia* mutated (ATM) mRNA leading to increased follicular atresia and DNA breaks in porcine granulosa cells (Lin et al., 2012).

Luteinizing hormone (LH) surge prior to ovulation triggers drastic molecular and morphological changes in granulosa cells leading to their luteinization and *corpus luteum* formation. Upon stimulation with human chorionic gonadotrophin (hCG), expression differences of several miRNAs have been observed in the ovarian follicles of different animals. Mouse miR-122, miR-132, and miR-30a were up-regulated and let-7b down-regulated upon hCG stimulation (Fiedler et al., 2008, Kim et al., 2010). Proposed indirect target for the first two miRNAs was the transcription factor C-terminal binding protein 1 (CTBP1) that may lead to dramatic changes in overall gene expression profile (Fiedler et al., 2008). The levels of miR-21, miR-132, miR-212, and miR-224 were increased in hCG-stimulated equine follicles; this was associated with reduced expression of the putative miRNA targets PTEN, RAS p21 protein activator, and SMAD4 (Schauer et al., 2013). miR-136-3p and mir-122 were both associated with LH receptor mRNA targeting in rat (Kitahara et al., 2013, Menon et al., 2013). When transition from follicular to *corpus luteum* stage was studied in sheep, miR-125b, let-7b, let-7c, miR-199a were shown to be descriptive of follicular stage. miR125b (targeting LIF) and miR145 (targeting CDKN1A) were down-regulated in granulosa cells upon luteinization (McBride et al., 2012). In cow, miR-378 was shown to be expressed in non-regressed *corpus luteum*, targeting interferon gamma receptor 1B mRNA and thus inhibiting apoptosis of progesterone-producing cells (Ma et al., 2011).

Follicle maturation, ovulation and *corpus luteum* formation require multidirectional intercellular communication between granulosa and theca cells, as well as the oocyte. An elegant study in cow showed that miRNA profile in CGC and the oocyte were altered depending whether the cells were cultured as a cumulus-oocyte complex or separately from each other (Abd El Naby et al., 2013). miR-210 was shown to be inhibited in cumulus cells by the proximity of the oocyte. Likewise, a group of miRNAs (miR-205, miR-150, miR-122, miR-146a, and miR-146b-5p) was over-expressed in the oocyte if the surrounding cumulus cells were removed. The cumulus cell miRNA profile may also depend on the meiotic maturation stage of the oocyte as shown in mice (Kim et al., 2013) and cow (Tesfaye et al., 2009). Intercellular communication between cumulus and mural granulosa cells in the

antral follicle requires overcoming considerable distance between these cell types that are separated by a fluid-filled antrum. Such communication has been described to take place via secreted vesicles packed with coding as well as non-coding RNA molecules in protein complexes (Valadi et al., 2007). Microvesicles and exosomes have been successfully purified from equine (da Silveira et al., 2012) and bovine (Sohel et al., 2013) ovarian FF and their uptake by granulosa cells was observed in culture. The difference in miRNA profiles was demonstrated between vesicles from follicles of old and young mares (da Silveira et al., 2012), as well as between follicles containing oocytes of different maturation states in the cow (Sohel et al., 2013), indicating that such form of intercellular communication may play a significant role in biological processes in the follicle.

Several studies have profiled the ovary-specific miRNA profile in animal models (Ahn et al., 2010, Huang et al., 2011, Li et al., 2011, Mishima et al., 2008, Ro et al., 2007, Torley et al., 2011, Tripurani et al., 2010). However, as the technical possibilities evolve, increasing amount of information is retrieved from high-throughput deep sequencing experiments. It has so far been well determined that miRNAs in the ovary participate in regulating IGF-1 signalling, cell cycle, TGF-β signalling, ephrin receptor signalling, steroid hormone metabolism, BMP signalling, VEGF signalling, pro-apoptotic processes, and pathways associated with axonal guidance (Hossain et al., 2009).

### *5.4.Studies of miRNAs in human reproduction*

Studies with human material aim to reveal species-specific differences in folliculogenesis, but more importantly to use the gained knowledge in the diagnosis of subfertility, select developmentally competent oocytes and embryos, and optimize follicle *in vitro* maturation protocols.

miRNAs in FF or follicular cells may fluctuate depending on the physiological parameters of the woman, as well as the condition or maturation status of the follicle. Four miRNAs in FF were determined to depend on the age of the woman: hsa-miR21-5p was exclusively expressed in the age group below 31 years, while hsa-miR-199b and hsa-miR-99b-3p were detected in women over 38 years. The level of hsa-miR-134 expression was significantly higher in FF from the older group. Signalling pathways targeted by these miRNAs include heparan-sulfate biosynthesis, extracellular matrix-receptor interaction, carbohydrate digestion and absorption, p53 signalling, and interactions between cytokines and their receptors (Diez-Fraile et al., 2014).

Two recent studies aimed to find FF miRNA markers to explain the molecular background of polycystic ovarian syndrome (PCOS) among *in vitro* fertilization patients (Roth et al., 2014, Sang et al., 2013). The size of the study groups as well as the methodology differed between these publications, which may provide explanation for no overlap between them. The levels of hsa-miR-132 and hsa-miR-320 were reported significantly lower in PCOS patients in one study (Sang et al., 2013), and those of hsa-miR-9, 18b, 32, 34c, and 135a displayed a significant increase in the PCOS group compared to oocyte donors in the other (Roth et al., 2014). At the same time a rat model of PCOS was created by 5α-dihydrotestosterone (DHT) treatment and miRNA profile of ovarian cortex confirmed the increase in

expression of miR-32 and decrease in miR-132 in DHT-treated mice, the latter with borderline statistical significance (Hossain et al., 2013). Taken together, the proposed PCOS markers may have a true potential value, but their validation is still necessary.

As there is constant infiltration of biomolecules across the follicular basal membrane, there is also a possibility for detecting follicular markers in peripheral blood plasma. This is especially true for miRNAs that are stabilized in vesicles or protein complexes. So far hsa-miR-181a in plasma has been associated with premature ovarian failure in humans (Zhang et al., 2013), but diagnostic markers are extensively being sought for infertility as well as for detecting ovarian cancer (Beach et al., 2014).

There is a potential use for miRNAs in improving IVF outcome, either as prognostic markers for oocyte viability measured in the cumulus cells, as proposed in animal studies (Kim et al., 2013, Tesfaye et al., 2009), or portraying the transcriptome of the first polar body that has been performed in human (Reich et al., 2011). One novel approach introducing miRNA mimics into follicle culture was shown to alter oocyte maturation stage (Kim et al., 2013), a knowledge that could be useful in practice for improving IVM outcome.

# AIMS OF THE STUDY

MGC and CGC are two granulosa cell sub-populations that differentiate from a single population during the secondary-antral stage transition of folliculogenesis (Figure 1). Differentiation leads to the gain of separate functions, the best studied of which are steroidogenesis by theca-MGC compartment and the regulation of oocyte maturation by CGC, just to mention a few. However, there are still several gaps in information regarding the inter-cellular communication between different somatic cell populations that is involved in the fine-tuning of folliculogenesis and oocyte development. Secondly, many of the molecular processes involved in folliculogenesis have been studied in animal models and the degree of species-specific differences, especially when comparing animal models to human, need to be elucidated. Finally, studies performed on human ovarian follicular material give further information on female reproductive health, the etiologies of infertility and the efficacy of COS regimens in individual patients.

The main aims of the present thesis are as follows:

1. To describe the mRNA profile of CGC and MGC isolated from hormonally stimulated IVF patients with the aim to predict novel molecular functions for the two cell populations.
2. To study the degree of co- and post-transcriptional modifications of signalling pathways in the granulosa cell types by investigating differential alternative splicing and determining the miRNA profile in MGC and CGC.
3. To analyse a panel of cytokines and apoptosis markers in the follicular fluid of hormonally stimulated IVF patients and correlate the results with patient physiological characteristics, COS and IVF outcome.
4. To examine the analysed cytokines as means of intercellular communication between MGC and CGC based on the previously described mRNA profile.

# MATERIALS AND METHODS

The following methods were used in this study:

- Archiving of the medical case history of each recruited patient (Publications I, II and III)
- Mural and cumulus granulosa cell isolation (Publications I, II, III)
- Follicular fluid isolation (Publication III)
- mRNA isolation from granulosa cells and cDNA synthesis (Publications I, II and III)
- miRNA isolation from granulosa cells and cDNA synthesis (Publication II)
- Gene expression analysis using Affymetrix GeneChip Human Gene 1.0 ST Array (Publication I)
- Gene expression analysis using high-throughput poly(A) RNA-seq (Publication II)
- Gene expression analysis using high-throughput small RNA-seq (Publication II)
- Gene expression analysis using real-time PCR (Publications I, II and III)
- Bioinformatic analysis for microarray, poly(A) RNA-seq, small RNA-seq and real-time PCR data (Publications I, II and III)
- Bioinformatic prediction of miRNA targets (Publication II)
- Gene ontology profiling of differentially expressed genes and miRNA targets (Publications I and II)
- Bioinformatic analysis for studying alternative splicing from poly(A) RNA-seq data (unpublished data, details provided in Appendix I)
- Multiplex flow cytometry analysis for detecting protein markers from follicular fluid samples (Publication III)
- Statistical analysis (Publication III)

# RESULTS AND DISCUSSION

## 1. mRNA expression differences between mural and cumulus granulosa cells (Publication I, publication II, unpublished data)

The aim of the current chapter is to describe the degree of difference in the expression levels of coding genes between MGC and CGC. Granulosa cells emerge from a single layer of cells in the primary follicle stage and differentiate into two sub-populations by the antral stage (Figure 1).

To study the molecular difference between the two sub-populations, two genome-wide approaches were used: Affymetrix microarray (publication I) and next-generation polyadenylated RNA sequencing (poly(A) RNA-seq) on Illumina platform (publication II). The advantages of microarray based technology are the standardized sample preparation and analysis protocols as well as cost efficiency. On the other hand, the RNA-seq method does not limit the number of different RNA molecules detected and allows the identification of unannotated RNA transcripts.

Besides the detection method used, there are additional differences in the set-up of the two studies. The number of patients recruited was 19 in the microarray study and 3 in the RNA-seq study; the patients did not overlap between the two groups. During the collection of the MGC samples for the RNA-seq study, the cells were depleted of CD45+ leukocytes to give a more relevant picture on the expression of immune-related genes in non-immune system cells. In spite of these differences the results between the two studies showed very high and statistically significant positive correlation ($r = 0.82$, $p < 2.2\times10^{-16}$, Supplemental figure 3 in publication II). Therefore, if not mentioned otherwise, the gene expression differences between MGC and CGC in the current chapter refer to the results of publication I.

Results obtained during these studies reflect women that have undergone COS with GnRH antagonist protocol, rFSH stimulation and ovulation trigger with hCG. Not only has it been shown that COS as such has an influence on the follicular transcriptome (CGC population studied in de los Santos et al., 2012), but differences are also obvious, depending on the protocol used. The effect of stimulation by rFSH or human menopausal gonadotrophin (hMG) on MGC transcriptome has been investigated in two studies (Brannian et al., 2010, Grondahl et al., 2009). Another publication demonstrates the differences in MGC and CGC gene expression upon ovulation triggering with either hCG or GnRH agonist (Borgbo et al., 2013). The relevance of these differences still needs to be revealed, since all the described stimulation protocols lead to successful folliculogenesis and potentially mature oocytes.

Additionally, our study concentrated on the pool of MGC and CGC from the whole cohort of stimulated follicles of a patient. Therefore we observed genes expressed in all follicles regardless of the maturation state of the corresponding oocytes. Differential transcriptome profile of CGC from individual follicles containing an oocyte of germinal vesicle or metaphase 2 stage has been recently determined (Yerushalmi et al., 2014).

According to our results, statistically significant difference in expression levels between MGC and CGC were observed for 15.5% of the analyzed genes, which is enough to discriminate well between the two cell types (Figures 1 and 2 in publication I). The result is not surprising, considering the already well-known differential roles of these cells.

When the top differentially expressed genes were enriched into networks according to their molecular function, the genes up-regulated in CGC turned out to be mainly involved in tissue development and intercellular communication (Table 1 in publication I). The network with the highest IPA score (refers to p-value of $10^{-48}$) is connected by a node depicting TGF-β family members, well reflecting the role of these genes in the oocyte-CGC communication necessary for COC expansion and oocyte meiotic maturation (reviewed in Gilchrist et al., 2008). The COC expansion process is also dependent on extracellular matrix (ECM) synthesis, remodelling and the expression of cell-surface ECM receptors. Proteins involved in these processes were strongly represented among CGC transcripts (Figure 3, Table 1 and 2 in publication I).

A great number of membrane transporters from the solute carrier family (SLC) proteins were exclusively expressed in CGC. In fact, none of the members of this protein family were differentially expressed in MGC > 2.5 fold (Table 2 in publication 1). The SLC proteins up-regulated in CGC are involved in the transport of amino acids (SLC1A3, SLC7A11, SLC38A1), oligopeptides (SLC15A1), nucleosides (SLC28A3) and choline (SLC44A5), which is an indication towards the metabolic communication between CGC and the oocyte (Collado-Fernandez et al., 2012).

The genes up-regulated in MGC are predicted to be involved in immune response and immunological diseases (Table 1 in publication I). However, upon sub-setting the transcripts according to folliculogenesis-related functions, genes related to immune response were strongly represented in both cell populations (Table 2 in publication I). This result will be further discussed in chapter 4 later in the thesis. One of the central proteins in the MGC network with the highest statistical significance (p-value $10^{-44}$) is the suppressor of cytokine signalling 3 (SOCS3), a well-known inhibitor of the JAK-STAT pathway involved in the signalling of several cytokines (reviewed in Carow et al., 2014).

In addition, transcription factors from the early growth response family proteins (EGR1-3) are highly expressed and represented in the MGC network. The role of EGR1 has been well studied in rodents (Espey et al., 2000, Russell et al., 2003, Yoshino et al., 2002) and in the bovine model (Sayasith et al., 2006), where it has been shown to be induced by FSH, LH and hCG. In return, EGR1 is necessary for LHR expression in luteinized MGC (Sayasith et al., 2006, Yoshino et al., 2002). In animal models the expression of EGR1 has been described as quick and transient: expression peaks at 4h after hCG stimulation in rats and at 6h in cow; and declines by 24h post-hCG. According to our results, the expression decline in human MGC takes considerably longer as we still observed a strong signal after 36h post-hCG stimulation.

The second group of transcription regulators highly expressed in the network with the top score in MGC belong to the nuclear receptor subfamily 4 group

A (three members: NR4A1-3). Although well studied in the processes of inflammation (reviewed in McMorrow et al., 2011), these proteins have an important role in regulating steroidogenesis in ovarian granulosa and theca cells of several species, including human (Li et al., 2010). NR4A members are strongly induced by LH (Park et al., 2003) and drive the cells towards the luteinized state by inhibiting aromatase expression (Wu et al., 2005) and inducing expression of proteins in the progesterone synthesis pathways (Havelock et al., 2005). Androgenic hormone synthesis and signalling is also modulated by NR4A1, including an increase in the expression of the testosterone biosynthesis pathway proteins and androgen receptor (AR) (Dai et al., 2012, Li et al., 2010).

As a conclusion, our data demonstrated that the CGC coding transcriptome is over-represented by genes involved in cell-cell interaction that is necessary for the processes involved in COC expansion. MGC, on the other hand, carry out differential roles in steroidogenesis and inflammation-related processes via expressing distinct families of transcription factors not seen in CGC. Our further interests in the study focus on the degree of differential co- and post-transcriptional mRNA regulation between MGC and CGC, especially on alternative splicing and function of differentially expressed miRNAs.

## 2. Gene expression regulation by alternative splicing in mural and cumulus granulosa cells (unpublished results based on data from publication II)

Typical large-scale gene expression study methods assume that the structure of mRNA molecules under investigation does not vary between samples. However, in biological systems this is rarely the case, especially when the samples differ substantially in nature: they are isolated from different tissues, cell-types, experimental conditions etc.

Alternative splicing is the most common diversification mechanism for mRNA molecules, giving rise to a wide variety of mRNA transcripts encoded from the same gene. It is estimated that 95% of multi-exon genes undergo alternative splicing in humans (Pan et al., 2008). The inclusion or exclusion of exons and/or introns into or from an mRNA molecule may substantially modify the stability and the capacity of protein translation from the mRNA strand or the properties of the translated protein itself. As a result, the whole signalling pathway containing the protein may be altered, thus transforming the properties or the fate of the cell (reviewed in Li et al., 2014).

The next goal was to obtain a preliminary insight into how much of the gene expression profile in human MGC and CGC populations could be influenced by alternative splicing. The RNA-seq data is a good source for answering such questions, as the sequencing reads generated by this method are potentially without bias, which is not the case with data derived from most microarray based or real-time PCR experiments that use pre-designed probes or primers for the detection of mRNA molecules. For analysing differential exon usage between MGC and CGC, an R/Bioconductor package DEXSeq was used (Anders et al., 2012). A detailed

description of the package is included as Appendix I. The main limitation of using this package is that only previously known mRNA isoforms available at some database can be analysed. Therefore, novel alternatively spliced mRNA molecules were not included in the current study.

As a result, differential exon usage between MGC and CGC was demonstrated for 1008 genes. Ensembl human genome version GRCh37 was used as a reference, which contains all known and predicted non-coding mRNA isoforms. Therefore, not all of the 1008 genes that differentiate between the two cell types generate alternatively spliced coding mRNA molecules. However, since the role of non-coding mRNAs in gene regulation is still poorly understood, it is not yet clear, how such cases, where alternative splicing generates non-coding mRNA molecules should be interpreted.

A subset of results on the differential exon usage between MGC and CGC is presented in Table I. The gene ontology terms shown were chosen due to their importance in the processes of folliculogenesis. As examples, sterol metabolism and steroid hormone receptor signalling are regulated in granulosa cells by differential splicing, as are processes involved in hypoxia, insulin-like growth factor receptor signalling pathway, extracellular matrix remodelling and cell differentiation. BMP receptor BMPR1B and epiregulin (EREG) involved in COC expansion are expressed in both cell types, but as different mRNA isoforms.

It is clear that discussing the roles of all proteins that demonstrate differential alternative splicing in human granulosa cells is out of the scope of the current study and functions of many of the isoforms are still not known today. It is however important to perceive the multi-level nature of gene expression data available for any biological system under study.

However, a good example of a clear difference in the alternative splicing pattern between MGC and CGC is the antagonist of IL1 receptor IL1RN (Figure 4). While the protein-coding isoform IL1RN-005 is clearly expressed in both cell types, the isoforms numbered 001-003 are exclusively expressed in CGC as no sequencing reads are observed in the counting bins specific for these transcripts. The mRNA isoform numbered 004 is not expressed in either cell type. Interestingly, IL1RN-005 is the only secretory isoform, while IL1RN-001, -002 and -003 are intracellular proteins that can be released only by certain cell-types (Evans et al., 2006). If not released, the intracellular isoforms may have unique roles in certain cells: in intestinal epithelial cell-line Caco-2, the intracellular form inhibits IL-1-induced secretion of IL-6 and IL-8 production (Garat et al., 2003). The role of IL1RN isoforms in human ovary still need to be elucidated.

**Table I.** A subset of gene ontology terms enriched for genes that demonstrated differential exon usage between MGC and CGC. The presented terms were chosen due to their relevance in the processes of folliculogenesis. Enrichment was performed in The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7.

| Category | Gene Ontology Term | No of Genes | Gene Symbols |
|---|---|---|---|
| Biological process | GO:0030518 steroid hormone receptor signalling pathway | 10 | NEDD4, DNAJA1, KDM3A, FHL2, YWHAH, RARA, RAN, TGFB1I1, CTNNB1, CALR |
| | GO:0001666 response to hypoxia and GO:0070482 response to oxygen levels | 15 | SOD2, VEGFA, ECE1, ITPR2, ACTN4, THBS1, PML, ADAM17, PLOD2, NR4A2, ADCK3, SMAD3, BNIP3, LONP1, SERPINA1 |
| | GO:0048009 insulin-like growth factor receptor signalling pathway | 4 | IGF1R, PIK3R1, IRS1, EIF2AK3 |
| | GO:0030198 extracellular matrix organization | 11 | NFKB2, APLP2, FKBP1A, CRISPLD2, COL4A2, GFOD2, SERPINH1, ACAN, ILK, ANXA2, COL5A2 |
| | GO:0016125 sterol metabolic process | 10 | MSMO1, IDI1, SCARB1, LIPE, SC5DL, MVK, PPARD, HMGCS1, HMGCR, INSIG1 |
| | GO:0048165 fused antrum stage, oogenesis and GO:0001550 ovarian cumulus expansion | 2 | BMPR1B, EREG |
| | GO:0045597 positive regulation of cell differentiation | 28 | ACT3, GNAS, KLF10, PDLIM7, ARHGDIA, SMAD3, SMAD4, ACIN1, BMPR1B, BDNF, CTNNB1, CLU, HIF1A, INHBA, INS, IL4R, IL6ST, JUND, MORF4I2, PNP, NDEL1, PPARD, PPARG, RARA, RUNX1, TGFB1I1, TNFRSF12A, RELA |
| Molecular Function | GO:0003707 steroid hormone receptor activity | 9 | PPARD, NR4A1, THRA, NR1D1, RARA, PPARG, NR4A2, ESRRA, NR2C2 |
| | GO:0035258 steroid hormone receptor binding | 7 | KDM3A, FHL2, YWHAH, RAN, TGFB1I1, CTNNB1, CALR |
| | GO:0004222 metalloendopeptidase activity | 11 | UQCRC1, PMPCB, PITRM1, ECE1, MMP19, YME1L1, UQCRC2, ADAM17, ADAMTS9, ADAM9, SPG7 |

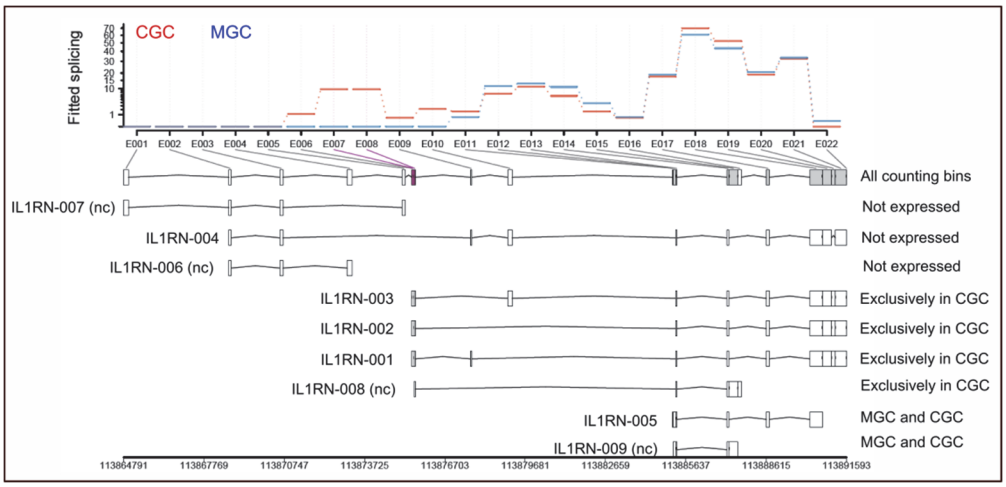


**Figure 4.** An example of differential exon usage at the 5' end of IL1RN gene in MGC and CGC. Counting bins that are differentially expressed between MGC and CGC are coloured in violet. The process how counting bins are generated from the known exons of a reference genome by DEXSeq package is described in detail in Supplementary figure 1 in Appendix 1. The mRNA isoforms are named according to Ensembl human reference genome version GRCh37. nc – non-coding mRNA isoform.

## 3. MicroRNA expression in human granulosa cells (Publication II, unpublished data)

MiRNAs are regulatory RNAs that have both, known and unexplored functions in gene expression regulation. In order to investigate, how mRNA expression could be regulated post-transcriptionally by miRNAs, we performed deep-sequencing analysis of small RNAs with the intention to detect miRNAs and of poly(A) RNA-seq with the aim to study potential miRNA targets in the same samples of MGC and CGC. We compared the acquired small RNA sequencing data with miRBase version 18 (www.mirbase.org).

### *3.1. Annotated microRNAs in granulosa cells and their predicted targets.*

Analysis of miRNAs with the highest expression levels did not reveal obvious differences between the two granulosa cell types (Publication II, Table 1). Clearly the most abundant miRNA in both populations was anti-apoptotic hsa-miR-21 (Carletti et al., 2010). Other functions for the most abundant miRNAs include aromatase targeting by hsa-let-7f (Shibahara et al., 2012) and cancer growth inhibition by hsa-miR-99a-5p (Cui et al., 2012). However, the functions of the latter two miRNAs have not been demonstrated in the ovarian follicle.

Looking at the whole list of detected annotated miRNAs, 90 of them with average or low expression levels were differentially expressed between MGC and CGC: 57 being more abundant in MGC and 33 in CGC (Publication II, Table 2). As transcripts of low expression levels tend to fluctuate between samples due to technical errors, we used analysis algorithm that takes this possibility into account.

We discarded all miRNAs with < 10 counts in all individual samples and performed empirical Bayes shrinkage method, the aim of which is to normalize the expression fluctuations at the lower end and making gaining statistical significance stricter for molecules with low expression levels (Robinson et al., 2007).

It was surprising that the signalling pathways targeted by the differentially expressed miRNAs in MGC and CGC partially overlapped (Figure 5). Based on that information, it may be assumed that TGF-β, ErbB signalling and heparan sulfate biosynthesis are pathways, where regulation by miRNAs is necessary also after the two granulosa cells populations have differentiated. At the same time, the expression of more than twice the number of miRNAs is upregulated upon differentiation in MGC as compared to CGC. This was predicted to lead to the post-transcriptional regulation of various signalling pathways involved in cellular adhesion, ligand-receptor interactions as well as endocytosis and several metabolic pathways.

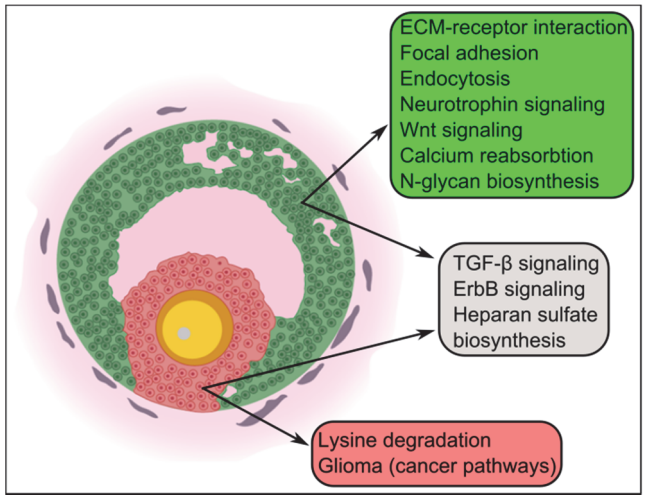


**Figure 5.** Enrichment of genes that were targeted by differentially expressed miRNAs in cumulus an mural granulosa cells (CGC and MGC, respectively) for pathway terms according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). The data shown is based on Table 3 in publication II. The oocyte is depicted in yellow, CGC in pink and MGC in green. The pathways targeted in both cell populations are presented in grey box.

### *3.2.Novel miRNAs and their targets.*

Rapid development of deep sequencing technology enables the identification of novel RNA molecules. Using small RNA-seq method, we detected nine novel miRNAs (unannotated according to miRBase version 18, Table 4 in Publication II), four of which were expressed at sufficiently high level to be confirmed by real-time PCR (Supplemental Figure 2B in publication II).

Out of the four novel miRNAs hsa-miR-548ba and hsa-miR-7973 are of special interest due to their predicted location of transcription origin from FSHR and aromatase gene introns, respectively (Figure 1 in publication III). Taking into account the fact that the samples under study were obtained from women after COS performed with rFSH, there is a possibility that the expression of these miRNAs

becomes detectable only in case of strong over-expression of their host genes and these might not be observed from samples obtained during unstimulated cycles. However, we have detected hsa-miR-548ba also in human endometrium samples (Saare et al., 2014), a tissue well known to express FSHR (La Marca et al., 2005), confirming the validity of our finding.

Secondly, based on the current knowledge, we cannot declare the dependence of hsa-miR-548ba and hsa-miR-7973 expression on their host genes. Further studies are needed to validate the biogenesis pathway of these two miRNAs: whether they are generated by splicing machinery as mirtons, spliceosome-independently as simtrons, or by RNA polymerase II or III from independent promoters according to the canonical pathway as depicted in Figure 3 (Monteys et al., 2010). We detected a few reads from poly(A) RNA-seq experiments in the locus of hsa-miR-548ba hairpin expression in two out of the six samples analyzed (Figure 6). It is, however, too difficult to predict the pri-miRNA transcript due to such a low coverage. We did not get a single read from poly(A) RNA-seq experiment overlapping hsa-miR-7973 locus (data not shown). As only one time-point was analysed in the current study, we hypothesize that the peak of expression of these two novel miRNAs could appear in an earlier stage of folliculogenesis, possibly coinciding with the expression of their host genes FSHR and aromatase in secondary follicles (Findlay et al., 1999, Oktay et al., 1997).

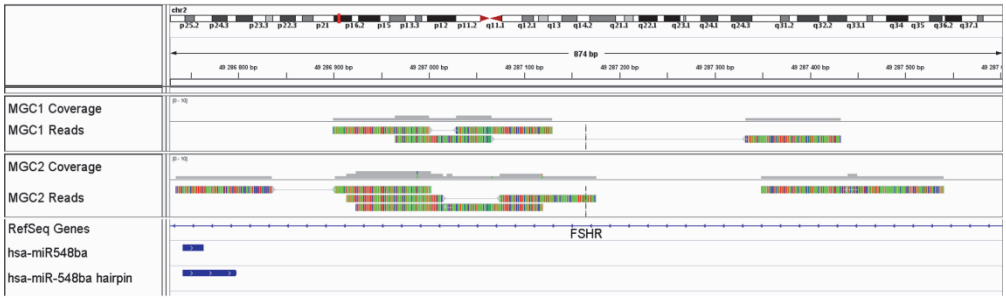


**Figure 6.** FSHR gene intron region depicting continuous coverage of paired-end reads from poly(A) RNA-seq that overlap with hsa-miR-548 hairpin and mature miRNA loci (depicted as blue rectangles in the bottom panel). Low coverage was detected only in two MGC samples (MGC1 in top and MGC2 in the middle panel). Paired-end reads are depicted as coloured rectangles connected by a thin line. The figure was created in Integrative Genomics Viewer version 2.3.23.

Hsa-miR-548ba is of interest due to its predicted targets being activin A receptor type IIB (ACVR2B) and bone morphogenetic protein receptor type II (BMPR2). Activin signalling is well known to increase FSHR expression (Minegishi et al., 1999, Myers et al., 2008), therefore finding a miRNA from FSHR intron that may be co-expressed with its host gene proposes a novel negative feedback loop in folliculogenesis. BMPR2 is a receptor component for the oocyte secreted factors GDF9, BMP15 or their heterodimer, which have important roles in several aspects of folliculogenesis: cumulus expansion, progesterone production, and oocyte meiotic maturation (reviewed in Gilchrist et al., 2008). As the hsa-miR-548 miRNA family

is relatively new in evolution, confirmed only in primates (Piriyapongsa et al., 2007), it is highly possible that regulation of the described pathways by miRNAs is species-specific.

## 4. Cytokines and apoptosis markers in follicular fluid (Publication III, unpublished results)

FF filling the antral cavity contains a mixture of proteins and metabolites infiltrated from plasma or secreted by follicular cells (Rodgers et al., 2010). We were further interested in a panel of cytokines and apoptosis markers that have been shown to possess several ovary-specific roles besides the ones investigated in relation to the immune system (Table II). The studied protein markers include 8 pro-inflammatory cytokines: IL-1β, IL-6, IL-18, IFN-α, IFN-γ, TNF-α, IL-12, and IL-23; anti-inflammatory cytokine G-CSF; 5 chemokines: MIP-1α, MIP-1β, MCP-1, RANTES, and IL-8; as well as 2 anti-apoptotic markers sAPO-1/FAS and CD44v6. As mentioned earlier in the thesis, most of these proteins have alternative roles in different tissues, therefore IL-6, G-CSF and TNF-α may also be added to the category of apoptotic markers, and CD44v6 is well studied for its role in promoting migration in cancer cells (reviewed in Heider et al., 2004).

To measure the concentrations of the above-mentioned proteins from small volumes of FF obtained from 153 IVF patients, we used a bead-based cytometric assay from Bender MedSystems (Young et al., 2008). The particular technology uses microbeads of variable diameter and fluorescence intensities allowing highly multiplexed analysis. Each type of bead is coated with a specific capturing antibody for the antigen of interest. After incubation of the beads with a biofluid sample, detection antibodies are included to the mix that add a second fluorescent label to the system. The beads are then analysed by flow cytometry. The bead size and intensity of fluorescence at 612 nm was used to distinguish between the different analytes and phycoerythrin signal at 575 nm was used for discriminating between empty beads and beads that carry the antigen. The number of antigen-coated beads was counted by the flow cytometer and protein concentrations were calculated according to standard curves. This technology has been shown to give more sensitive signals when compared to standard ELISA protocols due to the use of fluorescent signal over the colorimetric one. In addition, smaller volumes of body fluids can be used due to the multiplex nature of the test and the increased surface area of microbeads compared to the standard multi-well plates (Young et al., 2008).

All significant results are graphically presented in Figure 1 of publication III and in Table II of the current thesis. We found no associations between IL-6, G-CSF, MCP-1 and RANTES concentrations in the collected FF samples and the studied parameters.

**Table II.** Overview of the protein markers measured in the follicular fluid (FF) samples, their roles in the immune system, ovarian follicle and conclusion of obtained results in the current study.

| Marker | Role in the immune system | Role in the ovarian follicle | Our results |
|---|---|---|---|
| IL-1β | Pro-inflammatory cytokine | Angiogenesis (Levitas et al., 2000). MGC differentiation (Karakji et al., 1995). PGE synthesis (Hurwitz et al., 1995). | Down-regulated in TFI group |
| IL-6* | | CGC expansion, oocyte meiotic maturation (Liu et al., 2009). Anti-apoptotic protein (Maeda et al., 2007a, Maeda et al., 2007b). | No significant results. |
| IL-12 | | Not known. | Down-regulated in unexplained infertility. Positively correlates with the proportion of good quality embryos. |
| IL-18 | | Increases the number of ovulated oocytes and COC expansion in mice (Tsuji et al., 2001). | Down-regulated in unexplained infertility. Elevated in case of pregnancy. Positively associated with the number of fetuses at the first ultrasonography. Positively associated with parity. |
| IL-23 | | Not known. | Elevated in smoking women. Elevated in endometriosis patients. |
| IFN-α | | Steroidogenesis and granulosa cell proliferation in pig (Yasuda et al., 1992). | Down-regulated in TFI group. Elevated in case of pregnancy. |
| IFN-γ* | | Enhances FAS-mediated apoptosis (Quirk et al., 2000, Quirk et al., 1995). Inhibits granulosa luteinization (Best et al., 1994, Gorospe et al., 1988). Involved in autoimmune ovarian failure (Hill et al., 1990). | Elevated in endometriosis patients. |
| TNF-α* | | Granulosa proliferation (Son et al., 2004). Collagenase expression (Yang et al., 2004) and bioactivity (Johnson et al., 1999). | Elevated in endometriosis patients. |

**Table II.** Continues

| Marker | Role in the immune system | Role in the ovarian follicle | Our results |
|---|---|---|---|
| G-CSF* | Anti-inflammatory cytokine | Concentration in FF positively correlates with embryo quality (Ledee et al., 2013). Role not known. | No significant results. |
| IL-8 | Chemokine | Angiogenesis (Murayama et al., 2010, Yoshino et al., 2003), steroidogenesis (Shimizu et al., 2012). | Up-regulated in TFI and endometriosis patients. Elevated in case of pregnancy and positively correlated with serum progesterone level at OPU. Positively associated with parity. |
| MCP1 | | Not known | No significant results. |
| MIP-1α | | Not known | Up-regulated in PCOS. |
| MIP-1β | | Expression in bovine MGC increases with follicular growth (Skinner et al., 2008). Role not known. | Elevated in case of pregnancy. |
| RANTES | | Attraction of eosinophils to the early *corpus luteum* (Aust et al., 2000). | No significant results. |
| sAPO1/FAS | Apoptosis | Anti-apoptotic soluble protein. | Elevated in smoking women. |
| CD44v6** | | Not known. | Down-regulated in unexplained infertility. Up-regulated in PCOS. Elevated in smoking women. |

*Additionally involved in apoptotic processes in other tissues; ** Possesses various additional functions; CGC – cumulus granulosa cells; MGC – mural granulosa cells; OPU – ovarian puncture; PCOS – polycystic ovary syndrome; TFI – tubal factor infertility.

### *4.1.Markers correlating with the etiology of infertility*

Studies regarding the follicular milieu have not been widely carried out for all etiologies of infertility. The peritoneal fluid samples in case of endometriosis and hydrosalpingeal fluid in case of TFI are much more frequently under investigation. Therefore our study is one of the few aiming to find protein markers descriptive of various etiologies of infertility at the follicular level, potentially giving a further insight into the molecular mechanisms that may be altered in these situations.

Unexplained infertility was associated with lower levels of CD44v6 when compared to the reference group with male factor infertility, and lower IL-18 concentration, when compared to the TFI group. IL-18 appeared as a positive marker for IVF pregnancy, as discussed later. It is known that deviations from normal IL-18 signalling in the follicle leads to a diminished degree of COC expansion and reduced number of ovulated ova (Tsuji et al., 2001). Additionally, FF IL-18 has also been correlated with the number of retrieved oocytes after COS (Gutman et al., 2004). Based on the background information the IL-18 signalling pathway could play an important role during folliculogenesis, perturbations of which may lead to infertility.

The FF of PCOS patients contained higher levels of MIP-1α and CD44v6. The elevated level of MIP-1α may refer to the disturbed pro-inflammatory milieu in the follicles of PCOS patients. This chemokines is normally secreted at the site of injury, which is expected in case of tissue re-organisation prior to ovulation (reviewed in Maurer et al., 2004). For some unknown reason in PCOS patients the level of this chemokines is significantly higher than in other patient groups, a result that cannot be well interpreted with the knowledge currently available. In the bovine model, MIP-1α and one of its receptors CCR1 was shown to be up-regulated in the granulosa cells during follicle expansion (Skinner et al., 2008). The authors suggest that this chemokine may have therefore additional inflammation-independent roles during folliculogenesis, perhaps involved in inter-cellular communication.

Women with endometriosis were distinguished by increased IL-23 levels in their FF. The same cytokine has been previously shown to be elevated in the peritoneal fluid samples of endometriosis patients (Andreoli et al., 2011). IL-23 has been linked to several autoimmune diseases, an aspect that has been under close surveillance for endometriosis as well (reviewed in Eisenberg et al., 2012). This pro-inflammatory cytokine showed also other unfavourable associations regarding fertility in our study: higher concentrations of IL-23 were measured in the FF of smoking women as well as in samples obtained from women with secondary infertility. The latter aspect may also revert to the direction of autoimmune causes of infertility, but this claim needs further validation.

Additionally, the FF levels of IFN-γ and TNF-α were higher in the endometriosis group when compared to women with TFI, the latter result has been previously shown by others (Falconer et al., 2009). In addition, a functional experiment has demonstrated that MGC isolated from endometriosis patients secrete significantly higher levels of TNF-α in culture (Carlberg et al., 2000). Perturbations in the IFN-γ pathway have also been acknowledged previously in case of endometriosis: it has been shown that cells from endometriotic foci are considerably more resistant to IFN-γ-induced apoptosis compared to normal endometrial cells

(Nishida et al., 2005). Disturbances in survival and apoptosis pathways may therefore be another background mechanism in endometriosis patients at the follicular level.

The effect of TFI on processes involved in folliculogenesis has not been extensively studied. However, our results suggest that women with TFI are not completely comparable with women undergoing IVF due to the infertility of their partner. In fact, the TFI group of women was distinguished by the decreased follicular levels of IL-1β and IFN-α, when compared to the male factor infertility group. Considering the various roles attributed to these two cytokines in folliculogenesis and steroidogenesis (see Table II), it is clear that the TFI group of women need to be studied in further detail regarding their follicular environment.

Two markers, sAPO1/FAS and CD44v6 that are known as anti-apoptotic proteins were both elevated in the FF of smoking women. This result is not surprising, as it has previously been determined that the oxidative stress level in the follicle is markedly elevated (Paszkowski et al., 2002) and the DNA in CGC contains increasing number of breaks if the woman is a regular smoker (Sinko et al., 2005). Therefore the anti-apoptotic proteins are expectedly over-expressed in these follicles to counterbalance the stress evoked by the metabolites of cigarette smoking.

### *4.2.Markers correlating with stimulation and IVF outcome*

IL-12 was the only cytokine in our study that correlated with COS efficiency and embryo quality. The concentration of IL-12 in FF positively correlated with the number of fertilized oocytes and the proportion of good-quality embryos of a patient. Interestingly, a lot of controversial information regarding this cytokine is available in publications by other research groups. Several of those associate the high expression of IL-12 with a negative outcome in IVF: highly fragmented embryos (Ledee et al., 2008) or embryo implantation failure (Bedaiwy et al., 2007, Gazvani et al., 2000). Ostanin *et al.* concluded in their study that oocyte immaturity and low morphological quality coincide with multicomponent cytokine deficit, IL-12 being one of the components (Ostanin et al., 2007). Vujisic *et al.,* on the other hand, reported higher levels of IL-12/IL-23 common subunit p40 in FF samples that contained an oocyte (Vujisic et al., 2006). All these contradictory results refer to the need for further validation of IL-12 as an oocyte or embryo quality marker.

Three cytokines were associated with achieving clinical pregnancy after COS and IVF procedure. MIP-1β is a chemokines that has been confirmed as a positive marker for clinical pregnancy also by other researchers (Ostanin et al., 2007). It is possible that the inflammatory milieu at the pre-ovulatory stage is providing an optimal environment for the oocyte maturation, fertilization and further embryo development.

IL-8 and IL-18 appear to provide a longer lasting favourable background for a positive outcome for IVF, as these cytokines positively correlated with parity, as well as with positive clinical pregnancy outcome. The importance of IL-18 was already discussed above, as the diminished concentration of this cytokine in FF described the group of women with unexplained infertility. In addition, IL-18 levels in FF correlated with the number of foetuses detected by ultrasonography in case of

multiple embryo transfer, confirming the positive influence of this cytokine on the IVF outcome.

IL-8 correlated with serum progesterone levels at the day of OPU, this steroidogenic effect has been confirmed also in the bovine model (Shimizu et al., 2012). It has also been reported that IL-8 in MGC is stimulated by FSH and LH (Runesson et al., 2000). Therefore it is safe to conclude that IL-8 plays an important part in the luteinization of granulosa cells. Our gene expression results showed that IL-8 is expressed at significantly higher levels in MGC compared to CGC, meaning that luteinization processes are enhanced by MGC via an autocrine loop (Figure 2 and Table 2 in publication III). Another role of IL-8 in the growing follicle is that of a pro-angiogenic factor (Murayama et al., 2010). Our data revealed a positive correlation between IL-8 expression and the follicle diameter, which coincides well with functional results demonstrating that IL-8 is up-regulated upon follicular hypoxia in human (Yoshino et al., 2003).

### *4.3. Cytokines as intercellular signalling molecules in the human ovary*

Our results regarding the measurements of various cytokines and anti-apoptosis markers in the FF that demonstrated distinct profiles regarding the etiologies of female infertility, the outcome of COS and that of the IVF procedure were described above. The number of obtained statistically significant associations confirms the high importance of these markers in follicle physiology. It is not straightforward to confirm the source of the cytokines that were measured from the FF: leukocytes in the theca layer, theca cells themselves and both types of granulosa cells may be responsible for their production and secretion.

As there is not much knowledge about the role of cytokines in the intercellular communication, we were interested, which cytokines, their receptors and signalling modulators (if any) are differentially expressed between MGC and CGC. Statistically significant differential expression would give preliminary evidence that communication between these two sub-populations of granulosa cells exists via the described mediators. In the current study, data obtained from RNA-seq experiments (Publication II) is presented, because the MGC population used in the experiment was depleted of CD45+ leukocytes and the nature of the data also allows to study differential exon usage. The results are presented in Table III.

As already noted earlier (Publication I), the majority of the studied immune system components are more abundantly expressed in MGC (also represented in Figure 2 and Table 4 in publication III). As a few examples, IL-8 and both of its receptors are over-expressed in MGC, as are three of the four chemokines receptors involved in the binding of the studied ligands. However, the only chemokine with differential expression was RANTES that demonstrated higher levels in MGC, while other chemokines are equally expressed in MGC and CGC. Also IL-12 system containing a ligand, receptor and co-receptor demonstrated an autocrine loop in MGC in our study. IL-12A over-expression in MGC was below statistical significance in the RNA-seq study, but the result was statistically significant in the real-time PCR experiments (Figure 2 and Table 4 in publication III).

The IL-1β system represents a good example of communication between MGC and CGC populations. Although IL-1β is more strongly expressed in MGC, the secreted ligand seemed to have a different effect on MGC compared to CGC, since the IL-1 receptor subtypes expressed in these cell populations differ significantly: IL1R1 is more abundantly expressed in CGC, while IL1R2 conveys IL-1β signals into MGC. It is well known that IL1R2 lacks the cytoplasmic domain of IL1R1 and therefore acts as a decoy receptor inhibiting the IL-1 signal (Colotta et al., 1993, McMahan et al., 1991).

In addition, as mentioned earlier, the antagonist for IL1 receptors, IL1RN, is differentially spliced, when MGC and CGC are compared, meaning that the IL-1 signalling pathway is modulated differently in the two cell types. However, the full meaning of this interesting result needs to be revealed by functional studies.

**Table III.** Differential gene expression and alternative splicing of studied markers, their receptors, co-receptors and signalling modulators at mRNA level in human granulosa cells. Transcripts depicted in green were more abundantly expressed in MGC, those in red were over-expressed in CGC, and those in blue express different mRNA isoforms in MGC than in CGC. Transcripts in black were not differentially expressed. Statistical significance FDR<0.05 was used as a cut-off.

| Marker | Receptor | Co-receptor | Modulator |
|---|---|---|---|
| IL-1β | IL-1R1 or IL1R2 | IL-1RAP | IL-1RN |
| IL-6 | IL6R | IL6ST | |
| IL-12A and IL-12B | IL12RB1 | IL12RB2 | |
| IL-18 | IL-18R1 | IL-18RAP | IL18BP |
| IL-23 and IL-12B | IL12RB1 | IL23R | |
| IFN-α* | IFNAR1 | IFNAR2 | |
| IFN-γ | IFNGR1 | IFNGR2 | |
| TNF-α | TNFR1 or TNFR2 | | |
| G-CSF | CSF3R | | |
| IL-8 | CXCR1 or CXCR2 | | |
| MCP-1 | CCR2 | | |
| MIP-1α | CCR1 or CCR5 | | |
| MIP-1β | CCR1 or CCR5 | | |
| RANTES | CCR1 or CCR3 or CCR5 | | |
| FASLG | FAS | | |

*Refers to IFNA1 mRNA isoform.

Regarding IL-18 ligand-receptor system, it is surprising to find the ligand-binding receptor and the signal-transducing co-receptor expressed in different cell types. A possible explanation for such a result is that we have caught a time-window, where the expression levels of either of the receptors start to shift from one

granulosa cell type to the other. Time-lapse experiments are necessary to reveal the rearrangements in the IL-18 signalling system and to provide a clearer answer.

Data in Table 4 in publication III clearly reveals that it is not the equal expression levels between MGC and CGC, but rather high inter-patient variability in the transcription levels of several cytokine genes that rule out reaching the statistical significance. Good examples are TNF-α, MIP-1α and MIP-1β in MGC and IFN-γ in CGC. These cytokines serve as excellent cases for further study, as their expression in granulosa cells is probably influenced by the physiological background of the woman. In the current study, we already revealed that TNF-α and IFN-γ in the FF are up-regulated in IVF patients with endometriosis, MIP-1α is more abundant in the FF of women with PCOS and MIP-1β correlates positively with IVF outcome (Figure 1 in Publication III). Interesting future prospects would be to study the functional roles of these cytokines in follicle culture or in animal models to better understand, how cellular signalling in the ovary is modulated by the cytokines according to different etiologies of infertility and follicular physiology.

# CONCLUSIONS

The current thesis reached the following main conclusions:

- The results demonstrated that approximately 15% of the polyadenylated transcriptome is differentially expressed between MGC and CGC isolated from hormonally stimulated patients. MGC was distinguished from CGC by the up-regulation of immune system related pathways and transcription factor families NR4A and EGR that are associated with the expression of gonadotrophin receptors and genes involved in steroidogenic pathways. CGC was characterized by the enrichment of transcripts of signalling pathways involved in inter-cellular interactions: extra-cellular matrix proteins and corresponding receptors, TGF-β family members and solute carrier family gene products involved in inter-cellular molecular transport.
- Over 1,000 genes produced different alternatively spliced mRNA products, when MGC and CGC were compared, including genes in steroidogenic, insulin-like growth factor signalling, hypoxia, cell differentiation and cumulus expansion pathways.
- The two granulosa cell types were not distinguishable by the most abundantly expressed miRNAs, however, ninety miRNAs with moderate expression levels were differentially expressed. The predicted miRNA targets suggest that a large proportion of miRNA-regulated pathways overlap between MGC and CGC. However, miRNAs that were differentially expressed in MGC specifically targeted genes involved in cellular adhesion and various metabolic pathways.
- Nine previously unannotated miRNAs were discovered. Hsa-miR548ba in the FSHR gene intron and hsa-miR-7973 in the aromatase gene intron provide extended interest due to the roles of their host genes in folliculogenesis. Two of the predicted targets for hsa-miR-548ba are the TGF-β receptor family members ACVR2B and BMPR2 that play crucial part in the oocyte-cumulus communication leading to cumulus expansion prior to ovulation.
- Twelve of 16 cytokines and apoptosis markers investigated in the FF samples of IVF patients are useful in distinguishing women according to the etiology of infertility, and the outcome of ovarian stimulation and IVF. The level of IL-12 positively correlated with the overall embryo quality of a patient. The concentrations of MIP-1β, IL-8 and IL-18 in the FF were significantly higher in women that achieved clinical pregnancy after IVF.
- The presented data supports the involvement of the majority of the studied cytokines in inter-cellular signalling within or between the granulosa cell populations. The ligands and receptors of IL-12, IL-23, IL-8 and RANTES signalling pathways are significantly over-expressed in MGC, referring to autocrine signalling loops within this cell layer. At the same time, the IL-1β pathway is a good example as a mean of communication between MGC and CGC. However, functional experiments are needed to reveal the mechanisms, how these pathways are affected by different etiologies of infertility or other physiological parameters of the woman.

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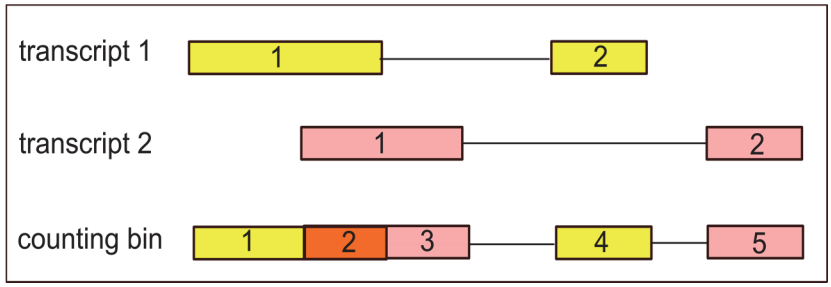
# APPENDIX I

## Analysis of differential exon usage with R/Bioconductor package DEXSeq

Data was generated from the same samples and according to the protocol described in Publication II.

In brief, total RNA was extracted from CGC and MGC, enrichment for polyadenylated RNA was performed and sequencing was carried out on Illumina HiSeq2000 platform with 101 base pair long paired-end reads obtained. TopHat algorithm was used for the alignment of reads to Ensembl human genome version GRCh37 and for splice-junction mapping. Analysis of differential exon usage was performed by R/Bioconductor DEXSeq algorithm (Anders et al., 2012).

DEXSeq algorithm creates counts for each exon determined in the reference genome from user sequencing data. If the exons of alternatively spliced isoforms overlap, they are divided into "counting bins" of overlapping and non-overlapping regions (see figure below). Sequencing reads generated by user are then allocated into these "counting bins" (see Supplementary Figure 1). Thereafter negative binomial generalized linear model is used for detecting differentially expressed exons between samples. The results are normalized according to the total library size and overall differential expression of the gene.




**Supplementary Figure 1.** Principle of creating "counting bins" from multiple potential transcript isoforms of a gene by the DEXSeq algorithm. An artificial "counting bin" is created for regions, where the known exons of different transcripts overlap (bin number 2 in the bottom row).

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# PUBLICATION I

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MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

# The differential transcriptome and ontology profiles of floating and cumulus granulosa cells in stimulated human antral follicles

**S. Kõks[1,2,3,†], A. Velthut[4,5,11,†], A. Sarapik[2,5], S. Altmäe[6,7], E. Reinmaa[6], L.C. Schalkwyk[8], C. Fernandes[9], H.V. Lad[8], U. Soomets[10], Ü. Jaakma[2,3], and A. Salumets[3,4,5,6]**

[1]Department of Physiology, University of Tartu, Tartu 50411, Estonia [2]Department of Reproductive Biology, Estonian University of Life Sciences, Tartu 51014, Estonia [3]Competence Centre on Reproductive Medicine and Biology, Tartu 50410, Estonia [4]Nova Vita Clinic, Centre of Infertility Treatment and Medical Genetics, Tallinn 74001, Estonia [5]Department of Obstetrics and Gynaecology, University of Tartu, Tartu 50406, Estonia [6]Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Tartu 51010, Estonia [7]Division of Obstetrics and Gynaecology, Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm SE-171 77, Sweden [8]Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, King's College London, London SE5 8AF, UK [9]Division of Psychological Medicine and Psychiatry, Institute of Psychiatry, King's College London, London SE5 8AF, UK [10]Department of Biochemistry, University of Tartu, Tartu 50411, Estonia

[11]Correspondence address. E-mail: avelthut@novavita.ee

**ABSTRACT:** Communication between various ovarian cell types is a prerequisite for folliculogenesis and ovulation. In antral follicles granulosa cells divide into two distinct populations of mural and cumulus granulosa cells (CGC), enveloping the antrum and surrounding the oocyte, respectively. Both cell types, with the mural compartment in excess, contribute to the floating granulosa cell (FGC) population in the follicular fluid. The aim of this study was to compare the transcriptomes of FGC and CGC in stimulated antral follicles obtained from 19 women undergoing IVF–ICSI procedure. FGC were obtained from follicular fluid during the follicle puncture procedure and CGC were acquired after oocyte denudation for micromanipulation. Gene expression analysis was conducted using the genome-wide Affymetrix transcriptome array. The expression profile of the two granulosa cell populations varied significantly. Out of 28 869 analysed transcripts 4480 were differentially expressed ($q$-value $< 10^{-4}$) and 489 showed $\geq$2-fold difference in the expression level with 222 genes up-regulated in FGC and 267 in CGC. The transcriptome of FGC showed higher expression of genes involved in immune response, hematological system function and organismal injury, although CGC had genes involved in protein degradation and nervous system function up-regulated. Cell-to-cell signalling and interaction pathways were noted in both cell populations. Furthermore, numerous novel transcripts that have not been previously described in follicular physiology were identified. In conclusion, our results provide a solid basis for future studies in follicular biology that will help to identify molecular markers for oocyte and embryo viability in IVF.

**Key words:** Affymetrix / gene expression / gene ontology / human granulosa cells / IVF

## Introduction

The oocyte, intra-follicular granulosa cells, and theca cells that surround the follicle are responsible for the dynamics of ovarian function including the balance of signals necessary for follicular and oocyte maturation and subsequent ovulation. In the mammalian follicle, granulosa cells surrounding the oocyte divide into two distinct cell populations by the antral stage of folliculogenesis. The cumulus granulosa cells (CGC) are in physical contact with the oocyte through adhesive and gap junction transzonal projections (Albertini *et al.*, 2001) and collectively form the cumulus-oocyte complex (COC). The CGC in this complex are responsible for the metabolism of glucose and the supply of pyruvate for oocyte energy production (Biggers *et al.*, 1967; Russell and Robker, 2007). In return, the oocyte provides mitogenic stimulation and controls the differentiation and expansion of CGC, a process that is necessary for post-ovulatory COC transport in the

[†]These authors contributed equally to this work.

oviduct and sperm capacitation (McNatty et al., 2005; Russell and Robker, 2007). In the antral follicle, the mural granulosa cells (MGC) are situated in a distinct environment from the CGC forming the inner lining of the follicular basal lamina. MGC are the first intra-follicular targets of the ovulation-inducing endocrine signals in the pre-ovulatory follicle, abundantly expressing the luteinising hormone receptor (LHCGR) (Peng et al., 1991). In contrast to the population of CGC, MGC are markedly influenced by molecular signalling from the theca cell layer and less so by the oocyte-secreted factors (Erickson and Shimasaki, 2000). A proportion of MGC are shed from the follicular wall into the follicular fluid and with a minority of CGC constitute the compartment of floating granulosa cells (FGC), also referred to as antral or luteinized granulosa cells.

Studies on transcripts expressed at a fixed time point provide an opportunity to determine the cell function in a distinct biological background. The signalling pathways involved in folliculogenesis and ovulation make up a complex network between the different ovarian cell types. Therefore, single gene expression experiments have been replaced by large-scale transcriptome studies that enable a more comprehensive and detailed analysis of active genes and their networks. Extensive work has been done to reveal the differential gene expression patterns of mammalian follicular cells isolated at different stages of their maturation (Diaz et al., 2007; Skinner et al., 2008). For human studies, ovum pick-up (OPU) in IVF provides an opportunity to collect FGC from the follicular fluid as well as CGC from the COC without additional inconvenience for the patient. However, the isolation of primary cells inevitably causes contamination with other tissues up to a variable degree (Beckmann et al., 1991; Quinn et al., 2006) and thus the results must be dealt with certain reservation when comparing studies using different cell purification methods.

OPU is performed 36 h after human chorionic gonadotrophin (hCG) administration, which is comparable to the endogenous luteinising hormone (LH) surge, and provides cells that correspond to the pre-ovulatory stage of folliculogenesis. Previous gene activity studies on either FGC or CGC obtained during IVF have shown correlative gene expression patterns for the ovarian stimulation regimen (Perlman et al., 2006; Grondahl et al., 2009), embryo cleavage rate and morphology (McKenzie et al., 2004; van Montfoort et al., 2008) and embryo viability (Feuerstein et al., 2007; Hamel et al., 2008). A report on pathway interactions between the human oocyte and surrounding CGC is available (Assou et al., 2006) as well as the transcriptome profiles of mouse COC during the different maturation stages (Hernandez-Gonzalez et al., 2006). However, a comparative study of floating and cumulus granulosa cell populations that are physically adjacent, yet functionally discrete, has not been provided.

Our current study describes the differential transcriptome profiles of FGC and CGC collected during follicle puncture from recombinant follicle-stimulating hormone (FSH) and gonadotrophin-releasing hormone (GnRH) antagonist-stimulated female infertility patients undergoing IVF with intracytoplasmic sperm injection (ICSI). Our study approach provides the first genome-wide overview of the specific features of FGC and CGC transcriptional profiles. In addition to being of interest for basic science in the field of follicular biology, our results may also be useful for identifying the molecular markers for oocyte and embryo competence as an approach towards improved embryo selection and IVF pregnancy success.

# Materials and Methods

## Patients and stimulation protocol

A total of 19 women, aged 32.2 $\pm$ 3.8 years (mean $\pm$ standard deviation), undergoing IVF–ICSI and embryo transfer at the Nova Vita Clinic (Tallinn, Estonia) in the spring of 2008 were enrolled. All patients had been suffering from infertility for at least 1 year before entering the study. The reasons for the couple's infertility were the following: eight male factor infertility, two polycystic ovarian syndrome (PCOS), two tubal occlusion patients, one with endometriosis and six cases of combined causes for infertility. In addition to male factor infertility these six latter patients were diagnosed for the following female infertility associated diseases: one endometriosis and tubal occlusion, two endometriosis, one tubal occlusion, one PCOS and one autoimmune thyroiditis. IVF–ICSI was chosen for patients due to male factor infertility or previous oocyte fertilization failure using the conventional IVF. The study was approved by the Ethics Committee of the University of Tartu and informed consent was obtained from all participants.

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). In average, 1667.1 $\pm$ 413.2 IU of FSH was used during the 9.3 $\pm$ 0.5 days of ovarian stimulation. All patients underwent OPU after 36 h of hCG administration (Ovitrelle, Merck Serono) with the puncture of follicles $\geq$15 mm in size. The total number of oocytes retrieved from each patient was 13.2 $\pm$ 5.6, out of which 12.2 $\pm$ 4.7 were considered mature at metaphase II stage. ICSI was used to fertilize the oocytes 4–6 h after OPU with 69.0% of oocytes fertilized. Up to three (1.9 $\pm$ 0.5) embryos were transferred using second ($n = 17$) or third ($n = 2$) day embryos, resulting in 26.3% ($n = 5$) of clinical pregnancies per embryo transfer with two of them being twins. One additional clinical pregnancy was achieved following frozen embryo transfer.

## Granulosa cell collection: CGC and FGC

FGC were obtained from follicular fluid of patients after OPU and the manual removal of COC and CGC aggregates devoid of the oocyte. The fluid from all follicles of a patient was pooled, centrifuged at 450*g* for 10 min, and the supernatant was removed. The cells were separated on a 50% density gradient of PureSperm 100 (Nidacon, Mölndal, Sweden) in Universal IVF Medium (MediCult, Jyllinge, Denmark), washed three times in Universal IVF Medium at 37°C, lysed with Qiagen RNeasy Mini kit lysis buffer (Qiagen, Hilden, Germany), and stored in liquid nitrogen for future use.

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 (MediCult). The CGC from all oocytes irrespective of their maturity were pooled and centrifuged at 450*g* for 5 min, the supernatant was discarded, and the cells were lysed and stored as described above.

## RNA preparation and quality evaluation

Total RNA from CGC and FGC from all 19 patients was extracted using the RNeasy Micro and RNeasy Mini Kit (Qiagen), respectively, according to the manufacturer's instructions. The quantity and purity of each RNA sample was assessed by spectrophotometer (NanoDrop ND-10, Thermo Fisher Scientific, Wilmington, DE, USA). The integrity of the

RNA was analysed using the RNA 6000 Nano Kit and RNA 6000 Pico Kit with the 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). All samples were of high quality with the absorbance wavelength ratio (A260/A280) of $\geq$1.9 and the RNA Integrity Number of $\geq$8.

## Microarray analysis

For double-stranded cDNA synthesis, 0.3 μg of total RNA was used as a template. Total RNA was reverse transcribed using T7-$(N)_6$ primers (WT cDNA Synthesis and Amplification Kit, Affymetrix, Santa Clara, CA, USA). cRNA was generated from the double-stranded cDNA template through *in vitro* transcription reaction and 10 μg of cRNA was reverse transcribed into cDNA using random primers (WT cDNA Synthesis and Amplification Kit, Affymetrix). In all samples, 5.5 μg of ssDNA was fragmented by incubation with a mixture of UDG and APE1 restriction endonucleases, biotinylated via a terminal transferase reaction, and hybridized to the Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix) for 16 h at 45°C and 60 rpm. Subsequently, each chip was washed and stained with the Affymetrix GeneChip Fluidics Station. Stained arrays were scanned at 532 nm with an Affymetrix GeneChip Scanner 3000, which generated CEL files for each chip. All protocols were followed according to standard Affymetrix instructions. The microarray probeset data are available at the Gene Expression Omnibus public repository (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE18559.

## Data processing and statistical analysis

After the images were processed using the Affymetrix Microarray Suite 5.0, the CEL file data were further analysed and assessed for quality control with the Affymetrix Expression Console software. For probeset summarization the Robust Multichip Analysis (RMA) was used. The normalized, background-subtracted and modelled expression data were further analysed in the statistical software package R (http://www.r-project.org/) and Bioconductor without further transformation (Ihaka and Gentleman, 1996). A paired *t*-test and the R command *t*-test (signal–tissue, paired = T) were used to analyse the differences in transcript expression levels of the cell populations. Correction for multiple testing was performed employing the 'qvalue' package and the *q*-values for each probeset were computed. In addition, we used 'limma' package to construct a volcano plot and heat map.

Using the Ingenuity Pathway Analysis (IPA) Software (Ingenuity Systems Inc, Redwood City, CA, USA), we performed the functional annotation of expression data. For pathway analysis, the probesets were filtered according to their *P*-values and grouped by the fold change. Two sets of genes were uploaded: one set contained the top 100 genes up-regulated in the CGC group and the second set contained the top 100 genes up-regulated in the FGC group. For clarification: transcript up-regulation in one cell population, as mentioned in this manuscript, automatically refers to the down-regulation of the same transcript in the other population in comparison. All genes in these sets had *P*-values from paired *t*-test $< 10^{-3}$ and were therefore considered statistically significant.

## cDNA synthesis and mRNA quantification by real-time PCR

For microarray validation, samples from 10 additional patients not included in the Affymetrix GeneChip Array analysis were used. These women, aged 34.3 $\pm$ 4.3 years, were enrolled in IVF cycle due to the following infertility etiologies: five patients with male factor, three with tubal occlusion and two with combined tubal occlusion and male factor infertility. The stimulation was conducted as described above. The length of recombinant FSH usage was 9.8 $\pm$ 1.1 days and the amount used was 2177.5 $\pm$ 987.6 IU. The total number of collected oocytes was 14.1 $\pm$ 4.7, out of which 10.9 $\pm$ 4.2 were considered mature and 54% were fertilized by ICSI. One ($n = 1$) or two ($n = 9$) embryos (1.9 $\pm$ 0.3) were transferred on Day 2 ($n = 9$) or 6 ($n = 1$). Clinical pregnancy rate was 20% and one additional pregnancy was achieved after frozen embryo transfer from the same IVF cycle.

Five hundred nanograms of total RNA from FGC and CGC samples were reverse-transcribed to cDNA using the $RT^2$ First Strand Kit (SABiosciences, Frederick, MD, USA) according to the manufacturer's protocols. $RT^2$ SYBR Green/ROX qPCR Master mix (SABiosciences), 400 nM of primers (Sigma-Aldrich), and 1/200 of the cDNA synthesis mixture was used for real-time PCR amplification of the mRNA from the following genes: FGG, NTS, FOSB, DUSP6, TNC, ULBP1, RYR2 and EDIL3. GAPDH amplification was used as an endogenous control. All primer sequences are presented in Supplementary Table SI. The reactions were performed on 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The results were analysed using the 7500 Software (Applied Biosystems) and the ΔΔCt relative quantification method. PCR efficiency for all primer pairs was determined using the standard curve method of serial cDNA template dilutions and taken into account during relative quantification calculations. The fold differences in mRNA expression levels between FGC and CGC samples were converted and presented in $\log_2$ scale. Paired student's *t*-test was used to analyse the difference in the mean relative gene expression levels of FGC and CGC samples and statistical significance of $P < 0.05$ was acquired for all transcripts.

# Results

Paired samples of CGC and FGC populations isolated from 19 patients showed distinct gene expression profiles. Out of 28 869 analysed genes, 4480 showed a difference in mRNA expression level with a *q*-value $< 10^{-4}$. Out of these, 489 genes were differentially expressed by $\geq$2-fold absolute difference with 222 genes

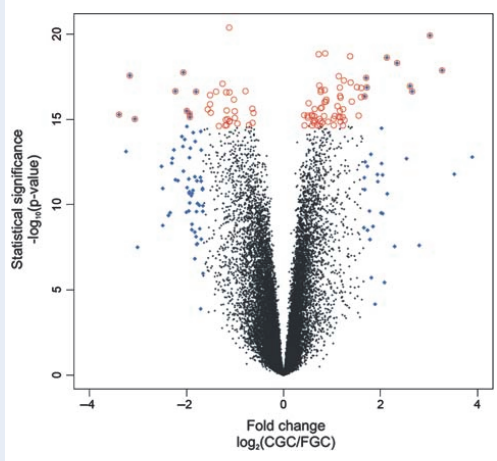


**Figure 1** Volcano plot depicting the fold differences in gene expression levels between the CGC and FGC. Coloured points refer to top 100 transcripts according to fold change (blue diamonds) and *P*-value (red circles).

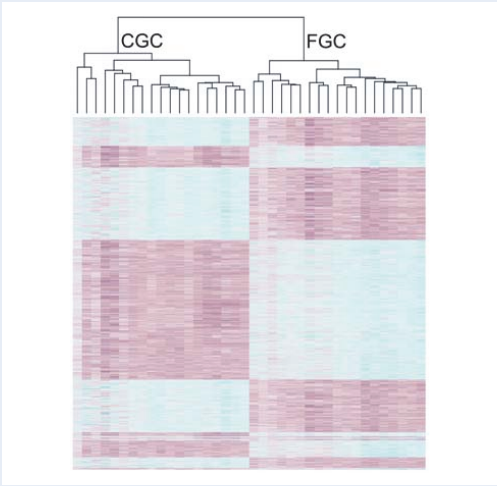


**Figure 2** Heat map and cluster dendrograms of gene clusters differentially expressed between CGC and FGC populations. Up-regulated genes are marked in red and down-regulated genes in blue.

up-regulated in FGC and 267 genes in CGC. A volcano plot of the combined expression data of all transcripts grouped by fold difference and *P*-values is depicted in Fig. 1. In addition, the FGC and CGC samples from each patient were analysed separately for transcripts that had statistically significant differences, resulting in two discrete hierarchical clusters on a heat map (Fig. 2). We also performed statistical analyses whereas omitting the eight patients that had been diagnosed with additional female infertility associated diseases other than tubal occlusion. The list of differentially expressed genes did not change, however, the statistical significance decreased slightly due to the smaller sample size (data not shown).

One hundred genes with the highest fold difference in expression levels between the FGC and CGC samples (Supplementary Table SII) were used for further study on cell population specific gene ontology profiles. Comparing the two cell populations, transcripts from the FGC and CGC groups were up-regulated 2.5–14.9-fold and 2.8–21.1-fold in absolute values, respectively. The aforementioned genes were categorized by molecular function ontology using the IPA Software and grouped into signalling networks. Network enrichment was assessed by the IPA network score. Four networks with the highest network scores for FGC and CGC are presented in Table I. A large portion of transcripts up-regulated in FGC are involved in immune response, hematological system function and organismal injury, although transcripts that predominate in the

**Table I List of cellular networks generated by the IPA Software**

| Molecules in network | Score | Focus genes | Top functions |
|---|---|---|---|
| (A) Floating granulosa cells | | | |
| BHLHB2, BTG2, CCL20, CITED1, DUSP6, EGR1, EGR2, EGR3, FOSB, HDC, IL18, LYZ, MSR1, NR4A1, NR4A2, NR4A3, NTS, OSBPL6, SHC4, SOCS3, TRIB | 44 | 21 | Cell cycle, immunological disease, cellular function and maintenance |
| A2M, ARHGDIB, AXUD1, CYBB, DHRS9, ENPP2, FGG, GM2A, IL1B, KCNMA1, NFKBIZ, NR1D1, PLD2, PPBP, PPM1L, PSMB9, S100A8, TREM1, UCP2, VCAM1 | 42 | 20 | Immune response, cell-to-cell signalling and interaction, hematological system development and function |
| ANPEP, AOX1, C4ORF7, CALCRL, DOCK8, ENPP3, EREG, FGL2, GPR109B, HLA-DPA1, LAPTM5, MBOAT5, MCC, MMP10, MOCOS, PLEK, RARRES2 | 39 | 17 | Immune response, cell-to-cell signalling and interaction, hematological system development and function |
| C13ORF15, CTSC, ENPP2, FREM2, GM2A, HIST2H2BE, HSD17B1, ID2, ID4, KLF4, LYVE1, RGS18, TMEM37, TNFRSF21 | 31 | 14 | Organismal injury and abnormalities, cell cycle, cellular assembly and organization |
| (B) Cumulus granulosa cells | | | |
| ADAM12, DLX5, DOK5, EPHB1, FBLN5, FBXO32, FOXG1, GFPT2, GSTA1, IGFBP5, ITGB3, LIPG, LTBP1, MMP16, RASD1, RGS4, RIMS2, THBS1, TIMP3, TNC, ULBP1, ULBP3, VCAN | 48 | 23 | Protein degradation, cell-to-cell signalling and interaction, tissue development |
| ABLIM3, ANKRD1, BDNF, BEX1, CACNA1C, CBLB, DTNA, E2F7, FABP3, GABRA5, GAL, GAP43, GRIN2A, IL7R, LRAT, MCAM, NDRG2, NEDD4, RYR2, TXNIP | 40 | 20 | Cell-to-cell signalling and interaction, nervous system development and function, behaviour |
| CORO2A, DUOX2, EDIL3, GLIPR1, GRIK2, HMCN1, IL7R, KLF12, MCAM, NDP, NT5E, PEG10, SLC38A1, SMOC2, TSPAN7, VCAN | 30 | 16 | Gene expression, cellular development, cancer |
| ALOX5, B3GALT2, B4GALT5, BICC1, CTSK, DAAM1, EXT1, FOXG1, HTRA1, MAOB, NFIB, SCN9A, SLC7A11, TNC, VCAN | 27 | 15 | Cellular development, cellular growth and proliferation, respiratory system development and function |

One hundred genes with the highest fold difference for floating granulosa cells (FGC) (A) and cumulus granulosa cells (CGC) (B) were analysed by the program and grouped into functional networks. The networks are characterized by scores indicating the degree of overlap between the inserted genes and the entire network generated by the software. Focus genes indicate the number of up-regulated transcripts in our experiment belonging to the specific pathway.

CGC are involved in protein degradation and nervous system function. Cell-to-cell signalling and interaction pathways were noted in both cell populations.

The networks with the highest IPA score for both FGC and CGC are depicted in Fig. 3. Twenty one differentially expressed transcripts in FGC mediate their signalling through the up-regulation of EGR1 and SOCS3 and are important in cell cycle control, immunological disease and cellular function and maintenance (Fig. 3A). The transcription factor CREB, a central molecule in this network, was not differentially expressed. Twenty three transcripts specific for CGC population are joined by transforming growth factor (TGF)-beta family members and the adhesion molecule ITGB3 (Fig. 3B), the latter being also up-regulated in CGC. This network is involved in protein degradation, cell-to-cell signalling and interaction and tissue development. The

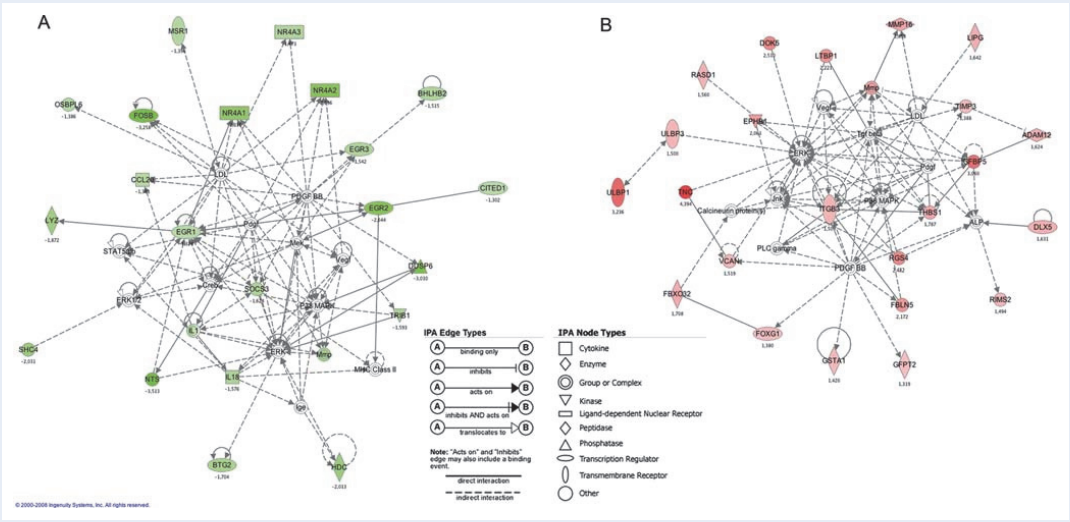


**Figure 3** Pathway networks with the highest IPA scores identified from the comparison of gene expression profiles between FGC and CGC. (**A**) Network with the highest IPA score in FGC is involved in cell cycle, immunological disease and cellular function and maintenance. (**B**) Pathway with the highest IPA score in CGC is associated with protein degradation, cell-to-cell signalling and interaction and tissue development. Up-regulated genes in our study are marked in colour. Transcripts in grey-scale were not differentially expressed.

**Table II List of differentially expressed transcripts grouped according to their function on the basis of the gene ontology database and published literature**

| Functional category | Genes |
|---|---|
| (A) *Floating granulosa cells* | |
| Folliculogenesis/cell differentiation | **A2M, ANPEP, BHLHB2**, **BTG2, DUSP6, EPB41L4B, EVI2B, FOSB, ID2, ID4, NR1D1, PLD2**, RARRES3, **SLC16A7** |
| Ovulation steroidogenesis | **EGR1, EREG, FGG, NTS**, NRIP1, PAI1, ACVR1B, AHR, CYP17A1, **CYP11A1**, FST**, HSD17B1, NR4A1-3, OSBPL6**, SCP2 |
| Hormone receptors | ESR1 |
| Prostaglandin synthesis | COX2, PLA2G4A, PTGES, PTGER4 |
| Extra-cellular matrix molecules | **FREM2**, PTX3, TSG6 |
| Extra-cellular matrix proteases | ADAMTS1, ADAMTS12, CTSL1, MMP9, **MMP10**, MMP15 |
| Cell cycle | **ESCO2, KLF4, PSMB9** |
| Angiogenesis | **CALCRL, COL15A1**, EDG7, **EGR3, ENPP2, FGL2, LYVE1, TRIB1, VCAM1** |
| Apoptosis | **AXUD1, EGR2, FRAG1, MSR1, TEK, TNFRSF21** |

*Continued*

**Table II** *Continued*

| Functional category | Genes |
|---|---|
| Immune response | **CCL20**, CD14, **CTSC, CYBB, ENPP3, HLA-DPA1, IFI30, IL1B**, IL6, **IL18, LYZ, MSR1, NFKBIZ, S100A8, SOCS3**, TRAF3, TRAM2, **TREM1, TRIM22**, TLR1,2,3,4,5,8, **UCP2** |
| Retinoid metabolism | **AOX1, BCDO2, DHRS9, RARRES2** |
| Cell migration | **ARHGDIB, DOCK8, PIK3C2B, SHC4** |
| Ion binding and transport | **KCNMA1, TMEM16E, TMEM37** |
| G-coupled receptor signalling | **GPR109B, RGS18** |
| Transcription factors/ mediators | **CITED1, HIST2H2BE, NPAS2** |
| Metabolism and homeostasis | **ACADSB, ACSS3, AK7, APOA1, GCNT4, GM2A, GK5, HDC, LAPTM5, MBOAT5, MOCOS, MOSC2** |
| Unknown function in ovary | **ANKRD22, PGA5, PGA3, PPM1L, PLEK, RBMS3, PPBP, EVI2B, MCC, DEPDC6, PGAP1, EPB41L4B, PHEX** |
| (B) *Cumulus granulosa cells* | |
| Folliculogenesis/cell differentiation | **ABLIM3**, ADM, **BEX1, BICC1, DLX5, EPHA5, EPHB1, FABP3, LTBP1, NDRG2** |
| Oocyte development | **BDNF, IGFBP5** |
| Steroidogenesis | AKR1C1, **CYP1B1**, CYP19A1, **GAL, GSTA1** |
| Hormone receptors | AR, PGR |
| Prostaglandin synthesis | AKR1C1, AKR1C2, AKR1C3, PLA2G4B, PLA2G10, PTGER2, PTGFR |
| Adhesion/cell–cell attachment | **ASAM**, GJA1, GJA5, GJA7 |
| Extra-cellular matrix molecules | CD44, **CILP, EPYC, FBLN5**, FN1, HAS2, **HMCN1, ITGB3, THBS1, TNC, TSPAN7, VCAN** |
| Extra-cellular matrix proteases | **ADAM12**, ADAMTS6, ADAMTS14, **CTSK, HTRA1, MMP16**, MMP19, TIMP2, **TIMP3** |
| Angiogenesis | **BMPER, EDIL3, MCAM, SMOC2** |
| Apoptosis | **GLIPR1** |
| Immune response | **ACE2, ALOX5, DUOX2**, IL1R1, **IL7R, IL17RB, IL18R1**, IL23R, **LXN, TXNIP, ULBP1, ULBP3** |
| Retinoid metabolism | **LRAT** |
| Cell migration | **GAP43, NT5E, ST8SIA6** |
| Wnt signalling | **DAAM1, NDP** |
| Ion binding and transport | **CACNA1C, CALB2, GABRA5, GRIN2A, RYR2, SCN9A** |
| G-coupled receptor signalling | **GPRC5B, GPR56, GPR177, RASD1, RGS4, RGS5** |
| Transcription factors | **AFF3, E2F7, FOXG1, KLF12, NFIB, PRB2** |
| Solute carrier family transporters | **SLC1A3, SLC7A11, SLC15A1, SLC28A3, SLC38A1, SLC44A5** |
| Metabolism and homeostasis | **B3GALT2, EXT1, LIPG, MAOB, PDE5A, PLCXD3, ST6GAL2** |
| Unknown function in ovary | **CLSTN2, CORO2A, DTNA, DPY19L4, FBXO32, FRMD5, KRTAP13-2, NEDD4, PEG10, PRB1, RHOBTB3, RIMS2, RPSAP52, STXBP5L** |

(A) Differentially up-regulated genes in FGC compared with CGC. (B) Differentially up-regulated genes in CGC compared with FGC. Transcripts in bold font were among the top 100 genes by fold difference in expression levels between the two cell types. All transcripts differ significantly in expression levels ($P$-value $< 0.05$) by at least 1.3-fold. Transcripts without published function or expression data from the ovaries and that did not fall under any available category were listed as 'unknown function in ovary'.

MAPK/ERK and p38 MAPK pathways as well as signalling via platelet-derived growth factor (PDGF) are central in both networks.

The 200 genes used for the IPA pathway analysis and a set of noteworthy differentially regulated genes that have been given importance in follicular development and ovulation in previous publications were categorized into distinct functional groups in Table II. Proteins from these differentially expressed transcripts have been shown to participate in folliculogenesis, ovulation and oocyte function, as well as in diverse cellular events like steroidogenesis, hormonal signalling, prostaglandin synthesis, cellular adhesion, the formation and modulation of the extracellular matrix (ECM), cell cycle control, angiogenesis, apoptosis, immune response, retinoid metabolism, cell migration, Wnt signalling, ion binding and transport, G-coupled receptor signalling, transcription control, solute carrier family transporter activity and metabolism and homeostasis. Most apoptosis-related molecules were found in the list of FGC-specific transcripts. To the contrary, the transcripts of cell-to-cell mediated gap junction proteins as well as Wnt signalling proteins were up-regulated in CGC. As expected, various genes involved in general homeostasis and metabolism were found among the differentially expressed transcripts of both cell

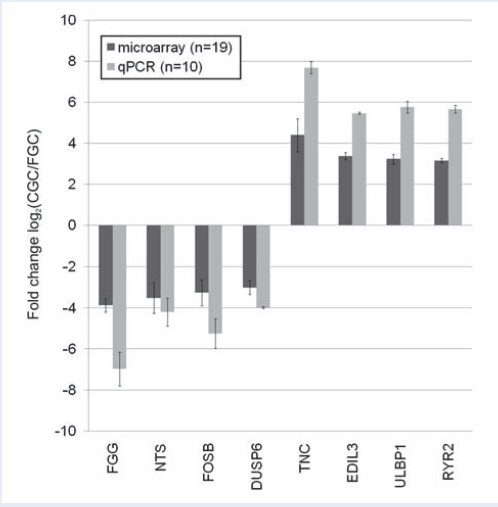


**Figure 4** Validation of microarray results by real-time quantitative PCR using the ΔΔCt method (mean ± SD).

populations. Gene transcripts that have not been previously described in folliculogenesis or to be expressed in the ovary were separated into the group titled 'unknown function in ovary'.

Ten FGC and CGC paired patients' samples not used for the microarray experiments were included in the validation of the microarray results. Four genes with the highest fold difference in expression level acquired by microarray technique were chosen for both FGC (FGG, NTS, FOSB and DUSP6) and CGC (TNC, EDIL3, ULBP1 and RYR2) and statistically different ($P < 0.05$) expression patterns between the two cell populations were confirmed by quantitative real-time PCR analysis (Fig. 4).

# Discussion

Our study represents the first attempt to describe the differential transcriptomes of two granulosa cell compartments from stimulated human pre-ovulatory follicles. The analysed granulosa cells were pooled from ≥15 mm follicles containing mature and immature oocytes and collected from patients with different infertility etiologies. The analysis of gene transcripts from FGC and CGC on a whole-genome microarray revealed the differential expression of nearly 500 genes with at least a 2-fold difference. The cut-off value for further discussion was set to 1.3-fold expression difference with statistical significance of $P < 0.05$ (included in Table II). These genes are involved in diverse aspects of folliculogenesis and oocyte maturation including gonadotrophin action, steroidogenesis, angiogenesis, ECM remodelling and immune response, as well as other pathways.

## Signalling via gonadotrophin receptors

The follicle stimulation protocol used during the preparatory phase of IVF includes regular FSH administration and a single injection of hCG to mimick the endogenous LH surge. We would therefore expect that 36 h after hCG injection, several previously described pathways downstream of FSH receptor (FSHR) and LHCGR would be active in granulosa cells. LHCGR mRNA has been detected in both granulosa cell populations in previous publications (Peng *et al.*, 1991; Assou *et al.*, 2006) and its expression levels in CGC are correlated with the oocyte maturation capability in several species (Yang *et al.*, 2005; Fu *et al.*, 2007; Kawashima *et al.*, 2008). Further, LHCGR expression in CGC has been demonstrated to vary greatly between individual human patients (Haouzi *et al.*, 2009) and to be sensitive in FGC to the stimulation protocol used in IVF (Grondahl *et al.*, 2009). Also the temporal fluctuation of transcript levels has been shown in FGC as LHCGR mRNA is degraded upon LH surge and up-regulated again after 24–48 h (Peegel *et al.*, 1994; Menon *et al.*, 2006). According to our results, no difference in FSHR and LHCGR expression was seen between FGC and CGC, the latter being in line with (Foong *et al.*, 2006). However, we expectedly observed significant up-regulation of the transcription factor EGR1 in FGC that is responsible for LHCGR expression in the rat (Yoshino *et al.*, 2002). The Sp1/Sp3 complex binding effectivity is mediated by LHCGR signalling and this complex is required for EGR1 transcriptional induction (Russell *et al.*, 2003a). Although Sp1 and Sp3 transcripts were expressed in comparable levels between the two cell populations, some known downstream targets of Sp1/Sp3 alone or in complex with EGR1, such as cathepsin L1, ADAMTS1 and epiregulin (EREG) were up-regulated in FGC, possibly as an outcome of FSH stimulation or the hCG surge (Sekiguchi *et al.*, 2002, Doyle *et al.*, 2004, Sriraman and Richards, 2004).

Several publications have shown that FSH and LH/hCG impart their action through the activation of kinase complex ERK1/2 (Das *et al.*, 1996; Maizels *et al.*, 2001) and that this pathway is important for, meiotic resumption of oocytes, COC expansion, FGC differentiation and *corpus luteum* formation (Su *et al.*, 2003; Fan *et al.*, 2008, 2009). The activity of ERK1/2 has been shown to be down-regulated by phosphatase DUSP6 (MKP3) in mouse granulosa cells (Fan *et al.*, 2008). Although active ERK1/2 signalling was proposed by the IPA software modelling in both cell types, DUSP6 expression was higher in FGC. Thus, the differential regulation of common pathways in these two closely related cell populations effectively shows the disparate signalling outcomes in response to identical hormonal stimuli. However, the specific role of DUSP6 in the gonadotrophin function on the FGC compartment remains to be elucidated.

## Adhesive and gap junction proteins

In addition to cellular communication via receptor–ligand interactions and molecular signalling pathways, physical connections between the cells are necessary for follicular development and oocyte maturation. Gap junction protein alpha (GJA or connexin) family members form transmembrane channels that connect adjacent cells for the exchange of ions, nucleotides, second messengers and small metabolites (Bruzzone *et al.*, 1996). In our experimental system GJA1, GJA5 and GJA7 (connexin 43, 40 and 45, respectively) were differentially up-regulated in CGC. GJA1 is necessary for granulosa cell layer expansion and failure to form multilaminar follicles severely affects the ability of the oocyte to fertilize (Ackert *et al.*, 2001). The temporal up-regulation of GJA1 transcription in porcine CGC occurs after the

mechanical rupture of CGC from MGC and the down-regulation at protein level coincides with oocyte maturation (Sasseville *et al.*, 2009). Therefore, GJA1 most likely affects the intercellular connections between individual granulosa cells as well as between granulosa cells and the oocyte. GJA7 partially co-localizes with GJA1 in rat granulosa cells and has been proposed to complement the role of GJA1 (Okuma *et al.*, 1996). In the same study GJA5 was localized to ovarian vascular endothelium by immunofluorescence method. Our experiments propose that GJA5 has a role in human CGC. Further, adipocyte-specific adhesion molecule (ASAM), a novel component of intercellular tight junctions (Raschperger *et al.*, 2004; Sze *et al.*, 2008), was significantly up-regulated in CGC and could possibly be involved in intercellular adhesion in human ovarian follicle.

## Steroidogenesis

After obtaining the responsiveness to LH and initiating progesterone production from cholesterol, the mural compartment of FGC in the late antral stage is referred to as luteinized granulosa. As expected, we observed active regulation of steroidogenesis in FGC. Sterol-carrier protein 2 (SCP2) is a cytoplasmic cholesterol transporter (reviewed in Seedorf *et al.*, 2000) that was differentially expressed in FGC in our study. This up-regulation has been previously correlated with the maturation of rat granulosa cell-line (Rennert *et al.*, 1991). The cholesterol side-chain cleavage enzyme (CYP11A1) responsible for the conversion of cholesterol to pregnenolone, as well as CYP17A1 that converts progesterone to androstenedione, and HSD17B1 converting estrone to 17β-estradiol were all up-regulated in FGC. Our results coincide with some previous studies (Ghersevich *et al.*, 2000; Bak *et al.*, 2009) that show HSD17B1 expression in murine granulosa cells being stimulated by Activin A via its receptor Alk 4 (ACVR1B) and Smad 2 signalling and modulated by follistatin activity. Interestingly, Alk 4 and follistatin were up-regulated in FGC in the current study. We also detected the down-regulation of enzyme AKR1C1 (20αHSD) and aromatase (CYP19A1) in the same cell population. Both processes have been considered as indicators of granulosa cell luteinization (Niswender *et al.*, 2000; Stocco *et al.*, 2001).

The only differentially expressed hormone receptors in our study were the elevated levels of androgen (AR) and progesterone (PGR) receptors in CGC, and estrogen receptor 1 (ESR1) in FGC. It has been proposed that the oocyte orchestrates the follicular development partly through the AR-mediated signalling (Hickey *et al.*, 2004). AR enhances the role of mitogens in porcine granulosa cells. Its function depends on the distance from the oocyte as during follicular development AR activity was maintained in the COC, although FGC lost responsiveness to androgens (Hickey *et al.*, 2004). PGR is up-regulated in porcine CGC when cultured in the presence of LH and FSH, resulting in the down-regulation of GJA1 expression and in the initiation of the oocyte meiotic maturation (Shimada and Terada, 2002). Supporting our results for ESR1, its expression has been previously observed in human MGC to be increasing along with the follicular development (Saunders *et al.*, 2000), but is barely noticeable in *corpus luteum* (van den Driesche *et al.*, 2008).

## Angiogenesis

During folliculogenesis, a capillary bed is formed around the growing follicles for the supply of oxygen and nutrients. After the LH surge a decrease in blood supply has been reported (Fraser, 2006). Although angiogenesis in the peri-follicular region is mostly associated with the layer of theca cells expressing angiogenic factors, several signalling molecules are also synthesized by granulosa cells (Fraser, 2006). One of these molecules up-regulated in FGC in our study is fibrinogen-like 2 (FGL2), a pro-coagulant prothrombinase protein exerting angiogenic effects (Su *et al.*, 2008). The calcitonine gene-related peptide (CALCA) signalling through its receptors CALCRL and RAMP1 has been described as a pro-angiogenic system in peri-implantation endometrial tissue (Dong *et al.*, 2007). CALCRL was differentially expressed in FGC in our experiment. Adrenomedullin (ADM), another ligand for CALCRL, was differentially expressed in CGC. ADM has been shown to be expressed and secreted from granulosa cells and is important for follicular maturation by affecting progesterone synthesis (Balasch *et al.*, 2004). Lysophosphatidic acid (LPA) is secreted into human follicular fluid and promotes the expression of angiogenic cytokines IL6, IL8 and VEGF (Chen *et al.*, 2008). We found the transcription of autotaxin (ENPP2), the enzyme that converts lysophosphatidylcholine to LPA (Umezu-Goto *et al.*, 2002), elevated in FGC. In addition, an LPA receptor EDG7 (or LPAR3) was selectively expressed in the same cell population, in agreement with previous results (Chen *et al.*, 2008).

## Prostaglandin synthesis

Prostaglandin $E_2$ ($PGE_2$) production from arachidonic acid (AA) is necessary for successful ovulation as shown in several animal models (Murdoch *et al.*, 1993). The rate-limiting enzymes in this process are phospholipase A2 (PLA2), cyclooxygenase (COX) 1 and 2 and PGE synthase (PTGES), all of which were up-regulated in primate FGC shortly after hCG stimulation (Duffy *et al.*, 2005). In our study, several PLA2 isoforms, responsible for AA generation, were differentially expressed in either cell population as specified in Table II. In agreement with a previous research in rodents, prostaglandin-E receptor (PTGER) subtype 2 was expressed in CGC, though PTGER4 was more abundant in the FGC population as were the genes of COX2 and PTGES, the three latter results being contrary to the earlier findings (Segi *et al.*, 2003). COX1 transcription levels did not differ significantly. Interestingly, we found several transcripts related to the production and signalling of prostaglandin F (PGF) expressed at higher levels in CGC. These include PGF synthase AKR1C3, receptor PTGFR, as well as enzymes AKR1C1 and AKR1C2 involved in PGF and PGE interconversion (Dozier *et al.*, 2008). PGF production by bovine CGC has been previously shown *in vitro* (Kobayashi *et al.*, 2007), but studies in human have been limited to FGC and *corpus luteum*. These differences in transcription levels suggest that prostaglandin synthesis in human ovarian follicles might be spatially regulated, but a thorough interpretation will require additional studies to reveal the functional significance of our preliminary results.

## ECM composition and remodelling

The cumulus matrix is an important component for the post-ovulatory transport of the COC complex and sperm capacitation, but also for the reduction of oocyte oxidative stress (reviewed in Russell and Robker, 2007). The COC matrix is constructed of hyaluronan (HA) that is cross-linked and stabilized by various ECM proteins. A substantial proportion of these proteins are synthesized in CGC although

others are produced in the mural compartment and subsequently transported into the COC matrix. We found the HA receptor CD44, as well as ECM proteins HAS2, fibronectin and tenascin C (TNC) expressed in CGC. Surprisingly, the matrix-stabilising proteins TSG6 and PTX3 in FGC and versican in CGC were up-regulated in our study, findings opposite of those previously obtained in mice (Fulop *et al.*, 1997, Russell *et al.*, 2003b, Salustri *et al.*, 2004). This result suggests a possible difference in the spatial regulation of matrix protein synthesis in antral follicles of different species.

Various proteinases and their regulators are involved in remodelling the ECM during follicular growth, ovulation, and the expansion of the COC matrix (reviewed in Ohnishi *et al.*, 2005). Matrix metalloproteinases (MMPs) have been shown to degrade several ovarian ECM components (Curry and Osteen, 2003). In our study, MMP16 and MMP19 as well as two MMP inhibitors, TIMP2 and TIMP3, were differentially up-regulated in CGC. Up-regulation of MMP9, MMP10 and MMP15 were observed in FGC. PAI-1 a protein from the plasminogen regulatory mechanism was differentially up-regulated in FGC. The role of plasminogen pathway in follicle rupture has been demonstrated (Peng *et al.*, 1993), and pathway inhibitory proteins PAI-1 and PAI-2 are expressed by cultured human FGC and CGC (Piquette *et al.*, 1993). A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family proteinases are also involved in intercellular and cell–matrix adhesion modulation. ADAMTS1 was up-regulated in FGC in our study and it has been reported to regulate the COC expansion by cleaving the COC matrix protein versican in mice (Russell *et al.*, 2003b). Our study supports a potential role in human follicle physiology for yet three other members: ADAMTS6 and ADAMTS14 were differentially expressed in CGC and ADAMTS12 in FGC.

### Inflammatory system

Ovulation is an inflammatory-like process involving the rupture of the follicular membranes and the ovarian surface epithelium as well as tissue repair by mechanisms comparable to wound healing. Recently, active innate immune system signalling has been demonstrated in the ovarian somatic cells of mice (Shimada *et al.*, 2006). Toll-like receptors (TLRs) are factors of the innate response involved in recognizing the 'non-self' from 'self' molecules. Studies of mouse COC have shown the expression of TLRs and downstream effector signalling molecules linking these pathways to COC expansion, oviductal migration and fertilization (Liu *et al.*, 2008). Contrary to the above-mentioned study, we observed a subset of TLR-related signalling components differentially up-regulated in FGC. TLR1, 2, 3, 4, 5 and 8 were all detected in this cell population. In addition, the TLR3/4-specific signalling molecules CD14, TRAM2 and TRAF3 were differentially expressed in FGC, as well as IL6, the target gene of TLR2/4 signalling. At present, the functions of TLRs in FGC have not been addressed, therefore subsequent studies will be needed to elucidate their roles in follicular biology.

Cytokines execute an important role in the regulation of ovarian function and oocyte quality (Vujisic *et al.*, 2006). Even though leukocytes are the primary source of cytokines, ovarian stromal cells have been shown to express cytokines and their receptors (Tsuji *et al.*, 2001). We detected interleukins IL1β and IL18 in FGC of human preovulatory follicles. The roles for the IL1 system are still under great controversy and the importance of IL1β and its receptors in oocyte maturation, COC expansion, and progesterone synthesis may be species specific. However, similar results from granulosa cells of several species have been shown in regards to IL1β-mediated inhibition of estrogen synthesis (Gerard *et al.*, 2004). IL18 and its receptor activity was shown in the mouse ovary and treatment of mice during ovarian stimulation with an α-IL18R blocking antibody reduced the number of ovulated ova and inhibited the expansion of CGC (Tsuji *et al.*, 2001). Interestingly, the receptors for interleukins that were differentially expressed in our study as IL1R1, IL7R, IL17RB, IL18R1 and IL23R showed up-regulation exclusively in CGC, suggesting a possible signalling loop between the intrafollicular somatic cells. We also detected the differential expression of chemokine CCL20 in FGC. The level of secreted CCL20 in the follicular fluid has been correlated with human oocyte maturity (Kawano *et al.*, 2004).

## Conclusion

The presented results are, to our knowledge, the first to describe the unique transcriptomes of FGC and CGC of stimulated human preovulatory follicles required to decipher the sophisticated cellular interplay at the studied time-point of follicular maturation. This research was performed on primary granulosa cells and the results were compared with studies where the methods for obtaining the cells may have varied leading to a dissimilar time-frame and different level of contaminating tissues. Further research is needed in this field. The comparison of data from independent studies could simplify the identification of new oocyte and embryo viability markers useful for improving pregnancy outcomes following IVF treatment.

## Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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# PUBLICATION II

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# Research Resource: Small RNA-seq of Human Granulosa Cells Reveals miRNAs in FSHR and Aromatase Genes

Agne Velthut-Meikas, Jaak Simm, Timo Tuuri, Juha S. Tapanainen, Madis Metsis, and Andres Salumets

Competence Center on Reproductive Medicine and Biology (A.V.-M., M.M., A.S.) Tartu 50410, Estonia; Center for Biology of Integrated Systems (A.V.-M., J.S.), Tallinn University of Technology, Tallinn 12618, Estonia; Department of Obstetrics and Gynaecology (A.V.-M., A.S.), University of Tartu, Tartu 51014, Estonia; Department of Obstetrics and Gynecology (T.T., J.S.T.), University of Helsinki and Helsinki University Central Hospital, Helsinki 00029, Finland; Department of Obstetrics and Gynecology and Clinical Research Center (J.S.T.), University of Oulu and Oulu University Hospital, Oulu 90014, Finland; Institute of Mathematics and Natural Sciences (M.M.), Tallinn University, Tallinn 10120, Estonia; and Institute of Biomedicine (A.S.), University of Tartu, Tartu 51014, Estonia

The granulosa cells in the mammalian ovarian follicle respond to gonadotropin signaling and are involved in the processes of folliculogenesis and oocyte maturation. Studies on gene expression and regulation in human granulosa cells are of interest due to their potential for estimating the oocyte viability and in vitro fertilization success. However, the posttranscriptional gene expression studies on micro-RNA (miRNA) level in the human ovary have been scarce. The current study determined the miRNA profile by deep sequencing of the 2 intrafollicular somatic cell types: mural and cumulus granulosa cells (MGCs and CGCs, respectively) isolated from women undergoing controlled ovarian stimulation and in vitro fertilization. Altogether, 936 annotated and 9 novel miRNAs were identified. Ninety of the annotated miRNAs were differentially expressed between MGCs and CGCs. Bioinformatic prediction revealed that TGF$\beta$, ErbB signaling, and heparan sulfate biosynthesis were targeted by miRNAs in both granulosa cell populations, whereas extracellular matrix remodeling, Wnt, and neurotrophin signaling pathways were enriched among miRNA targets in MGCs. Two of the nine novel miRNAs found were of intronic origin: one from the aromatase and the other from the FSH receptor gene. The latter miRNA was predicted to target the activin signaling pathway. In addition to revealing the genome-wide miRNA signature in human granulosa cells, our results suggest that posttranscriptional regulation of gene expression by miRNAs could play an important role in the modification of gonadotropin signaling. miRNA expression studies could therefore lead to new prognostic markers in assisted reproductive technologies. **(*Molecular Endocrinology* 27: 1128–1141, 2013)**

Mammalian ovarian follicles undergo substantial changes including recruitment, extensive expansion, maturation, rupture of follicular membranes during ovulation, development into corpus luteum, and final atresia. These processes are controlled by gonadotropin secretion from the pituitary and intertwining signaling networks between the oocyte and the somatic cells in the ovary.

Inside the follicle 2 somatic cell types can be clearly distinguished: the mural granulose cells (MGCs) and cu-

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Abbreviations: ACVR2B, activin A receptor type IIB; BDNF, brain-derived growth factor; CGC, cumulus granulose cell; Chr, chromosome; COC, cumulus-oocyte complex; ECM, extracellular matrix; FDR, false discovery rate; FSHR, FSH receptor; hCG, human chorionic gonadotropin; HS3ST1, heparin sulfate 3-*O*-sulfotransferase; HSPG, heparan sulfate proteoglycan; KEGG, Kyoto Encyclopedia of Genes and Genomes; ICSI, intracytoplasmic sperm injection; IPA, Ingenuity Pathway Analysis; IVF, in vitro fertilization; MGC, mural granulosa cell; miRNA, micro-RNA; NGS, next-generation sequencing; OPU, ovarian puncture; poly(A) RNA, polyadenylated RNA; pre-miRNA, precursor miRNA.

mulus granulosa cells (CGCs). These cells derive from the same population of early follicles, but differentiate into 2 subpopulations separated by distance by the preovulatory stage (1, 2). CGCs remain close to the oocyte with the inner layer forming adhesive and gap junction transzonal projections that allow trafficking of metabolites between the somatic cells and the oocyte (3, 4). The oocyte secretes several signaling molecules that influence mitogenic processes and the differentiation of CGC (reviewed in Ref. 5). Collectively, oocyte and CGCs form the cumulus-oocyte complex (COC) that remains intact during ovulation up to fertilization (6, 7). MGCs receive weaker signals from the oocyte due to their distance: these cells establish the inner lining of the follicular basal lamina and are responsible for the steroidogenic activity of the maturing follicle (1). In addition, MGCs express receptors for LH necessary for triggering the final maturation of the follicle and ovulatory processes (8).

The intrafollicular cells can be collected without additional inconvenience during oocyte collection procedure from women undergoing in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI), and the data obtained from the collected material give valuable information on the regulation of final stages of follicle maturation. By determining the signals that trigger the processes of folliculogenesis during IVF cycles, new ways to improve the efficiency of stimulation as well as new markers for predicting oocyte viability can be found.

The signaling cascades underlying the processes during folliculogenesis have been extensively studied on several model organisms as well as human samples (9–14). However, advances in the knowledge regarding posttranscriptional regulation of mRNAs would make it possible to better predict the actual composition of signaling molecules in the cell. A group of highly conserved posttranscriptional regulators of gene expression are mature micro-RNAs (miRNAs). These are small RNAs, on average 21 nucleotides long, that act by binding to target mRNAs in the RNA-induced silencing complex followed by translational suppression, or mRNA degradation (reviewed in Ref. 15). Preceding the described final outcome, miRNAs undergo several stages of processing. Up to several kilobases long, primary miRNAs are transcribed from the miRNA genes, processed into hairpin-like precursor miRNAs (pre-miRNAs) by DGCR8/Drosha protein complex, and ultimately cut into final length by endoribonuclease Dicer. The last stages of miRNA maturation involve the loading of the short RNA duplex into RNA-induced silencing complex and the degradation of the star strand partially complementary to the mature miRNA sequence (reviewed in Ref. 16). During the last years, alternative pathways of miRNA generation have been revealed, showing that some pre-miRNAs derive from short introns after alternative splicing, and others do not require processing by Dicer (reviewed in Refs. 17 and 18).

The modification of signaling pathways by miRNAs may have considerable importance during ovarian folliculogenesis: abnormal follicle recruitment and maturation were observed in conditional knock-out mice, in which the Dicer1 gene was deleted from all cells expressing the anti-Müllerian hormone receptor (Amhr), including the ovarian granulosa cells (19). In addition, increased follicular atresia, reduced ovulation rates, and compromised oocyte and embryo integrity were observed in these mice by 2 independent investigators (19, 20). Another study reporting an inbred mouse line with hypomorphic Dicer1 evidenced decreased progesterone secretion from corpora lutea leading to infertility (21). However, the ovulation processes were not hampered in this model.

High-throughput miRNA profile analysis from different ovarian somatic cell types appears rare among the publications so far. To our knowledge, one sequencing study on human postmortem ovarian homogenate has been published (22). There is slightly more data from model organisms comparing the miRNA profiles between the bovine ovarian and testis tissues (23) and different stages of the corpus luteum (24), ovine follicle homogenates from various stages of folliculogenesis (25), porcine atretic and normal follicles (26), and cultured rat granulosa cells upon FSH stimulation (27).

The aim of the current study was to fill the gap in information regarding the miRNA profile in human follicular granulosa cells from IVF patients. We performed our study using next-generation sequencing (NGS) that enabled us to determine annotated as well as novel, yet unannotated, miRNAs. Our goal was to examine the degree of difference between MGCs and CGCs regarding their miRNA profile and to predict the potential targets of annotated and novel miRNAs in either cell type. In addition, the polyadenylated RNA (poly[A] RNA) population from the same samples was sequenced in order to acquire further biological confirmation for the predicted miRNA targets.

## Materials and Methods

### Patients and stimulation protocol

The study was approved by the Ethics Committee of the University of Tartu in Estonia, and informed consent was obtained from all participants. Three women, aged 31.3 ± 3.1 years (mean ± SD), undergoing ICSI and embryo transfer at Nova Vita Clinic (Tallinn, Estonia) were enrolled. Due to male factor infertility, all patients had been unable to conceive for at least 1 year before entering the study.

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, Schering-Plough, Kenilworth, New Jersey). On average, 1316.7 ± 401.0 IU of FSH was used during the 9.0 ± 1.0 days of ovarian stimulation. All patients underwent ovarian puncture (OPU) of follicles ≥15 mm in size after 36 hours of human chorionic gonadotropin (hCG) administration (Ovitrelle, Merck Serono). The total number of oocytes retrieved from each patient was 17.3 ± 7.1, of which 17.0 ± 7.5 were considered mature at metaphase II stage (97.0 ± 5.2% of all). ICSI was used to fertilize the oocytes 4–6 hours after OPU with 73.4% of oocytes fertilized. Up to 2 (1.7 ± 0.6) second (n = 2) or third (n = 1) day embryos were transferred, resulting in one patient achieving a clinical pregnancy.

The control group used for confirming the differential and novel miRNA expression consisted of samples from 8 additional IVF patients with the following characteristics: age 31.0 ± 6.2 years, FSH amount used was 1528.0 ± 653.8 IU, the number of retrieved oocytes was 12.0 ± 9.1, of which 75.0 ± 22.2% were mature and 66.7 ± 10.9% fertilized normally. All women underwent IVF-ICSI due to male factor infertility, and 2 of them had been additionally diagnosed with tubal infertility.

## Granulosa cell isolation

MGCs were obtained from follicular fluid after OPU following the manual removal of COC 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 3 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, California), lysed with QIAGEN miRNeasy Mini kit lysis buffer (QIAGEN, Hilden, Germany), and stored in liquid nitrogen for future use.

CGCs were collected and processed as described in detail in our previous publication (13).

## RNA extraction and quality control (QC)

Total RNA and small RNA from MGCs and CGCs were extracted using the miRNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions. The quantity and purity of each RNA sample were assessed with spectrophotometer NanoDrop ND-10 (Thermo Fisher Scientific, Wilmington, Delaware). RNA integrity was analyzed using the RNA 6000 Nano Kit and Small RNA Kit with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). All samples were of high quality with the absorbance wavelength ratio (A260/A280) of ≥1.9 and the RNA Integrity Number of ≥8.6. On average, 1 μg of RNA was used as starting material for poly(A) RNA library preparation, and 100 ng for small RNA library preparation.

## Library preparation and sequencing

Library preparation and sequencing was performed at Biomedicum Functional Genomics Unit at the University of Helsinki in Finland. The detailed protocol is described in Supplemental Materials and Methods published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. In brief, separate libraries were prepared for small RNA and poly(A) RNA population from the same samples. Unique adapters from NEXTflex DNA Barcodes (Bioo Scientific Corp, Austin, Texas) were ligated to separate samples for further indexing, after which all 6 samples were pooled. After PCR amplification and cluster generation, paired-end sequencing of 101 bp read length was performed with HiSeq 2000 (Illumina, Inc, San Diego, California) for poly(A) RNA libraries, and 36-bp single reads were acquired on Genome Analyzer IIx (Illumina) from small RNA libraries. Small RNA libraries were sequenced twice, and the acquired data are further considered as technical replicates.

## Sequencing data analysis of poly(A) RNA samples

The pipeline for sequencing data analysis is described in detail in Supplemental Materials and Methods. The number of raw reads for each gene acquired via the described pipeline was used as input for R/Bioconductor package EdgeR (28, 29) for performing differential expression analysis between MGC and CGC samples. Genes with less than 0.1 counts per million in all samples were discarded, and false discovery rate (FDR) less than 5% was considered as statistically significant after multiple testing correction.

## Sequencing data analysis of small RNA samples

Raw filtered data was submitted to miRDeep2 software (30) that integrates adapter trimming, sequence alignment, and miRNA annotation according to miRBase version 18. Further, novel miRNAs are predicted by miRDeep2 upon the detection of potential mature, star and loop sequences from the read pool, and hairpin formation stability of the potential miRNA precursor according to the RNAfold algorithm (31). Default parameters were used in all steps. Similarity search between potential novel miRNAs and human mature miRNA sequences in the miRBase database was performed by BLASTN algorithm online (www.mirbase.org). Significant multiple alignments were visualized in Jalview v.2.8 software (32).

Differential miRNA expression analysis was performed by EdgeR as described for poly(A) RNA data analysis, except that the reads for technical replicates were summed, and miRNAs with less than 10 raw counts in all samples were discarded.

All data obtained via NGS is available at Gene Expression Omnibus data repository, accession number GSE46508 (http://www.ncbi.nlm.nih.gov/geo/).

## Validation of miRNA expression by real-time RT-PCR

A selection of miRNAs showing statistically significant expression level differences between MGCs and CGCs in sequencing data was validated by predesigned real-time RT-PCR assays (Exiqon, Vedbaek, Denmark). cDNA was synthesized from 20 ng of small RNA from 8 patients from the control group using the Universal cDNA Synthesis Kit, and real-time RT-PCR was performed in triplicates with Universal RT SYBR Green Mastermix according to the manufacturer's protocols (Exiqon). 7900 HT real-time PCR System and SDS 2.3 software were used to run the reactions (Applied Biosystems, Foster City, California). Eight annotated miRNAs were tested: hsa-miR-30a-5p, hsa-miR-142-5p, hsa-miR-126-3p, hsa-miR-223-3p, hsa-miR-

874, hsa-miR196a-5p, hsa-miR129-5p, hsa-miR-129-3p, normalized for hsa-miR-132–3p and analyzed for expression differences between MGCs and CGCs according to the ΔΔCt method.

Validation of 4 novel miRNAs was performed on the above-described conditions with custom made real-time PCR assays (Exiqon). In addition, synthetic RNA oligonucleotides with 5′-end phosphate group corresponding to the mature sequences of the tested miRNAs were used as positive controls (Integrated DNA Technologies, Inc, Coralville, Iowa).

### Bioinformatic prediction of miRNA targets

Two approaches were used for predicting annotated miRNA target genes and pathways. First, the lists of differentially expressed miRNAs were uploaded into DIANA miRPath version 2.1 (33), a web-based software that is updated to miRBase v18 and uses the microT-CDS algorithm for calculating miRNA binding sites in the 3′-untranslated region as well as the coding region of the mRNAs. It further predicts enriched signaling pathways of the submitted miRNA targets using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (34). Because the list of MGC miRNAs was longer than that of CGC miRNAs, only the top 33 miRNAs were used in order not to analyze significantly more targets from MGCs. Conservative parameters were chosen for acquiring the union list of predicted target genes (a priori method): MicroT score threshold was set to 0.9, *P* value was set to .05, and both the Benjamini and Hochberg FDR and the conservative statistics options were used. Subsequently, the acquired KEGG pathways were studied for their enrichment of genes that were differentially expressed at mRNA level from our poly(A) RNA NGS experiment. Pathways with less than 10% of genes providing proof from the experiment were discarded because they were considered to turn up in the analysis by chance.

The second approach was used to analyze data from our sequencing experiments only. Analysis was performed by Ingenuity Pathway Analysis (IPA) Software (Ingenuity Systems, Inc., Redwood City, California) and is described in detail in Supplemental Materials and Methods.

In order to predict targets for novel miRNAs, DIANA microT v3.0 (35, 36) that accepts novel potential miRNA mature sequences as input was used. All predicted targets with a score greater than 7.35 for each novel miRNA were further submitted to The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (37, 38), where the list was further enriched for gene ontology terms according to biologic process, cellular compartment, or molecular function. FDR less than 5% was used as a cut-off for statistical significance in DAVID in all cases.

## Results

### NGS outcome parameters and the description of the most abundant miRNAs

In addition to studying the miRNA profile of ovarian somatic cells, we also attempted to predict their potential targets and confirm these using experimental data. For that reason, small RNA and poly(A) RNA sequencing was performed from the same samples, and technical data from the experiments are summarized in Supplemental Table 1.

**Table 1.** Top 10 Lists of Most Abundant miRNAs in MGCs (A) and CGCs (B)

| A | | B | |
|---|---|---|---|
| miRNA | Average rpm in MGCs | miRNA | Average rpm in CGCs |
| *hsa-miR-21-5p* | 69080.68 | *hsa-miR-21-5p* | 89927.85 |
| *hsa-let-7f-5p* | 40089.97 | *hsa-miR-99a-5p* | 28689.39 |
| *hsa-miR-451a* | 16549.71 | *hsa-let-7f-5p* | 14219.34 |
| hsa-miR-30a-5p | 15931.49 | *hsa-miR-26a-5p* | 10606.24 |
| *hsa-miR-99a-5p* | 15257.67 | hsa-let-7a-5p | 8057.85 |
| *hsa-let-7g-5p* | 14633.43 | *hsa-miR-451a* | 7119.30 |
| *hsa-miR-26a-5p* | 12858.71 | *hsa-miR-191-5p* | 6382.17 |
| hsa-miR-27b-3p | 8324.83 | hsa-miR-22-3p | 5731.12 |
| hsa-miR-486-5p | 7730.59 | hsa-miR-146b-5p | 5424.98 |
| *hsa-miR-191–5p* | 6858.22 | *hsa-let-7g-5p* | 5324.91 |

Expression is presented as average reads per million reads (rpm). Common miRNAs in two lists are printed in italic.

Altogether, we identified 1039 annotated mature miRNAs with at least 1 raw read in at least 1 sample: 936 in MGCs and 883 in CGCs (Supplemental Table 2). The most abundant miRNAs in either cell population are presented in Table 1. Seven miRNAs are common between these lists, and the remaining 3 can be found among the top 22 most abundant miRNAs of the other cell type (Supplemental Table 2). Hsa-miR-21-5p was clearly the most abundant miRNA in both MGCs and CGCs. From poly(A) sequencing data we identified mRNAs corresponding to 22 629 genes in MGCs and 22 554 genes in CGCs. Among those, we found evidence of hairpin sequences for several miRNAs detected from the small RNA library (Supplemental Table 2). The full list of transcripts is available at Gene Expression Omnibus data repository.

Based on both miRNA and mRNA expression profiles, the samples clustered together on a multidimensional scaling plot according to the individual patients and the cell type from which the RNA was extracted (Supplemental Figure 1, A and C). This result was also confirmed upon hierarchical clustering on heat maps (Supplemental Figure 1, B and D).

### Differentially expressed annotated miRNAs and their targeted pathways

Ninety miRNAs were differentially expressed with statistical significance: 33 revealed higher expression levels in CGCs and 57 in MGCs (Table 2). The expression levels of 8 miRNAs were also validated by real-time RT-PCR and normalized for hsa-miR-132-3p for which the smallest relative standard deviation was calculated from NGS

**Table 2A.** Differentially Expressed miRNAs According to FDR < 0.05. A: List of miRNAs Up-regulated in MGC.

| No. | miRNA | FC (MGC/CGC) | FDR |
|---|---|---|---|
| 1 | hsa-miR-548ap-5p | 5.97 | 0.0068 |
| 2 | hsa-miR-548j | 5.97 | 0.0068 |
| 3 | hsa-miR-539-3p | 3.71 | 0.0007 |
| 4 | hsa-miR-142-5p | 3.56 | 0.0002 |
| 5 | hsa-miR-144-5p | 3.37 | 0.0024 |
| 6 | hsa-miR-126-5p | 3.30 | 0.0060 |
| 7 | hsa-miR-126-3p | 3.17 | 0.0007 |
| 8 | hsa-miR-487a | 3.05 | 0.0114 |
| 9 | hsa-miR-454-5p | 2.95 | 0.0068 |
| 10 | hsa-miR-223-3p | 2.87 | 0.0009 |
| 11 | hsa-miR-624-5p | 2.87 | 0.0047 |
| 12 | hsa-miR-30a-5p | 2.83 | 0.0029 |
| 13 | hsa-miR-335-5p | 2.80 | 0.0068 |
| 14 | hsa-miR-889 | 2.77 | 0.0041 |
| 15 | hsa-miR-10b-3p | 2.76 | 0.0374 |
| 16 | hsa-miR-154-5p | 2.72 | 0.0210 |
| 17 | hsa-miR-655 | 2.69 | 0.0271 |
| 18 | hsa-miR-4732-5p | 2.68 | 0.0308 |
| 19 | hsa-miR-32-5p | 2.64 | 0.0167 |
| 20 | hsa-miR-451a | 2.62 | 0.0136 |
| 21 | hsa-let-7i-3p | 2.59 | 0.0392 |
| 22 | hsa-miR-10b-5p | 2.57 | 0.0068 |
| 23 | hsa-miR-20b-5p | 2.56 | 0.0068 |
| 24 | hsa-miR-30a-3p | 2.55 | 0.0068 |
| 25 | hsa-miR-196b-5p | 2.54 | 0.0271 |
| 26 | hsa-miR-363-3p | 2.52 | 0.0103 |
| 27 | hsa-miR-429 | 2.49 | 0.0302 |
| 28 | hsa-miR-194-5p | 2.46 | 0.0121 |
| 29 | hsa-miR-223-5p | 2.43 | 0.0400 |
| 30 | hsa-miR-379-5p | 2.42 | 0.0128 |
| 31 | hsa-miR-584-5p | 2.41 | 0.0128 |
| 32 | hsa-miR-656 | 2.40 | 0.0374 |
| 33 | hsa-miR-106a-5p | 2.38 | 0.0075 |
| 34 | hsa-miR-144-3p | 2.38 | 0.0271 |
| 35 | hsa-miR-146a-5p | 2.36 | 0.0128 |
| 36 | hsa-miR-487b | 2.35 | 0.0149 |
| 37 | hsa-miR-142-3p | 2.34 | 0.0308 |
| 38 | hsa-miR-324-5p | 2.34 | 0.0374 |
| 39 | hsa-miR-377-3p | 2.32 | 0.0257 |
| 40 | hsa-miR-369-3p | 2.31 | 0.0271 |
| 41 | hsa-miR-374a-5p | 2.28 | 0.0257 |
| 42 | hsa-miR-409-5p | 2.25 | 0.0084 |
| 43 | hsa-miR-548b-5p | 2.24 | 0.0308 |
| 44 | hsa-miR-1185-2-3p | 2.23 | 0.0480 |
| 45 | hsa-miR-29b-3p | 2.20 | 0.0257 |
| 46 | hsa-miR-382-3p | 2.20 | 0.0402 |
| 47 | hsa-miR-494 | 2.19 | 0.0125 |
| 48 | hsa-miR-98 | 2.19 | 0.0233 |
| 49 | hsa-miR-199b-5p | 2.17 | 0.0257 |
| 50 | hsa-miR-10a-5p | 2.14 | 0.0271 |
| 51 | hsa-miR-335-3p | 2.11 | 0.0404 |
| 52 | hsa-miR-411-5p | 2.07 | 0.0271 |
| 53 | hsa-let-7f-2-3p | 2.02 | 0.0385 |
| 54 | hsa-miR-96-5p | 1.96 | 0.0271 |
| 55 | hsa-miR-483-3p | 1.94 | 0.0302 |
| 56 | hsa-miR-340-5p | 1.93 | 0.0437 |
| 57 | hsa-miR-16-5p | 1.87 | 0.0302 |

(*Continued*)

**Table 2B:** List of miRNAs Up-regulated in CGCs

| No. | miRNA | FC (MGC/CGC) | FDR |
|---|---|---|---|
| 1 | hsa-miR-129-2-3p | −6.58 | 0.0000 |
| 2 | hsa-miR-129-5p | −5.57 | 0.0000 |
| 3 | hsa-miR-1273e | −3.86 | 0.0010 |
| 4 | hsa-miR-4488 | −3.62 | 0.0082 |
| 5 | hsa-miR-4461 | −3.61 | 0.0068 |
| 6 | hsa-miR-181a-2-3p | −3.60 | 0.0001 |
| 7 | hsa-miR-1290 | −3.46 | 0.0010 |
| 8 | hsa-miR-34c-3p | −3.41 | 0.0121 |
| 9 | hsa-miR-196a-5p | −3.31 | 0.0009 |
| 10 | hsa-miR-4792 | −3.17 | 0.0091 |
| 11 | hsa-miR-874 | −3.11 | 0.0006 |
| 12 | hsa-miR-3651 | −3.07 | 0.0009 |
| 13 | hsa-miR-135a-5p | −3.05 | 0.0098 |
| 14 | hsa-miR-873-3p | −3.04 | 0.0047 |
| 15 | hsa-miR-1291 | −2.93 | 0.0011 |
| 16 | hsa-miR-876-5p | −2.70 | 0.0154 |
| 17 | hsa-miR-181a-3p | −2.66 | 0.0065 |
| 18 | hsa-miR-1275 | −2.62 | 0.0241 |
| 19 | hsa-miR-4497 | −2.43 | 0.0352 |
| 20 | hsa-miR-181c-3p | −2.29 | 0.0257 |
| 21 | hsa-miR-320c | −2.25 | 0.0245 |
| 22 | hsa-miR-23b-5p | −2.24 | 0.0302 |
| 23 | hsa-miR-378g | −2.18 | 0.0462 |
| 24 | hsa-let-7c | −2.14 | 0.0271 |
| 25 | hsa-miR-320d | −2.12 | 0.0344 |
| 26 | hsa-miR-1292 | −2.08 | 0.0480 |
| 27 | hsa-miR-125b-5p | −2.04 | 0.0245 |
| 28 | hsa-miR-181b-5p | −2.01 | 0.0302 |
| 29 | hsa-miR-320b | −1.99 | 0.0271 |
| 30 | hsa-miR-202-3p | −1.92 | 0.0257 |
| 31 | hsa-miR-1180 | −1.91 | 0.0346 |
| 32 | hsa-miR-4485 | −1.87 | 0.0305 |
| 33 | hsa-miR-181a-5p | −1.73 | 0.0374 |

FC, fold change in $\log_2$ scale.

results. Samples from 8 women distinct from the NGS experiment were used. According to real-time PCR experiment, 6 miRNAs were differentially expressed with statistical significance, whereas the remaining 2 showed the same trend as in NGS experiment (Supplemental Figure 2A). Differential expression of poly(A) RNAs was compared with the results of our previous study on the Affymetrix GeneChip Human Gene 1.0 ST Array (13). A high positive correlation value of 0.82 was achieved when comparing the 2 datasets with a $P$ value $<2.2 \times 10^{-16}$ (Supplemental Figure 3).

Besides observing a number of miRNAs that were differentially expressed between MGC and CGC, we were interested in the signaling pathways and biological functions that could potentially be targeted by them. Because there are tens of algorithms available for the bioinformatic prediction of miRNA targets, we decided for two different approaches. Firstly, we used DIANA miRPath that is updated to miRBase v.18, the same version that was used for annotating known miRNAs from our NGS data. We allowed the software to predict miRNA target

genes, performed the enrichment of KEGG pathways from the predicted targets and then compared the results with our poly(A) RNA data (Table 3). This approach allows to observe additional targets that may have been missed by poly(A) RNA NGS due to small sample size and the lack of time-scale data.

Interestingly, there are several common signaling pathways that are targeted by the differentially expressed miRNAs: those involved in TGF-$\beta$ and ErbB signaling as well as heparan sulfate biosynthesis. From our poly(A) RNA data we find evidence that in MGCs, already the first essential steps of heparan sulfate biosynthesis may be inhibited. More specifically, xylosyltransferase I that transfers the first saccharide unit to the target peptide and exostosin 1 responsible for further polysaccharide chain elongation were down-regulated in MGC on mRNA

**Table 3.** KEGG Pathways Enriched for Targets of miRNAs That Were Differentially Expressed in MGC (A) and CGC (B) According to DIANA miRPath v.2.1

**A**

| KEGG Pathway | FDR-Adjusted *P* Value | No. of Targeted Genes | No. of miRNAs Targeting the Pathways | Genes Confirmed by Poly(A) RNA NGS |
|---|---|---|---|---|
| ECM-receptor interaction | 1.14E-16 | 29 | 22 | ITGB8, ITGB3, ITGA5, THBS1, COL5A1, COL1A2, FN1 |
| *TGF-β signaling pathway* | 1.35E-09 | 37 | 24 | THBS1, PITX2, ACVR1, ACVR2B, E2F5, LTBP1, SMAD7 |
| Endocytosis | 5.22E-09 | 72 | 26 | CBL, CAV1, NEDD4L, RAB31, PSD3, FLT1, NEDD4, DNM3, SMAD7, ADRB1, ASAP2 |
| *Glycosaminoglycan biosynthesis - heparan sulfate* | 1.11E-06 | 10 | 9 | EXT1, XYLT1 |
| Focal adhesion | 0.0005 | 65 | 27 | ITGB8, THBS1, CAV1, ITGB3, ITGA5, MAPK8, COL5A1, FLT1, COL1A2, FN1 |
| Neurotrophin signaling pathway | 0.0025 | 46 | 26 | SORT1, BDNF, YWHAQ, RPS6KA5, MAPK8, CAMK2B |
| Wnt signaling pathway | 0.0035 | 46 | 26 | FZD7, WNT16, APC, FZD3, MAPK8, CAMK2B, WIF1, DAAM1 |
| Axon guidance | 0.0065 | 40 | 25 | PLXNA2, EPHA5, CXCL12, SEMA6B, EPHA3, NCK2, SEMA3A, NTN4, GNAI1, EFNA1 |
| *ErbB signaling pathway* | 0.0070 | 30 | 25 | CBL, NCK2, MAPK8, CAMK2B |
| Endocrine and other factor-regulated calcium reabsorption | 0.0251 | 15 | 16 | KL, DNM3 |
| *N*-Glycan biosynthesis | 0.0290 | 13 | 13 | ST6GAL2, MGAT3 |
| Amoebiasis | 0.0308 | 28 | 20 | ARG2, COL5A1, COL1A2, FN1 |
| Mucin type *O*-glycan biosynthesis | 0.0342 | 7 | 9 | GALNT3 |

**B**

| KEGG Pathway | FDR-Adjusted *P* Value | No. of Targeted Genes | No. of miRNAs Targeting the Pathways | Genes Confirmed by Poly(A) RNA NGS |
|---|---|---|---|---|
| *Glycosaminoglycan biosynthesis - heparan sulfate* | 2.60E-07 | 6 | 6 | HS3ST1 |
| Lysine degradation | 4.80E-06 | 14 | 13 | TMLHE, SUV39H1 |
| *TGF-β signaling pathway* | 0.0006 | 27 | 14 | ID4, DCN, BMP8A, TGFBR2 |
| *ErbB signaling pathway* | 0.0395 | 26 | 18 | TGFA, PAK7, SHC4, ERBB4, EREG |
| Glioma | 0.0423 | 17 | 13 | TGFA, IGF1, SHC4, |

Italic text represents common pathways between MGC and CGC. NGS, next-generation sequencing.

**Table 4.** Novel miRNAs Predicted from Small RNA Sequencing Data by miRDeep2

| miRNA Identification | Mature Sequence | Average Read Count in MGCs | No. of Samples Detected in MGCs | Average Read Count in CGCs | No. of Samples Detected in CGCs | Predicted Hairpin Genomic Coordinates and Strand | Predicted Hairpin Genomic Region |
|---|---|---|---|---|---|---|---|
| **Chr1.1** | uuucaggucuggggcugaaaccu | 18.3 | 3 | 48.7 | 3 | chr1:113424681..113424744:+ | Intergenic |
| **Chr2.1**[#] | aaagguaacugugauuuuugcu | 46.3 | 3 | 114 | 3 | chr2:49286742..49286798:+ | FSHR gene intron |
| **Chr15.1a**[##] | ugugacccuagaauaauuac | 34.3 | 3 | 11 | 1 | chr15:51606231..51606307:+ | CYP19A1 gene intron |
| **Chr15.1b**[###] | ugugacccuagaauaauuac | 34.3 | 3 | 11 | 1 | chr15:51606228..51606304:− | CYP19A1 gene intron |
| **Chr19.1**[$] | aggcugugaugcucuccugagccc | 43 | 3 | 217.7 | 3 | chr19:11606358..11606437:− | Intergenic |
| Chr1.2 | aguuggagagcauuagacuga | 0 | 0 | 3.5 | 2 | chr1:213020182..213020250:+ | Intergenic |
| Chr19.2[$$] | auccuagucacggcacca | 19 | 1 | 3 | 1 | chr19:55634592..55634660:− | Intergenic |
| Chr3.1* | ugcccugagacuuuugcuc | 12.7 | 3 | 4 | 1 | chr3:127305953..127306019:− | Intergenic |
| Chr3.2** | uucccagccaacgcacca | 2 | 1 | 0 | 0 | chr3:176232891..176232940:+ | Intergenic |
| Chr4.1*** | ucugguguauagcguugcuca | 0 | 0 | 4 | 1 | chr4:21466322..21466381:− | KCNIP4 gene, intronic region |

Two versions of Chr1.1 (miRNA on chromosome 1.1) mature sequences were observed with similar prevalence, the difference in 5′-end is underlined. Chr15.1a and Chr15.1b share the same mature sequence. miRNAs in bold were studied in detail (see text).

[#], hsa-miR-548ba.

[##], hsa-miR-7973-1.

[###], hsa-miR-7973-2.

[$], hsa-miR-7974.

[$$], hsa-miR-7975.

*, hsa-miR-7976.

**, hsa-miR-7977.

***, hsa-miR-7978.

level. At the same time, 3-*O*-sulfotransferase (HS3ST1), which performs one of the many possible modifications at a later stage of heparan sulfate biosynthesis, was downregulated in CGC via predicted miRNA targeting.

Although the number of miRNAs uploaded to the software was equal, the number of targets and target pathways was considerably greater for MGCs than for CGCs. Extracellular matrix (ECM) proteins, endocytosis pathways as well as signaling via neurotrophins and Wnt pathways were specifically targeted by miRNAs from MGCs.

As a second approach, we only used data from our own poly(A) RNA NGS experiment to perform the enrichment of miRNA targets. The IPA software merges several target prediction algorithms into 1 environment. The differentially expressed genes from poly(A) RNA NGS results that were considered as highly predicted or experimentally validated targets for differentially expressed miRNAs in our data were further analyzed for their enrichment according to their molecular functions in gene ontology database and signaling pathways from KEGG and Reactome (Supplemental Table 3A).

For MGCs, the results acquired by the described method confirm well the outcome from miRPath. ECM-receptor interaction and axon guidance referring to neurotrophin signaling were also enriched by this approach. In addition, targets were grouped significantly into molecular functions involved in cytoskeletal protein binding, ion binding, and protein kinase activity (Supplemental Table 3A).

In contrast, in CGCs the molecular functions enriched were rather involved in phosphatase activity and transcription factor activity, including steroid hormone receptor activity. For example, estrogen receptor-$\alpha$ (ESR1) is a potential predicted miRNA target in CGCs. Enrichment of signaling pathways was not as successful for CGC as it was for MGC data. Only amino acid metabolism is predicted to be specifically targeted by miRNAs in CGCs according to Reactome (Supplemental Table 3B).

## Prediction of novel miRNAs and their targeted pathways

A great advantage of the NGS technology is the possibility of identifying novel transcripts. Therefore, our next aim was to predict novel, unannotated miRNAs from the small RNA data by miRDeep2 software. miRDeep2 predicts the probability of a sequence being a novel miRNA according to its surrounding genomic context and its potential to fold into hairpin-like structure with low free energy characteristic of miRNAs. Although there were several unannotated short sequences in our data, we introduce nine different potential novel miRNAs that were present in both technical replicates of at least 1 sample with a minimal average read count of 2 (Table 4). None of the novel miRNAs aligned with any other classes of small RNA, rRNA, or coding sequences. We further concentrated on 4 potential novel miRNAs with the highest expression values and the most frequent appearance among samples. Those miRNAs aligned on different chromosomes: 1, 2, 15, and 19 and, for the sake of clarity, are further referred to according to the chromosome (Chr)

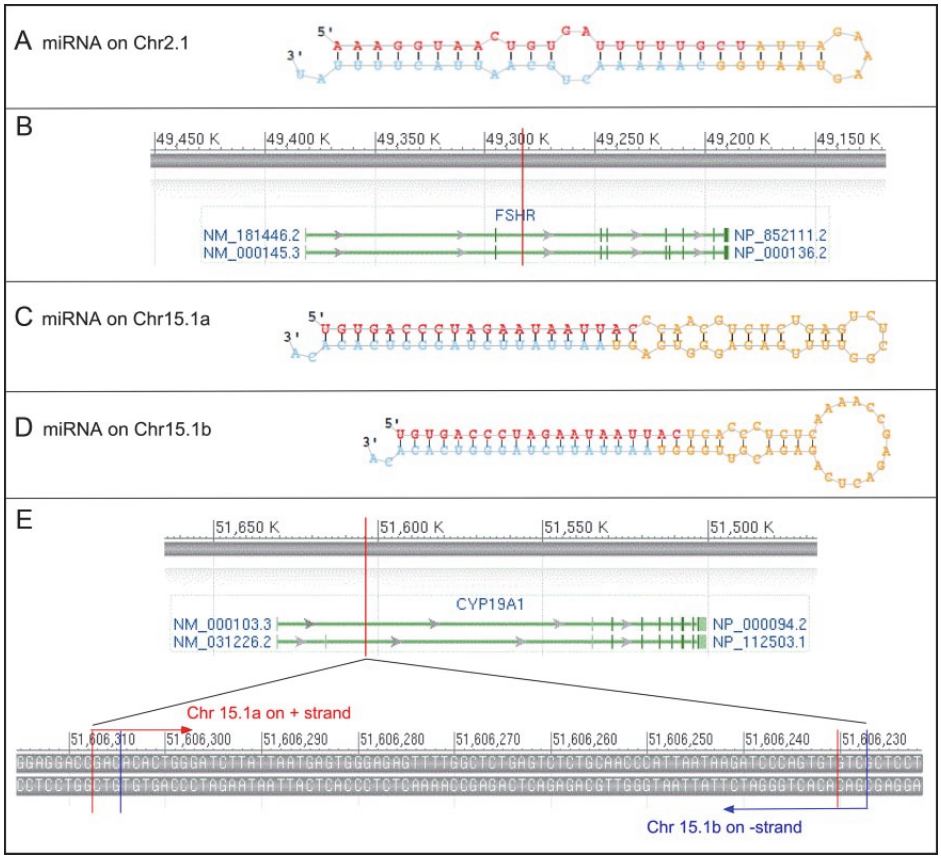


**Figure 1.** Hairpin Structures and Graphical Representations of pre-miRNA Genomic Locations for Novel miRNAs on Chr2.1, Chr15.1a and 15.1b. A, Hairpin structure of miRNA on Chr2.1. B: Graphical representation of FSHR gene. The transcription site for miRNA on Chr2.1 in the intronic region is marked in red. C and D: Two possible hairpin structures for miRNA on Chr15.1 noted as Chr15.1a and 15.1b. E: Two possible genomic locations for miRNA on Chr15.1 in the intronic region of CYP19A1 gene. Genomic location of miRNA on Chr15.1a is marked in red and Chr15.1b in blue in the subset.

number. We validated the expression of those 4 novel miRNAs by real-time RT-PCR. These miRNAs are expressed in both MGCs and CGCs, and only miRNA on Chr19 was more abundant in CGCs with statistical significance (Supplemental Figure 2B).

miRNAs on Chr1.1 and Chr19.1 are both transcribed from intergenic regions. For miRNA on Chr1.1, two alternative mature reads were observed with similar frequency: a 23-nucleotide read with 3 uridine residues in the 5′-end or a 22-nucleotide read with 2 uridine residues (Table 4). The hairpin of Chr2.1 pre-miRNA structure is depicted in Figure 1A, and its sequence aligned to the intronic region of the FSH receptor (FSHR) gene (Figure 1B). miRNA on Chr15.1 is predicted to be transcribed from an intron of the aromatase gene (CYP19A1). Interestingly, there are 2 possible genomic locations for this miRNA in the same intron, one from the plus (miRNA on Chr15.1a) and the other from the minus strand (miRNA on Chr15.1b), giving rise to 2 slightly different possible hairpins with the same mature sequences (Figure 1, C–E).

We searched for high-similarity sequences for the predicted novel miRNAs in the miRBase online search tool. High similarity with several members of the hsa-miR-548 family was found for the sequence of miRNA on Chr2.1. The highest BLASTN score of 78 was acquired by pairwise alignment between miRNA on Chr2.1 and hsa-miR-548d-5p, hsa-miR-548w, and hsa-miR-548ag as well as hsa-miR548ay-5p. In all of the cases, 3 mismatches appeared in the alignment, and discrepancies in the 3′-ends of the sequences were observed (Figure 2). No other predicted novel miRNAs showed similarities to any annotated human mature miRNAs.

Subsequently we analyzed the potential targets of the

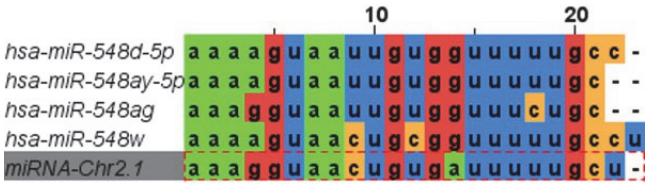


**Figure 2.** Sequence Alignment between miRNA on Chr2.1 (shaded) and Four Members of the hsa-miR-548 Family. BLASTN score for each pair-wise alignment with Chr2.1 was 78 and e-value was 0.2.

novel miRNAs using DIANA microT v3.0, a web-based software that accepts unannotated user-defined miRNA sequences as input. The potential targets predicted for the 4 miRNAs are available as supplemental material (Supplemental Table 4). Fifty-five targets were predicted for miRNA on Chr1.1, 132 for miRNA on Chr2.1, 20 for miRNA on Chr15.1, and 91 for miRNA on Chr19.1. The enrichment of targets according to gene ontology (biologic processes, molecular functions, and cellular compartments) is presented in Table 5. The targets of miRNA on Chr1.1 are predicted to be cytoskeleton proteins associated with cell morphogenesis. Targets of miRNA on Chr2.1 are involved in response to carbohydrate stimulus, peptide secretion, and gene silencing. One of the predicted targets of this miRNA is DICER mRNA that is further translated into a central protein in miRNA biogenesis. Interestingly, activin A receptor type IIB (ACVR2B) and SMAD2, a component of activin-signaling cascade, are common genes in most of the en-

**Table 5.** Gene Ontology Terms Enriched by Predicted Novel miRNA Targets

| miRNA Identification | Category | Term | Count | Genes | FDR % |
|---|---|---|---|---|---|
| Chr1.1 | Cellular component | Cytoskeleton | 14 | LOC651610, DLGAP1, KAZ, TOPBP1, AKAP9, PCM1, TTN, ATM, RAB3IP, PPP1R9A, PAK2, MACF1, MYOM1, EDA, DLG2 | 0.09 |
| | | Cytoskeletal part | 10 | LOC651610, DLGAP1, PPP1R9A, AKAP9, TOPBP1, MYOM1, TTN, PCM1, DLG2, ATM, RAB3IP | 1.49 |
| | Biological process | Cell projection morphogenesis | 6 | MACF1, ONECUT2, NTNG1, VAX1, PCM1, RAB3IP | 1.09 |
| | | Cell part morphogenesis | 6 | MACF1, ONECUT2, NTNG1, VAX1, PCM1, RAB3IP | 1.32 |
| | | Cellular component morphogenesis | 7 | MACF1, ONECUT2, NTNG1, VAX1, TTN, PCM1, RAB3IP | 1.50 |
| | | Cilium assembly | 3 | ONECUT2, PCM1, RAB3IP | 4.47 |
| Chr2.1 | Biologic process | Pancreas development | 5 | ACVR2B, ONECUT2, NEUROD1, SMAD2, PROX1 | 0.17 |
| | | Postembryonic development | 6 | ACVR2B, MORC3, BCL11B, SMAD2, MECOM, BCL2L11 | 0.28 |
| | | Response to glucose stimulus | 5 | ACVR2B, NEUROD1, SMAD2, FKBP1B, PTEN, PTENP1 | 0.47 |
| | | Response to hexose stimulus | 5 | ACVR2B, NEUROD1, SMAD2, FKBP1B, PTEN, PTENP1 | 0.55 |
| | | Response to monosaccharide stimulus | 5 | ACVR2B, NEUROD1, SMAD2, FKBP1B, PTEN, PTENP1 | 0.55 |
| | | Posttranscriptional regulation of gene expression | 8 | CPEB2, MORC3, IMPACT, DICER1, QKI, SMAD2, LIN28B, PTEN, PTENP1 | 1.26 |
| | | Response to carbohydrate stimulus | 5 | ACVR2B, NEUROD1, SMAD2, FKBP1B, PTEN, PTENP1 | 1.40 |
| | | Insulin secretion | 4 | ACVR2B, NEUROD1, SMAD2, FKBP1B | 1.45 |
| | | Protein amino acid phosphorylation | 14 | CDK19, ITK, WNK1, BMPR2, MOBKL1A, ABI2, SMAD2, CDK6, PRKG1, ACVR2B, PAK2, MORC3, COL4A3BP, LOC100132369, EIF2AK2 | 1.45 |
| | | Peptide hormone secretion | 4 | ACVR2B, NEUROD1, SMAD2, FKBP1B | 4.21 |
| | | Peptide secretion | 4 | ACVR2B, NEUROD1, SMAD2, FKBP1B | 4.84 |
| | | Gene silencing by miRNA, production of miRNAs | 3 | DICER1, SMAD2, LIN28B | 4.96 |
| Chr15.1 | None | | | | |
| Chr19.1 | Biologic process | Cellular component morphogenesis | 9 | RAB8A, TNR, PCNT, NFASC, CNTN2, PIP5K1C, TTN, GAS7, CDH23 | 0.51 |
| | | Cell projection morphogenesis | 7 | RAB8A, TNR, PCNT, NFASC, CNTN2, PIP5K1C, GAS7 | 1.09 |
| | | Cell part morphogenesis | 7 | RAB8A, TNR, PCNT, NFASC, CNTN2, PIP5K1C, GAS7 | 1.37 |
| | | Cell morphogenesis | 8 | RAB8A, TNR, PCNT, NFASC, CNTN2, PIP5K1C, GAS7, CDH23 | 1.42 |
| | | Positive regulation of transcription, DNA-dependent | 9 | CIITA, PPARA, FOXK1, ZMIZ2, MAML1, PAX8, TEAD1, FOXO1, NR5A2 | 1.70 |
| | | Cell projection organization | 8 | RAB8A, TNR, PCNT, NFASC, CNTN2, PIP5K1C, GAS7, CDH23 | 1.72 |
| | | Positive regulation of RNA metabolic process | 9 | CIITA, PPARA, FOXK1, ZMIZ2, MAML1, PAX8, TEAD1, FOXO1, NR5A2 | 1.80 |
| | | Positive regulation of transcription from RNA Pol II promoter | 8 | CIITA, PPARA, ZMIZ2, MAML1, PAX8, TEAD1, FOXO1, NR5A2 | 1.80 |
| | | Regulation of transcription from RNA Pol II promoter | 11 | CIITA, PPARA, FOXK1, ZMIZ2, MAML1, PAX8, TEAD1, FOXO1, MDM4, NR5A2, KCNIP3 | 1.83 |
| | | Positive regulation of transcription | 9 | CIITA, PPARA, FOXK1, ZMIZ2, MAML1, PAX8, TEAD1, FOXO1, NR5A2 | 4.79 |
| | Molecular Function | Transcription activator activity | 9 | CIITA, PPARA, FOXK1, ZMIZ2, MAML1, PAX8, TEAD1, FOXO1, NR5A2 | 0.43 |

riched pathways targeted by miRNA on Chr2.1 referring to its potential role in the modulation of activin signaling. miRNA on Chr19.1 potentially silences transcripts related to cell morphogenesis, similarly to miRNA on Chr1.1. In addition, it may inhibit the translation of a group of transcription activators. Due to the small number of targets predicted for miRNA on Chr15.1, no gene ontology terms became enriched.

## Discussion

In the current study we presented, to our knowledge, for the first time the differential miRNA expression profiles of intrafollicular somatic cell populations, MGCs and CGCs, from human stimulated preovulatory follicles. Using NGS technology, we could detect annotated and novel miRNAs that provide new information on the basic biologic processes in the follicle via gene expression modulation.

Our data reveal that it is the miRNAs expressed at low levels that differentiated most significantly between CGCs and MGCs, and the most abundant miRNAs were highly similar between the cell types. In comparison with previous publications, there is a clear difference in the expression levels of abundant miRNAs, depending on whether follicular material or whole ovarian homogenates are analyzed (reviewed in Ref. 39). Therefore our results coincide best with those obtained from sheep follicular and luteal tissues (40): hsa-miR-21 being the most abundant miRNA, and hsa-miR-143-p, highly expressed in the ovarian homogenates of several mammals, is further behind in our top expression lists (22, 23, 41–43).

The mouse analog of hsa-miR-21 plays a role in granulosa cell survival during the transition from the follicular to luteal stage and is up-regulated by hCG-induced ovulation (44). Inhibition of this miRNA therefore leads to cell apoptosis (44). The second most abundant miRNA, hsa-let-7f, has been described as a tumor suppressor targeting aromatase mRNA in breast cancer cell lines (45). Aromatase, a key enzyme in estradiol biosynthesis, is evidently expressed in the ovarian follicle, proposing new potential roles for hsa-let-7f in the modulation of steroidogenesis. The third abundant miRNA in our data, hsa-miR-451a, is a proof of Dicer-independent miRNA biogenesis in granulosa cells. It has been the model miRNA for studying alternative pathways for miRNA generation, although by now it is clear that several other miRNAs are processed without Dicer (46). This finding may explain, in part, the relatively modest severity of the ovarian phenotype observed in conditional Dicer1 knock-out mice (19, 20).

The miRNA database miRBase is rapidly expanding with hundreds of new miRNA sequences added with every update. Therefore we were looking for unannotated miRNAs from our data, because human granulosa cells have not been studied in this regard. We identified 9 different novel miRNAs, 4 of which were expressed at a sufficient level for successful validation. Interestingly, 2 of the novel miRNAs (miRNA on Chr2.1 and miRNA on Chr15.1) are predictively transcribed from introns of 2 genes of high importance for folliculogenesis: FSHR and CYP19A1 (the gene encoding aromatase), respectively. It has been shown that by the end of follicle growth the expression of these 2 genes diminishes (47, 48). We currently have no knowledge, whether those miRNAs are coexpressed with their host genes or whether they are transcribed from independent promoters (49, 50). We also have no information on the role of the 2 miRNAs regarding the posttranscriptional regulation of their host genes. Both synergistic and antagonistic influences on the host gene mRNAs have been shown for intronic miRNAs (51, 52), and experimental validation is therefore necessary for each individual case. Our preliminary bioinformatic analysis did not confirm direct targeting of the host mRNAs by the 2 miRNAs. However, miRNA on Chr2.1 may be involved indirectly in the inhibition of FSHR expression. ACVR2B and SMAD2 are two members of the activin signaling cascade that are both predicted targets of miRNA on Chr2.1. Activin signaling leads to increase in FSHR mRNA expression in granulosa cells in the expansion stages of folliculogenesis (53, 54). If the elevated expression on FSHR mRNA leads to the coexpression of miRNA on Chr2.1 from FSHR intron, a negative feedback loop by targeting ACVR2B may be initiated, contributing to decreased FSHR expression by the preovulatory stage. Modulation of activin signaling by miRNAs has recently been studied in mouse, where mmu-miR-145 was experimentally confirmed to target ACVR2B mRNA in granulosa cells and inhibit their proliferation (55).

miRNA on Chr2.1 has high sequence similarity with the members of the hsa-miR-548 miRNA family. This is a group of miRNAs relatively new in evolution, only distinguishable in the primate lineage, that evolved together with a class of miniature inverted-repeat transposable elements Made1 (56). Our data therefore present further evidence that several new miRNAs will be discovered in known and unknown miRNA families when more cell and tissue types are investigated with sufficient depth.

In addition to providing new high-throughput data on miRNA expression, we were interested in determining the differentiating miRNA profiles and their roles between the human MGCs and CGCs. It is well known that a single miRNA may have several targets, and therefore

analyzing a group of miRNAs according to the ontology profiles of their targeted mRNAs is a common approach (57). However, there is no common tool for miRNA target prediction, and all available ones suffer from a high degree of both false-positive and false-negative results. Better prediction algorithms evolve together with experimental data on miRNA-mRNA binding sites. For higher confidence in estimating targets by bioinformatic methods, the use of mRNA data from the same system for comparison is suggested (57, 58). This approach is also not a golden standard, because not all posttranscriptional gene expression modifications can be evidenced on mRNA level, and several observed changes may be indirect (reviewed in Ref. 59). Therefore, although reducing the number of false predictions by using high-throughput mRNA and miRNA data together, the results should still be judged with caution. Another approach is to report overlapping data from several target prediction algorithms. This methodology may lead, on the other hand, to increased number of false-negative results with less true targets predicted.

Finding available miRNA target prediction software that is up to date with current miRBase is another challenge, as new miRNAs are constantly discovered and the database enlarges quickly. Therefore we used DIANA miRPath v.2.1 that is updated to miRBase v.18, the same version that was used for our small RNA NGS data analysis. Using this software, we only lost some miRNAs from analysis due to using very strict thresholds. As a second approach, we used IPA software that combines TargetScan, TarBase, and miRecords databases with our poly-A RNA seq results. With this method we lost data and obtained results for 25 miRNAs in CGCs and 19 miRNAs from MGCs (33 were uploaded in both cases).

TGF$\beta$, ErbB, and heparan sulfate synthesis pathways were similarly targeted by miRNAs in MGCs and CGCs, although differentially expressed lists of miRNAs were used as input for bioinformatic analysis. The enrichment of these pathways is not surprising because their role in the fine-tuning of oocyte-somatic cell communication has appeared essential for follicular somatic cell differentiation and ovulation. The TGF$\beta$ family members Gdf9 and Bmp15 are the best studied mammalian oocyte-secreted factors that retain CGCs their specific molecular functions and inhibit their differentiation into MGCs (reviewed in Ref. 60). Recently, heparan sulfate proteoglycans (HSPGs) were shown to mediate the signaling of Gdf9 to CGCs in the mouse (61). The ErbB-signaling pathways modulate LH induction involved in COC expansion and follicular membrane rupture. More specifically, in mice the oocyte-secreted factors up-regulate the ErbB family member epidermal growth factor receptor expression in CGCs to enable these cells to respond to LH-induced signaling from MGCs (62). On the other hand, mouse CGCs were shown to express ErbB family ligands Areg and Nrg1, which promote oocyte developmental competence as well as progesterone production by MGCs (63). HSPG synthesis is up-regulated in MGCs of mammalian preovulatory follicles, and this is necessary for tissue remodeling during basal membrane rupture at ovulation (reviewed in Ref. 64). MGCs secrete HSPGs that bind antithrombin to maintain the fluidity of follicular fluid up to COC expulsion (65, 66). Interestingly, follicular fluid HSPGs contain 3-O-sulfated chains with unusually high frequency (66). According to our data, HS3ST1, the enzyme responsible for the side-chain addition, may be down-regulated by miRNAs in CGCs, proposing one more differential role for the granulosa cell types in the ovarian follicle. In MGCs we identified miRNAs targeting several ECM proteins: laminins, integrins, collagens, and fibronectin 1, providing further evidence of the fine tuning of tissue remodeling.

Members of the Wnt- and neurotrophin-signaling pathways were 2 differentially enriched groups of genes potentially targeted by miRNAs in MGCs. The down-regulated neurotrophin confirmed by the poly(A) RNA NGS in MGCs was brain-derived growth factor (BDNF), which corroborates with the results obtained from experimental data with human follicular cells. More precisely, CGCs, but not MGCs, secrete BDNF, the ligand for TrkB receptor that among follicular cells is expressed exclusively on oocytes (67–69). The signaling cascade upon TrkB activation is involved in the oocyte cytoplasmic maturation and the resumption of meiosis before ovulation (67–69). Although BDNF expression is significantly induced by LH and hCG in both granulosa cell populations in vitro, MGCs are incapable of secreting the protein (67, 69, 70).

The role of Wnt signaling in CGCs is not definitely clear in humans. This pathway is involved in cellular patterning, proliferation, survival, and modulation of LH stimulation in rodents and cows (71). However, conflicting results exist regarding the expression of some Wnt-signaling pathway proteins upon LH stimulation when rodent and human granulosa cells were compared (72–73). In addition, we have described several Wnt pathway genes expressed in human granulosa cells that have never been studied in relation to ovarian functions (demonstrated in the current study and in Ref. 13).

In conclusion, the current study provides new information on the posttranscriptional regulation of gene expression in the human stimulated preovulatory luteinizing

follicle. We have clearly shown the similarities and differences in miRNA expression between CGCs and MGCs and provided a bioinformatic prediction of their roles in various signaling pathways. Novel miRNAs from the introns of FSHR and aromatase genes provide new evidence of their mRNA processing, but their role during folliculogenesis remains to be determined. Importantly, the miRNA profile in granulosa cells may possess high potential as a new marker for successful folliculogenesis and oocyte developmental capacity.

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Address all correspondence and requests for reprints to: Agne Velthut-Meikas, Competence Centre on Reproductive Medicine and Biology, Tiigi 61B, 50410 Tartu, Estonia. E-mail: agnevelthut@gmail.com.

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# PUBLICATION III

Sarapik A, **Velthut A**, Haller-Kikkatalo K, Faure GC, Béné MC, de Carvalho Bittencourt M, Massin F, Uibo R, Salumets A. Follicular proinflammatory cytokines and chemokines as markers of IVF success. Clin Dev Immunol. 2012;2012:606459. doi: 10.1155/2012/606459. Epub 2011 Oct 5. PubMed PMID: 22007253; PubMed Central PMCID: PMC3189459.

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*Clinical Study*

# Follicular Proinflammatory Cytokines and Chemokines as Markers of IVF Success

**Aili Sarapik,[1] Agne Velthut,[2,3] Kadri Haller-Kikkatalo,[1,3,4,5] Gilbert C. Faure,[6] Marie-Christine Béné,[6] Marcelo de Carvalho Bittencourt,[6] Frédéric Massin,[6] Raivo Uibo,[1,3] and Andres Salumets[3,4]**

[1] *Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila 19, Tartu 50411, Estonia*
[2] *Institute of Gene Technology, Tallinn University of Technology, Akadeemia Street 15, Tallinn 12618, Estonia*
[3] *Competence Centre on Reproductive Medicine and Biology, Tiigi 61b, Tartu 50410, Estonia*
[4] *Department of Obstetrics and Gynecology, University of Tartu, L. Puusepa 8, 51014 Tartu, Estonia*
[5] *Women's Clinic, Tartu University Hospital, L. Puusepa Street 1a, 50406 Tartu, Estonia*
[6] *Laboratoire d'Immunologie, Université Henri Poincaré, BP 184, Vandoeuvre lès Nancy, 54500 Nancy, France*

Correspondence should be addressed to Kadri Haller-Kikkatalo, kadri.haller-kikkatalo@ut.ee

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Cytokines are key modulators of the immune system and also contribute to regulation of the ovarian cycle. In this study, Bender MedSystems FlowCytomix technology was used to analyze follicular cytokines (proinflammatory: IL-1$\beta$, IL-6, IL-18, IFN-$\gamma$, IFN-$\alpha$, TNF-$\alpha$, IL-12, and IL-23;, and anti-inflammatory: G-CSF), chemokines (MIP-1$\alpha$, MIP-1$\beta$, MCP-1, RANTES, and IL-8), and other biomarkers (sAPO-1/Fas, CD44(v6)) in 153 women undergoing *in vitro* fertilization (IVF). Cytokine origin was studied by mRNA analysis of granulosa cells. Higher follicular MIP-1$\alpha$ and CD44(v6) were found to correlate with polycystic ovary syndrome, IL-23, INF-$\gamma$, and TNF-$\alpha$ with endometriosis, higher CD44(v6) but lower IL-$\beta$ and INF-$\alpha$ correlated with tubal factor infertility, and lower levels of IL-18 and CD44(v6) characterized unexplained infertility. IL-12 positively correlated with oocyte fertilization and embryo development, while increased IL-18, IL-8, and MIP-1$\beta$ were associated with successful IVF-induced pregnancy.

## 1. Introduction

Immunological abnormalities have been implicated in female reproductive failure, but whether these represent a cause or effect is unknown [1, 2]. According to our previous research, cellular and, particularly, humoral autoimmunologic perturbations are responsible for development of female infertility. Disturbances in the humoral immune system may lead to impairments in ovarian folliculogenesis [3–5], a long and complex process in which both the endocrine and immune systems play significant roles.

Cytokines, originally identified as products of immune cells, are important mediators of immune responses. These proteins are able to stimulate or inhibit cell growth, regulate cell differentiation, induce cell chemotaxis, and modulate the expression of other cytokines. However, recent research has indicated that cytokines are synthesized by a broad range of nonimmune cell types, including the normal ovarian cells. Cytokine function in the ovary has been described as promoting processes of follicular growth, steroidogenesis, recruitment and activation of leukocytes necessary for ovulation and tissue remodelling during ovulation, luteinization, and luteolysis [6].

To gain a more detailed understanding of the cytokines involved in female fertility and their role in pregnancy outcome, we assessed 16 different follicular cytokines during infertility treatment. In particular, we evaluated the cytokines for Th1/proinflammation (interleukin- (IL-) 1$\beta$, IL-6, IL-12, IL-18, IL-23, interferon (IFN)-$\gamma$, IFN-$\alpha$, and tumor necrosis factor-(TNF-) $\alpha$) and anti-inflammation (granulocyte colony stimulating factor (G-CSF)), the principal chemokines (macrophage inflammatory protein- (MIP-) 1$\alpha$, MIP-1$\beta$, monocyte chemotactic protein- (MCP-) 1, regulated on activation, normal T expressed and secreted

(RANTES) and IL-8), and other biomarkers (soluble apoptosis antigen (sAPO)-1/Fas and CD44 variant isoform CD44(v6)) secreted into the follicular fluid. The cytokines chosen for evaluation were shown in our previous study to be appreciably expressed in follicular granulosa cells at the mRNA level [7]; moreover, the importance of these particular cytokines in ovarian function has been proposed by others [8].

IL-1$\beta$, IL-6, and IL-18 are key mediators of inflammation and mediate many pathways of the normal immune response [9–11]. Human IFN-$\alpha$ comprises a family of extracellular signalling proteins with demonstrated antiviral, antiproliferating, and immunomodulatory activities [12]. The type II interferon IFN-$\gamma$ is another proinflammatory cytokine and has been implicated in the development of a variety of autoimmune diseases [13]. IL-12 regulates cell-mediated immune responses. The p40 subunit of IL-12 is shared with IL-23 and is essential for recruitment and activation of many inflammatory cell types. Both of these cytokines interact with the innate and adaptive immune systems [14]. TNF-$\alpha$, an acute phase protein, is critically involved in innate immune responses caused by pathogen exposure [15] but can also mediate noninfectious inflammatory processes such as autoimmunity and cancer [16].

Among the chemokines examined in this study, IL-8 is a neutrophil-specific factor involved in inflammatory processes and angiogenesis [17]. MIP-1$\alpha$ and MIP-1$\beta$ are known as CC chemokines, and both act as chemoattractants for T cells and monocytes to mediate beneficial inflammatory processes, such as wound healing [18]. Meanwhile, MCP-1 and RANTES are potent chemoattractants of monocytes and T lymphocytes [19].

The other biomarkers examined in this study are established immunomodulators. G-CSF acts as a growth factor for haematopoietic cells [20]. APO-1 regulates tissue homeostasis by acting as the receptor for Fas ligand, the binding of which triggers a signaling cascade that leads to apoptosis inhibition [21]. And CD44(v6), a splice variant of the CD44, is a transmembrane glycoprotein associated with cell adhesion and has mostly been investigated in tumours [22].

In recent decades, above-mentioned cytokines have become the subject of studies examining normal mammalian reproduction [23], which have indicated a significant role for these factors in supporting female fertility. Thus, we carried out a simultaneous (multiplex) examination of these cytokines and biomarkers in follicular fluid of infertile women in order to assess their effects on oocyte and embryo quality and on pregnancy outcome of *in vitro* fertilization (IVF) treatment. The approach of cytokine profiling using multiplex assays offers a promising tool for identifying condition-specific biomarker panels with high accuracy [24]. We employed the Bender MedSystems FlowCytomix platform, which uses antibody-coated autofluorescent beads to simultaneously measure corresponding analytes from small sample volumes and low concentrations [24], facilitating time- and cost-efficient high-throughput screening. In addition, we sought to determine the origin of the secreted cytokines by performing mRNA analysis from two distinct follicular somatic cell populations: mural and cumulus granulosa cells (MGC and CGC, resp.).

## 2. Materials and Methods

*2.1. Patients.* The Ethics Committee on Human Research of the University of Tartu approved this study, and informed consent was obtained from all patients. The study group consisted of 153 women, aged 33.3 ± 4.5 years (mean ± standard deviation), who underwent IVF at Nova Vita Clinic between 2007 and 2010. IVF with intracytoplasmic sperm injection (ICSI) was performed in all women in this cohort, and the case was either male factor infertility or previous oocyte fertilization failure. The causes of infertility were distributed as follows: male factor infertility (43.8%, $n = 67$), tubal factor infertility (TFI; 28.8%, $n = 44$), polycystic ovary syndrome (PCOS; 5.2%, $n = 8$), endometriosis (15.0%, $n = 23$), unexplained infertility (4.6%, $n = 7$), and other reasons (2.6%, $n = 4$).

Ovarian hormonal stimulation was conducted according to a protocol of gonadotrophin-releasing hormone (GnRH) antagonist (Cetrotide; Merck Serono, Geneva, Switzerland) administered with recombinant follicle-stimulating hormone (Gonal-F; Merck Serono or Puregon, Schering-Plough, Kenilworth, NJ, USA). ICSI was performed at 4–6 h after oocyte pickup (OPU), resulting in a 68.9% fertilization rate. ICSI-derived embryos were cultured up to 48 h, after which good-quality embryos were identified by the presence of at least four blastomeres and ≤20% fragmentation. The rate of good-quality embryos was calculated as the proportion (%) of good-quality embryos out of all fertilized oocytes. Two embryos were chosen for transfer to the uterus, and 25.5% of clinical pregnancies resulted per embryo transfer. Clinical confirmation of intrauterine pregnancy was made using an ultrasound scan performed at the 6th or 7th week after transference. Follicular fluid samples from each individual were taken from a single follicle on the day of OPU and stored at −80°C until further use.

*2.2. Flow Cytometry Analysis.* Altogether, 16 biomarkers (divided into two 8 plexes) were evaluated from the individual follicular fluid samples by using a commercially available FlowCytomix Human Basic Kit Assay (Bender MedSystems, Vienna, Austria) and following the manufacturer's instructions. Quantitation measurements were performed by flow cytometer instrument FC 500 and accompanying CXP Software (Beckman Coulter, Calif, USA). The first 8 plex consisted of: IL-23, sAPO-1/Fas, MIP-1$\beta$, MIP-1$\alpha$, CD44(v6), IL-8, G-CSF, and RANTES. The second 8-plex consisted of IL-12p70, IFN-$\gamma$, MCP-1, IL-6, IFN-$\alpha$, IL-18, Il-1$\beta$, and TNF-$\alpha$. Samples were prepared for processing by first thawing follicular fluids and centrifuging the whole volume at 450 g for 10 min. The resulting supernatants were used for analysis. FlowCytomix Pro 2.3 Software was used to perform calculations (Bender MedSystems). Standard curves for each biomarker were generated with manufacturer-supplied reference analyte (pg/mL concentrations). The concentration of a biomarker was calculated as

Table 1: List of primers used for real-time PCR analysis.

| Gene | Forward primer | Reverse primer | NCBI reference |
|---|---|---|---|
| G-CSF [25] | GCTTGAGCCAACTCCATAGC | CAGATGGTGGTGGCAAAGTC | NM_001178147.1, NM_172220.2, NM_172219.2, NM_000759.3 |
| IL-23A [26] | TGTTCCCCATATCCAGTG | TCCTTTGCAAGCAGAACTGA | NM_016584.2 |
| IFN-$\gamma$ | TGATGGCTGAACTGTCGCCAGC | CTGGGATGCTCTTCGACCTCGA | NM_000619.2 |
| MIP-1$\alpha$ | TCAGAAGGACACGGGCAGCAGA | TCAGCAGCAAGTGATGCAGAGAAC | NM_002983.2 |
| sAPO-1/Fas | CCAAGTGCAAAGAGGAAGTGAAGAG | TGGTTTTCCTTTCTGTGCTTTCTGC | NM_152871.2 |
| CD44(v6) | GCTACCACAGCCTCAGCTCA | ACCTCGTCCCATGGGGTGTGA | NA* |

*The forward primer was designed to cross the junction between exons 5 and 11, characteristic for only CD44(v6)-soluble splice isoform not described in NCBI database.

mean fluorescent intensity divided by single median standard curve.

*2.3. Gene Expression Analysis.* Gene expression studies of the measured cytokines were performed by real-time PCR of mRNA isolated from MGC and CGC from six patients. MGC were obtained from follicular fluid after OPU, and CGC were collected 4 h after OPU during oocyte denudation with bovine type IV-S hyaluronidase (Sigma-Aldrich, St-Louis, Mo, USA). The detailed isolation protocol has been previously published [7]. For leukocyte elimination, the MGC pool was incubated with CD45-coated magnetic beads (Dynabeads; Invitrogen, Oslo, Norway) for an additional 1 h at 4°C, followed by magnet-based cell sorting (DynaMag-15; Invitrogen) according to the manufacturer's protocol. Total RNA was extracted, and real-time PCR analysis was performed using either commercially available real-time PCR arrays (products PAHS-011A and PAHS-021A; SABiosciences, Frederick, Md, USA) or in-house designed and synthesized primers when the desired transcripts were not included in the kits or the quality of amplification, and melting curves were not satisfactory. Primers for Fas were designed to exclusively detect the soluble isoform, and those for CD44 were designed to amplify only exon 11 (the variable region 6). All primer sequences used in this study are listed in Table 1.

For double-stranded cDNA synthesis, 1 $\mu$g of high-quality total RNA was treated with DNase (Fermentas, Burlington, ON, Canada) and reverse transcribed to cDNA using the RT$^2$ First Strand Kit (SABiosciences) according to the manufacturer's protocols. RT$^2$ SYBR Green/ROX qPCR Master Mix (SABiosciences) and cDNA template were added to the array and product amplification was performed on a 7500 real time PCR System (Applied Biosystems, Foster City, Calif, USA). Those real-time PCR reactions using in-house primers were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7900HT real-time PCR instrument (Applied Biosystems).

Results were analyzed with instrument-specific software using the $\Delta\Delta$Ct relative quantification method. Three housekeeping genes were used for normalization of the amplification data: beta actin, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein RPL13A.

*2.4. Statistical Analysis.* The R2.3.1 A Language and Environment (Free Software Foundation, Boston, Mass, USA) was used to perform *t*-, Mann-Whitney *U*- and proportion tests and adjusted simple regression analysis. A $P$ value <0.05 was considered as indicative of statistical significance.

## 3. Results

Table 2 summarizes the clinical data and infertility treatment parameters, while Table 3 lists detected concentrations of the tested biomarkers. Figure 1 summarizes the main associations observed between the clinical data and the levels of follicular biomarkers, according to analysis by adjusted regression models.

*3.1. Associations between Infertility Cause and Levels of Biomarkers in Follicular Fluid.* Patients characterized by male factor infertility represented the reference group in simple regression analysis, unless otherwise stated. Our results indicated that women with TFI had lower concentrations of IL-1$\beta$-adjusted $r = -12.6$ pg/mL, $P = 0.037$), and lower IFN-$\alpha$ levels were also significantly associated with TFI when the status of current smoking was included in the model (adjusted $r = -13.9$ pg/mL, $P = 0.046$).

PCOS patients were characterized by significantly higher follicular levels of CD44(v6) (age-adjusted $r = 2072.7$ pg/mL, $P = 0.010$) and MIP-1$\alpha$ (adjusted for age, cause of infertility, and follicular count in prestimulatory ovary $r = 3111.7$ pg/mL, $P = 0.007$). Women with endometriosis presented with higher levels of IL-23 than male factor infertility patients (adjusted by follicular number prior to stimulation $r = 157.1$ pg/mL, $P = 0.025$). Moreover, when compared to TFI patients, women with endometriosis had higher levels of follicular TNF-$\alpha$ (age-adjusted $r = 2.6$ pg/mL, $P = 0.047$) and IFN-$\gamma$ (adjusted by follicular number prior to stimulation $r = 16.4$ pg/mL, $P = 0.030$). In women with unexplained infertility, significantly lower levels of follicular CD44(v6) were measured, as compared to male factor infertility patients (age-adjusted $r = -1888.4$ pg/mL, $P = 0.025$). However, when compared to TFI patients, the unexplained infertility patients also had lower levels of IL-18 (age-adjusted $r = -186.7$ pg/mL, $P = 0.021$).

Table 2: Clinical and IVF treatment parameters of patient groups.

| | Male factor infertility ($N = 67$)[a] | Tubal factor infertility ($N = 44$) | Polycystic Ovary Syndrome ($N = 8$) | Endometriosis ($N = 23$) | Unexplained infertility ($N = 7$) | Other reasons ($N = 4$) | Total ($N = 153$) |
|---|---|---|---|---|---|---|---|
| Health parameters | | | | | | | |
| Age (years[†]) | 32.6 ± 4.3 | 34.7 ± 5.0[b] | 34.8 ± 3.2 | 31.8 ± 3.9 | 32.7 ± 2.9 | 36.0 ± 5.9 | 33.3 ± 4.5 |
| Infertility[‡] | | | | | | | |
| Primary | 42 (62.7%, 50.0–73.9) | 15 (34.1%, 20.9–50.0)[c] | 6 (75.0%, 35.6–95.5) | 18 (78.3%, 55.8–91.7) | 6 (85.7%, 42.0–99.2)[c] | 2 (50.0%, 15.0–85.0) | 89 (58.2%, 49.9–66.0) |
| Secondary | 25 (37.3%, 26.1–50.0) | 29 (65.9%, 50.0–79.1)[c] | 2 (25.0%, 4.5–64.4) | 5 (21.7%, 8.3–44.2) | 1 (14.3%, 0.8–58.0) | 2 (50.0%, 15.0–85.0) | 64 (41.9%, 34.0–50.0) |
| Parity (N[†]) | 0.3 ± 0.5 | 0.5 ± 0.7[b] | 0.3 ± 0.5 | 0.1 ± 0.3 | 0.1 ± 0.4 | 0.5 ± 0.6 | 0.3 ± 0.6 |
| S-FSH (U/L[†]) | 7.6 ± 2.3 | 8.2 ± 3.2 | 7.0 ± 1.9 | 7.5 ± 2.7 | 6.9 ± 0.3 | 6.5 ± 2.2 | 7.7 ± 2.6 |
| Smoking[‡] | | | | | | | |
| Never smoker | 52 (78.8%, 66.7–87.5) | 32 (76.2%, 60.2–87.4) | 6 (75.0%, 35.6–95.5) | 20 (87.0%, 65.3–96.6) | 5 (71.4%, 30.3–94.9) | 2 (66.7%, 12.5–98.2) | 117 (77.5%, 69.8–83.7) |
| Past smoker | 7 (10.6%, 4.7–21.2) | 4 (9.5%, 3.1–23.51) | 1 (12.5%, 0.7–53.3) | 1 (4.3%, 0.2–24.0)[c] | 1 (14.3%, 0.8–58.0) | 1 (33.3%, 1.8–87.5)[c] | 15 (9.9%, 5.9–16.1) |
| Current smoker | 7 (10.6%, 4.7–21.2) | 8 (19.0%, 9.1–34.6) | 1 (12.5%, 0.7–53.3) | 2 (8.7%, 1.5–29.5) | 1 (14.3%, 0.8–58.0) | 0 | 19 (12.6%, 7.9–19.2) |
| Treatment parameters[‡] | | | | | | | |
| OPU S-E2 (pmol/L) | 4497.1 ± 7950.9 | 2978.0 ± 1858.6 | 4195.5 ± 3117.8 | 3259.7 ± 1991.7 | 2960.0 ± 1723.2 | 2164.8 ± 1236.3 | 3711.8 ± 5429.4 |
| OPU S-progesterone (nmol/L) | 36.0 ± 22.5 | 26.1 ± 16.7[b] | 34.2 ± 16.2 | 35.9 ± 17.0 | 40.3 ± 19.2 | 33.5 ± 26.9 | 33.3 ± 19.9 |
| Total dose of FSH (IU) | 1992.0 ± 704.0 | 2297.1 ± 925.0 | 1743.8 ± 525.6 | 1919.6 ± 726.3 | 2142.9 ± 574.2 | 2475.0 ± 656.7 | 2073.9 ± 702.8 |
| Follicular diameter (mm) | 21.4 ± 2.9 | 21.5 ± 3.3 | 21.7 ± 2.0[b] | 19.9 ± 2.0 | 20.3 ± 1.6 | 19.3 ± 1.1[b] | 21.1 ± 2.9 |
| Oocytes (N) | 11.6 ± 7.1 | 9.1 ± 5.5[b] | 13.6 ± 9.3 | 11.1 ± 6.6 | 9.4 ± 8.3 | 11.3 ± 7.4 | 10.9 ± 6.8 |
| Mature oocytes (N)[d] | 9.2 ± 6.0 | 6.9 ± 4.5[b] | 9.0 ± 7.8 | 9.6 ± 5.3 | 7.7 ± 6.8 | 8.5 ± 6.1 | 8.4 ± 5.7 |
| 2 PN-stage oocytes (N) | 6.6 ± 5.0 | 4.6 ± 3.0[b] | 6.0 ± 6.2 | 6.3 ± 4.0 | 4.7 ± 3.7 | 3.8 ± 4.1 | 5.8 ± 4.3 |
| Good-quality embryos (N)[e] | 3.8 ± 3.6 | 2.9 ± 2.3 | 2.9 ± 2.9 | 3.6 ± 3.1 | 2.4 ± 2.6 | 1.0 ± 2.0 | 3.3 ± 3.1 |
| Rate of quality embryos (%)[f] | 57.3 ± 30.0 | 56.4 ± 32.3 | 46.3 ± 38.4 | 52.9 ± 32.7 | 51.7 ± 33.6 | 14.8 ± 25.6 | 54.8 ± 31.6 |
| Transferred embryos (N) | 1.8 ± 0.5 | 1.8 ± 0.7 | 1.4 ± 0.7 | 1.9 ± 0.5 | 1.6 ± 0.8 | 1.3 ± 1.0 | 1.8 ± 0.6 |
| Status of pregnancy[‡] | | | | | | | |
| hCG negative | 44 (65.7%, 53.0–76.6) | 29 (65.9%, 50.0–79.1) | 6 (75.0%, 35.6–95.6) | 16 (69.6%, 47.0–85.9) | 5 (71.4%, 30.3–94.9) | 3 (75.0%, 21.9–98.7) | 103 (67.3%, 59.2–74.5) |
| Intrauterine | 17 (25.4%, 15.9–37.7) | 11 (25.0%, 13.7–40.6) | 2 (25.0%, 4.5–64.4) | 5 (21.7%, 8.3–44.2) | 2 (28.6%, 5.1–69.7) | 1 (25.0%, 1.3–78.1) | 38 (24.8%, 18.4–32.6) |
| Biochemical | 5 (7.5%, 2.8–17.3) | 4 (9.1%, 3.0–22.6) | 0 | 2 (8.7%, 1.5–29.5) | 0 | 0 | 11 (7.2%, 3.8–12.8) |
| No ultrasound performed | 1 (1.5%, 0.1–9.1) | 0 | 0 | 0 | 0 | 0 | 1 (0.7%, 0.0–4.1) |
| Fetuses (N[†]) | 0.3 ± 0.6 | 0.4 ± 0.7 | 0.3 ± 0.5 | 0.3 ± 0.6 | 0.3 ± 0.5 | 0.3 ± 0.5 | 0.3 ± 0.6 |

[†] Continuous variables are provided as mean ± standard deviation. [‡] Categorical variables are provided as absolute numbers (percentage, 95% confidence interval of percentage). Differences between study groups: [a]Reference group; [b]$t$-test, $P < 0.05$; [c]Proportion test, $P < 0.05$. Associations between different parameters assessed by adjusted regression models are provided in the text. [d]Number of oocytes which reached meiosis II stage at 4–6 h after oocyte retrieval. [e]Number of embryos with at least four blastomeres and <20% fragmentation on the second day after-ICSI. [f]The proportion (%) of good-quality embryos obtained from all 2PN fertilized oocytes. Abbreviations: hCG: human chorionic gonadotrophin; ICSI: intracytoplasmic sperm injection; OPU: oocyte pick-up day; PN: pronucleus; S-E2: serum estradiol; S-FSH: serum follicle-stimulating hormone.

Table 3: Biomarkers in the follicular fluid of patient groups.

| | Male factor infertility ($N = 67$)[a] | Tubal factor infertility ($N = 44$) | Polycystic ovary syndrome ($N = 8$) | Endometriosis ($N = 23$) | Unexplained infertility ($N = 7$) | Other reasons ($N = 4$) | Total ($N = 153$) |
|---|---|---|---|---|---|---|---|
| Biomarkers (pg/mL)[†] | | | | | | | |
| G-CSF | 82.5 (0–2464.0) | 48.1 (0–4986.0) | 104.1 (0–3156.0) | 122.2 (0–4809.0) | 118.5 (0–463.4) | 23.7 (0–341.6) | 89.7 (0–4986.0) |
| IL-1$\beta$ | 0 (0–236.8) | 0 (0–53.6) | 0 (0–29.0) | 0 (0–110.3) | 0 (0-0) | 0 (0–143.1) | 0 (0–236.8) |
| IL-6 | 0 (0–18.7) | 0 (0–10.7) | 0 (0–16.2) | 0 (0–37.2) | 0 (0-0) | 0 (0–8.4) | 0 (0–37.2) |
| IL-12p70 | 0 (0–24.9) | 0 (0–6.1) | 0 (0–8.1) | 0 (0–21.0) | 0 (0-0)[b] | 0 (0–8.1) | 0 (0–24.9) |
| IL-18 | 311.0 (0–722.0) | 290.2 (0–812.5) | 463.4 (0–648.5) | 283.3 (44.6–874.3) | 199.1 (0–255.5)[b] | 310.9 (110.8–767.0) | 297.2 (0–874.3) |
| IL-23 | 282.3 (0–1069.0) | 208.7 (0–1280.0) | 237.4 (0–746.4) | 388.8 (0–1160.0) | 408.5 (0–557.8) | 120.3 (0–260.3) | 260.3 (0–1280.0) |
| IFN-$\alpha$ | 0 (0–150.7) | 0 (0–107.6) | 0 (0–93.5) | 0 (0–114.2) | 0 (0-0) | 0 (0–161.9) | 0 (0–161.9) |
| IFN-$\gamma$ | 0 (0–111.2) | 0 (0–74.5) | 0 (0–60.4) | 0 (0–111.2) | 0 (0-0) | 9.5 (0–147.5) | 9.5 (0–147.5) |
| TNF-$\alpha$ | 0 (0–30.7) | 0 (0–10.8) | 0 (0–5.3) | 0 (0–21.0) | 0 (0-0) | 0 (0–58.8) | 0 (0–58.8) |
| IL-8 | 307.3 (119.4–4857.0) | 367.2 (117.4–1117.0)[b] | 417.6 (236.9–1032.0) | 473.6 (172.8– 1879.0)[b] | 424.3 (343.8–1472.0) | 416.2 (172.8–2851.0) | 371.2 (117.4–4857.0) |
| MCP-1 | 1019.0 (594.2–2046.0) | 1054.0 (416.1–2564.0) | 1067.0 (656.4–1572.0) | 1033.0 (373.8–2780.0) | 992.9 (818.1–1265.0) | 801.7 (198.4–1598.0)[b] | 1016.0 (198.4–2780.0) |
| MIP-1$\alpha$ | 143.6 (0–5766.0) | 80.6 (0–15990.0) | 555.8 (0–19840.0) | 227.6 (0–18230.0) | 52.3 (0–3383.0) | 136.3 (0–1788.0) | 130.8 (0–19840.0) |
| MIP-1$\beta$ | 52.3 (6.0–1254.0) | 48.2 (11.5–433.2) | 38.7 (17.59–96.4) | 51.7 (17.1–120.9) | 40.7 (36.8–64.5) | 63.5 (25.7–967.0) | 48.4 (6.0–1254.0) |
| RANTES | 97.4 (0–705.1) | 97.4 (0–908.3) | 50.1 (0–189.3) | 146.6 (0–438.8) | 77.4 (12.2–182.7) | 74.1 (2.6–1428.0) | 97.4 (0–1428.0) |
| sAPO-1/Fas | 129.0 (0–564.2) | 169.4 (0–9589.0) | 152.1 (0–4469.0) | 94.9 (0–520.4) | 96.8 (64.3–292.5) | 98.8 (0–226.8) | 129.0 (0–9589.0) |
| CD44(var6) | 8426.0 (5063.0–14030.0) | 8554.0 (5348.0–20610.0) | 10750.0 (6394.0–12130.0)[b] | 8219.0 (5535.0–11770.0) | 6619.0 (5168.0–9348.0)[b] | 6836.0 (5611.0–10200.0) | 8219.0 (5063.0–20610.0) |

[†] Concentrations are provided as medians (minimum – maximum value). Differences between study groups: [a]Reference group; [b]Mann-Whitney $U$-test, $P < 0.05$. Associations between different parameters assessed by adjusted regression models are provided in the text.

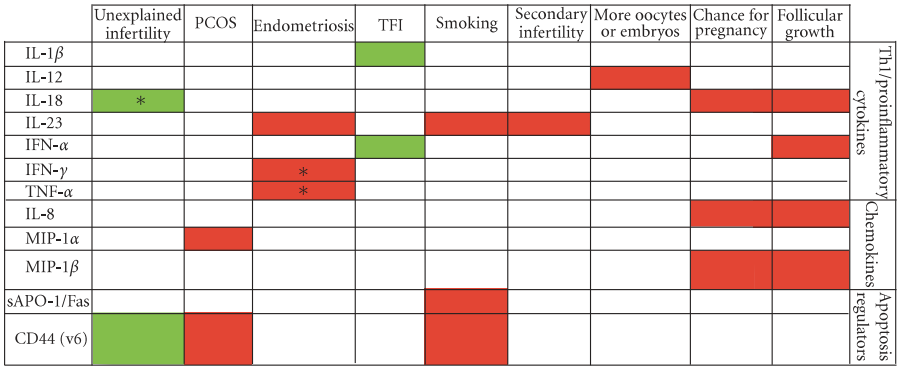


Figure 1: Associations between biomarkers and infertility parameters. Red boxes indicate positive association, green boxes negative association; empty boxes indicate no association found by adjusted regression analysis. Male factor infertility was chosen as a reference group, but in cases marked with $*$. TFI was used as a reference. Abbreviations are as mentioned in the text.

Active smoking was associated with elevated follicular CD44(v6) levels (adjusted for age and cause of infertility $r = 1227.8$ pg/mL, $P = 0.019$ versus never smokers group) and sAPO-1/Fas levels (adjusted $r = 464.9$ pg/mL, $P = 0.031$ versus never-smokers group). Similarly, follicular IL-23 levels were higher in women who reported history of smoking or current smoking, as compared to never-smokers, regardless of age or cause of infertility (adjusted $r = 107.6$ pg/mL, $P = 0.043$). In addition, an elevated IL-23 concentration was associated with women experiencing secondary infertility rather than primary infertility (regardless of the cause of infertility; adjusted $r = 94.6$ pg/mL, $P = 0.043$).

*3.2. Associations between Infertility Treatment Parameters and Biomarker Levels in Follicular Fluid.* A positive association was determined to exist between the concentration of follicular IL-12 and the number of fertilized oocytes (adjusted $r = 0.15$ pg/mL per every additional 2PN oocyte, $P = 0.007$) and the proportion of good-quality embryos (adjusted $r = 0.22$ pg/mL per every additional embryo, $P = 0.006$), when the data were adjusted for age, cause of infertility, and follicular size. Achieving intrauterine pregnancy was associated with higher levels of follicular MIP-1$\beta$, as compared to hCG-negative patients (adjusted for age and cause of infertility $r = 48.0$ pg/mL, $P = 0.047$). In addition, follicular MIP-1$\beta$ and IFN-$\alpha$ levels were both positively associated with the diameter of a follicle (adjusted $r = 7.8$ pg/mL, $P = 0.037$ and $r = 2.4$ pg/mL for every millimeter in diameter, $P = 0.023$, resp.), regardless of age or cause of infertility.

The concentration of IL-8 in follicular fluid was positively associated with intrauterine pregnancy (adjusted for age, cause of infertility, rate of good-quality embryos transferred, and endometrial thickness $r = 207.5$ pg/mL, $P = 0.051$), and also with parity (adjusted for age and cause of infertility $r = 150.6$ pg/mL for every child born, $P = 0.039$). Not surprisingly, IL-8 was also associated with higher levels of serum progesterone after ovarian stimulation (adjusted $r = 4.7$ pg/mL, $P = 0.031$).

Follicular IL-18 levels appeared to be positively correlated with several outcomes, including increased chance for intrauterine pregnancy (adjusted for the cause of infertility $r = 71.6$ pg/mL, as compared to hCG-negative patients, $P = 0.054$), number of fetuses detected by ultrasonography (adjusted for age, cause of infertility, number of embryos transferred, rate of good-quality embryos among them, and endometrial thickness $r = 67.2$ pg/mL for every additional fetus, $P = 0.020$), and with increased parity (adjusted for age and cause of infertility $r = 60.7$ pg/mL for every child to give birth, $P = 0.038$). Interestingly, the levels of both follicular IL-8 and IL-18 increased as follicles grew (adjusted for age and cause of infertility $r = 40.2$ pg/mL, $P = 0.005$, and $r = 1\ 3.1$ pg/mL for every additional millimeter in diameter, $P = 0.022$, resp.).

*3.3. mRNA Analysis of the Measured Protein Transcripts from MGC and CGC.* Our mRNA expression analysis demonstrated that most of the studied transcripts were more abundantly expressed in MGC (Figure 2). G-CSF and sAPO-1/Fas were not differentially expressed in the two cell types. Both of the interferons examined were found to be more highly expressed in CGC, although this result was not statistically significant (Table 4). When the abundance of intracellular transcripts was analyzed, the mRNA levels were found to differ by several orders of magnitude and were characterized by substantial interpatient variability (Table 4).

## 4. Discussion

In the current study, we evaluated the expression of 16 different biomarkers in the follicular fluid of infertile women by using multiplex assay from Bender MedSystems. These biomarkers included Th1/proinflammatory and anti-inflammatory cytokines, chemokines and antiapoptotic biomarkers that had previously been implicated in ovarian function by our previous study [7]. Ultimately, we found

Table 4: Relative* mRNA abundance of measured proteins in cumulus and mural granulosa cells.

| Biomarkers | CGC ± SD | MGC ± SD | *P* value (paired *t*-test) |
|---|---|---|---|
| G-CSF | 0.000216 ± 0.000151 | 0.000207 ± 0.000109 | 0.930 |
| IL-1$\beta$ | 0.001025 ± 0.000640 | 0.070199 ± 0.108075 | 0.178 |
| IL-6 | 0.000082 ± 0.000059 | 0.003981 ± 0.006412 | 0.209 |
| IL-12A | 0.000104 ± 0.000047 | 0.000209 ± 0.000083 | 0.022 |
| IL-18 | 0.001506 ± 0.000958 | 0.007472 ± 0.002346 | < 0.001 |
| IL-23A | 0.000009 ± 0.000004 | 0.000025 ± 0.000014 | 0.016 |
| IFN-$\alpha$ | 0.000236 ± 0.000186 | 0.000044 ± 0.000021 | 0.127 |
| IFN-$\gamma$ | 0.000064 ± 0.000045 | 0.000027 ± 0.000021 | 0.157 |
| TNF-$\alpha$ | 0.000133 ± 0.000150 | 0.001899 ± 0.002363 | 0.117 |
| IL-8 | 0.022214 ± 0.009590 | 0.487650 ± 0.431439 | 0.045 |
| MCP-1 | 0.003409 ± 0.004521 | 0.008618 ± 0.009842 | 0.239 |
| MIP-1$\alpha$ | 0.000015 ± 0.000011 | 0.000451 ± 0.000718 | 0.191 |
| MIP-1$\beta$ | 0.001029 ± 0.000864 | 0.055073 ± 0.071566 | 0.122 |
| RANTES | 0.000650 ± 0.000282 | 0.014258 ± 0.021169 | 0.293 |
| sAPO-1/FAS | 0.000024 ± 0.000014 | 0.000024 ± 0.000016 | 0.979 |
| CD44(v6) | 0.000024 ± 0.000006 | 0.000045 ± 0.000028 | 0.158 |

* As compared to the average of three housekeeping gene transcripts: beta actin, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein RPL13A.
Abbreviations: CGC: cumulus granulosa cells; MGC: mural granulosa cells; SD: standard deviation.

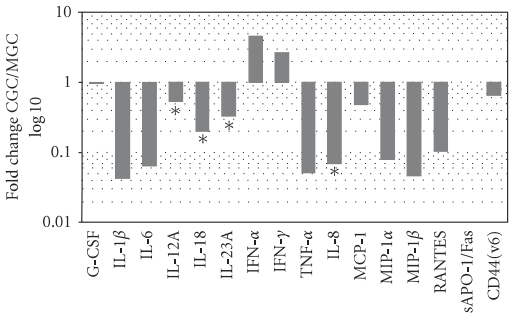


Figure 2: Differential expression of measured protein transcripts in cumulus and mural granulosa cells. ∗ Differences in expression were statistically significant. CGC: cumulus granulosa cells, MGC: mural granulosa cells.

that 12 of the 16 examined biomarkers were associated with a cause of infertility or IVF treatment outcome.

The mammalian ovulation event can be considered from the perspective of an inflammatory reaction, with proinflammatory cytokines produced and functionally interacting throughout the process [10]. For example, IL-1$\beta$ has been evidenced to participate in ovulation induction by facilitating follicular rupture [27]. The fact that we found lower values of IL-1$\beta$ in TFI patients indicates that impairment of folliculogenesis might have occurred and contributed to the infertility of these women. IL-18 is known to induce cytokines that are important for both folliculogenesis and ovulation, including IL-1$\beta$, TNF-$\alpha$, and IFN-$\gamma$ [28]. Our finding that levels of IL-18 were relatively low in unexplained infertility patients might then reflect an underlying perturbed immunological profile for this infertility cause. Importantly, IL-18 has been suggested to favor ovarian folliculogenesis; a positive correlation has been reported between the level of follicular IL-18 and the number of retrieved oocytes and implantation success in women with different etiologies of infertility [29, 30]. Our finding that follicular growth positively correlates with IL-18 levels indirectly supports the role of IL-18 in follicle maturation. Furthermore, our finding that higher follicular IL-18 was associated positively with parity indicates that this cytokine may increase the chance for pregnancy.

The elevated levels of follicular IFN-$\gamma$ found in our group of endometriosis patients is in agreement with previous results obtained with serum samples [27]. Increased production of IFN-$\gamma$ may reflect the immune system's efforts to overcome apoptosis inhibition and to decrease cell proliferation in the case of endometriosis [31]. Additionally, while a temporary increase in the concentration of IFN-$\gamma$ seems to be essential for ovulation, IFN-$\gamma$ levels that exceed normal physiologic concentrations may inhibit ovulation and contribute to early pregnancy loss [28]. IFN-$\alpha$ is synthesized primarily in response to infection, but the IFN-$\alpha$ signaling pathways have also been demonstrated to be involved in reproduction processes, even in the absence of detectable infection [12]. The fact that the group of healthy women (with male factor infertility) in our study possessed higher levels of IFN-$\alpha$ than did women with TFI further supports a positive role for IFN-$\alpha$ in reproduction. In addition, the positive correlation that was identified between follicular IFN-$\alpha$ levels and follicular diameter was in accordance with previous IFN-$\alpha$ data from preovulatory granulosa cells [32].

We observed elevated levels of TNF-$\alpha$ in endometriosis patients, as compared to TFI patients. It is possible that this finding simply reflects increased TNF-$\alpha$ serum levels

that had infiltrated into the follicular fluid [33] or increased secretion by granulosa cells induced by the inflammatory pelvic milieu in endometriosis [34]. TFI patients' expression of follicular TNF-$\alpha$ has also been previously suggested to be below the threshold of standard detection systems [6]. TNF-$\alpha$ in IVF has already been the subject of much study by infertility researchers. Some authors have concluded that follicular TNF-$\alpha$ might deteriorate the microenvironment in the follicle, thereby negatively affecting oocyte and embryo quality [35]. Still others have proposed a positive role of TNF-$\alpha$ regarding oocyte quality, and ovulation [36]. Overall, the roles of TNF-$\alpha$ in female reproduction are likely to be complex and dynamically involved in the different stages of folliculogenesis [37].

Previous studies examining IL-12 in the follicular fluid have yielded contradictory results. Nevertheless, a majority of the findings have indicated that IL-12 is associated with a negative effect on folliculogenesis, oocyte quality and implantation [9, 20, 38]. We failed to detect any correlation between the follicular level of IL-12 and the pregnancy outcome of IVF. Nonetheless, there was a positive association identified between IL-12 and the quality of oocytes and embryos. Our results are similar to a study published by Ostanin et al. [39], wherein the authors reported that follicular concentration of IL-12 was elevated in women who produced more high-quality oocytes. IL-12 is a Th1 cytokine that can become cytotoxic at high levels. It is, therefore, not unexpected that high concentrations of IL-12 in the follicular fluid might impair the natural process of folliculogenesis and ovulation [38]. However, in the current study, the mean concentration of IL-12 was found to be more than 10-fold lower than that reported in studies that had concluded deleterious function of IL-12 on reproduction [9, 20, 38]. Thus, we suggest a dose-dependent role for IL-12 in the follicles.

We also determined that endometriosis was associated with increased levels of follicular IL-23. Given that IL-23 is known to participate in autoimmune diseases by promoting inflammation, a hallmark of endometriosis, this result was not surprising. Impaired follicular fluid microenvironment characterized by elevated inflammatory cytokines may in fact be the cause for poor oocyte quality, which in turn could lead to poor IVF outcome in patients with endometriosis [19]. The detrimental effect of IL-23 on fecundity is further supported by our findings of higher levels of IL-23 in women who smoked or who suffered from secondary infertility.

MIP-1$\alpha$ is a marker for ongoing acute or chronic inflammatory host responses [18, 40]. Dahm-Kähler et al. [41] failed to detect MIP-1$\alpha$ in follicular fluids of unstimulated menstrual cycles, leading to their conclusion that MIP-1$\alpha$ is not produced under physiological conditions. Our contradictory findings of elevated levels of MIP-1$\alpha$ in PCOS patients may reflect a character of increased inflammation in stressed ovaries. To date, very few studies have appeared in the literature that investigate the function of MIP-1$\beta$ in female reproduction, although this chemokine has been suggested to promote folliculogenesis and pregnancy establishment [39]. Such a positive role was also supported by our finding that higher follicular fluid MIP-1$\beta$ levels correlated with follicular growth and achieving pregnancy.

The correlation of IL-8 concentration with follicular growth is in accordance with previously reported results. When taking into consideration that IL-8 has also been detected in unstimulated cycles [38], the involvement of this chemokine in the natural process of folliculogenesis and ovulation can be assumed [42]. Moreover, a recent study showed that lower serum levels of IL-8 correlated with a higher risk for extrauterine pregnancy [43]. Thus, our finding of higher follicular fluid IL-8 in cases of normal intrauterine pregnancy seems sensible. Nonetheless, two previous studies demonstrated no correlations between follicular fluid IL-8 concentration and IVF cycle parameters or pregnancy results [38, 42]. The discrepant results obtained from these studies and our own could be due to differences in sample sizes, patient groups examined, or detection methods used; this issue needs further investigation.

sAPO-1/Fas mediates apoptosis inhibition, which is important in preventing oocytes from succumbing to atresia during follicular maturation [21]. Increased sAPO-1/Fas levels have also been associated with enhanced activity of smoking-induced antiapoptotic signaling pathways in the oral cavity, which leads to epithelial hyperplasia [44]. In our study, we detected higher levels of sAPO-1/Fas in active smokers. Thus, our findings suggest that a compensatory increase of sAPO-1/Fas was established in the apoptosis-favoured environment of the follicles in active smokers. A similar effect has also been proposed for CD44(v6) in the ovary, where macrophage membrane-expressed CD44 protein has been shown to participates in clearance of apoptotic granulosa cells [44, 45]. Our findings of lower levels of CD44(v6) in unexplained infertility and higher levels in PCOS and active smokers might reflect impaired apoptosis mechanisms in the ovaries of these patients.

Considering that cytokines likely affect ovarian function, one could argue about the source of these immunomodulatory factors in follicular fluid. The ovulatory process is comparable to a classical local inflammatory reaction, and leukocytes have been shown to participate actively in the cyclic events of the ovary [6]. However, it is unlikely that migrating leukocytes producing proinflammatory cytokines represent the principal mechanism by which ovarian folliculogenesis is regulated [6]. Increased levels of serum-derived cytokines in follicular fluid have been demonstrated in endometriosis [33]. In addition, upregulated expression of proinflammatory cytokines by granulosa cells has been detected in cases of infertility [7]. Here, we confirmed our previous findings from the Affymetrix GeneChip platform using real-time PCR analysis to monitor mRNA expression in different conditions of infertility, as compared to levels expressed in conditions of normal fertility. To the best of our knowledge, our results represent the first description of the human granulosa cell expression profile of IL-12A, IL-23A, IL-18, MIP-1$\alpha$, MIP-1ß, IFN-$\alpha$, IFN-$\gamma$, and sAPO-1/FAS. MIP-1ß has been studied in the mouse cumulus-oocyte complex, where its expression increased in response to experimental exposure to hyaluronan fragments, and the related signal was determined to be mediated by Toll-like receptors [46]. On the other hand, luteinizing hormone induction of IFN-$\alpha$ was shown in rats and determined

to function as a modulator of steroidogenesis and MGC differentiation [47]. It is well known that cytokines and apoptosis networks functionally interact with one another in a variety of mammalian, and human, tissues. Therefore, our results also indicate a strong role of these proteins in human follicular physiology.

In conclusion, we discovered that various infertility etiologies are accompanied by distinct intrafollicular cytokine profiles. Furthermore, some of the cytokines evaluated, such as IL-12, were determined to influence oocyte fertilization and embryo quality, while others, such as IL-18, IL-8, and MIP-1$\beta$, were found to be correlated with successful pregnancy following IVF treatment. Collectively, these factors appear to be promising prognostic markers for IVF success and should be evaluated as such by future prospective studies.

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# ABSTRACT

Female gametogenesis is a long process that depends on hormonal influence from the pituitary gland and communication between the oocyte and various somatic cells in the ovary. During post-pubertal gametogenesis the meiotic and cytoplasmic maturation of the oocyte coincides with structural changes in the ovarian follicle, where oocyte development takes place. While the oocyte matures, the follicle expands considerably, becomes filled with follicular fluid (FF) and surrounded by blood capillaries. The one layer of granulosa cells in the early stages of follicle development starts to proliferate and differentiates into two distinct populations: the mural and cumulus granulosa cells (MGC and CGC, respectively) with distinct functional properties by the pre-ovulatory stage.

The knowledge of follicle and oocyte maturation have led to assisted reproductive technologies, including controlled ovarian stimulation and *in vitro* fertilization (IVF) that are helpful for many infertile couples. However, there is considerable room for development in these technologies, as only about 30-35% of IVF cycles culminate with a live birth. Therefore, information regarding the molecular processes involved in follicle development and oocyte maturation, as well as the extent of how these processes are influenced by the individual physiological properties of a woman is useful for treating more infertile patients.

The aim of the thesis was to give a thorough overview of differential gene expression between MGC and CGC by using several genome-wide methods. Beside gene expression at mRNA level, post-transcriptional gene regulation by alternative splicing, miRNA expression and mRNA targeting was studied to better understand the potential functions of these two granulosa cell population in human follicles just before ovulation. Secondly, 16 protein markers including cytokines and apoptosis-related proteins were studied in the FF of IVF patients with various etiologies of infertility and IVF outcome to reveal how the environment of oocyte maturation is modified by physiological background of a woman.

The thesis concludes that at mRNA level the gene expression profile between the two cell populations differs significantly, leading to various potential molecular functions that can be attributed to these cells. However, at the miRNA level the differential expression was not as significant and the miRNAs that were differentially expressed targeted the same molecular pathways to a large extent. Only in MGC, during the path of differentiation, the over-expressed miRNAs have switched to targeting several cell adhesion and metabolic pathways as compared to miRNAs in CGC. New miRNA molecules were identified in granulosa cells that are derived from the introns of two important genes in folliculogenesis: follicle stimulating hormone receptor and aromatase.

Fourteen of the proteins measured in the FF were found to be useful in discriminating the studied group of women according to the etiology of infertility, the outcome of ovarian stimulation or IVF procedure. Of potential further clinical use, IL-12 concentration was descriptive of embryo quality and MIP-1β, IL-8 and IL-18 correlated with the pregnancy outcome of the IVF cycle. In addition, several cytokines were found to be potentially involved in intercellular communication in the follicle according to our gene expression experiments and should be further studied in this regard.

# KOKKUVÕTE

Naise sugurakkude areng sõltub nii ajuripatsi poolt eritatud hormoonidest kui ka munaraku ja seda ümbritsevate somaatiliste rakkude omavahelistest interaktsioonidest. Puberteedijärgse sugurakkude arengu käigus toimuvad üheaegselt munaraku meiootiline ja tsütoplasmaatiline küpsemine ning struktuursed muutused munasarja folliikulis, kus toimub munaraku areng. Ajal, mil munarakk valmib, suurenevad märkimisväärselt folliikuli mõõtmed, see täitub follikulaarvedelikuga ning ümbritsetakse kapillaaride poolt. Folliikuli arengu varajastes etappides ühe kihina munarakku ümbritsevad granuloosrakud jagunevad intensiivselt ning moodustavad ovulatsioonieelseks staadiumiks kaks eraldi rakupopulatsiooni: muraalse ja kumuluse granuloosa, millel on erinevad rollid folliikuli arengu viimastes etappides ja ovulatsioonil.

Olemasolevad teadmised folliikuli ja munaraku arengust on viinud mitmete meditsiiniliste tehnoloogiate kasutuselevõtuni nagu munasarjade stimulatsioon ja kehaväline viljastamine (IVF), mida kasutatakse rutiinselt viljatusravis. Ent vaid 30-35% IVF protseduuridest on tänapäeval tulemuslikud, lõppedes elussünniga. Seetõttu on ravi efektiivsuse tõstmiseks äärmiselt oluline munaraku ja folliikuli arengu molekulaarsete protsesside ning naise individuaalse füsioloogia mõju parem mõistmine.

Käesoleva uuringu eesmärgiks oli anda põhjalik ülevaade muraalse ja kumuluse granuloosa geenikspressiooni mustritest, et mõista erinevate granuloosrakkude seni kirjeldamata rolle inimese munasarjas. Lisaks ülegenoomsetele geenikspressiooni uuringutele mRNA tasemel uuriti transkriptsioonijärgset geeniregulatsiooni alternatiivse splaissingu, mikroRNA-de (miRNA-de) ja nende sihtmärk-mRNA-de tuvastamise kaudu. Teiseks suuremaks eesmärgiks oli leida follikulaarvedelikust markereid, mis kirjeldaksid munasarja tasemel viljatuse põhjuseid ja IVF protseduuri tulemuslikkust. Selleks määrati erinevate viljatuse põhjustega IVF patsientide follikulaarvedelikust 16 valgu tase, mille hulgas olid tsütokiinid ja apoptoosiga seotud valgud, ning uuriti naise individuaalsete füsioloogiliste näitajate seost munaraku küpsemise keskkonnaga.

Uuringus leiti, et mRNA tasemel erinevad kaks granuloosa rakupopulatsiooni teineteisest märkimisväärselt ning nendele rakkudele omistati uusi seni avaldamata molekulaarseid funktsioone. Seevastu miRNA ekspressioonimustrid olid uuritud rakkudel enamjaolt sarnased, omades sihtmärke samades signaaliradades. Muraalse granuloosa populatsioonis oli siiski suurenenud nende miRNA-de tase, mis reguleerivad rakkude füüsilisi kontakte väliskeskkonnaga ja mitmeid ainevahetuse protsesse. Mõlemas rakupopulatsioonis leiti uusi seni tuvastamata miRNA molekule, millest kaks pärinevad kahe folliikuli arengus ülitähtsa geeni, folliikuleid stimuleeriva hormooni ja aromataasi, intronitest.

Follikulaarvedelikust mõõdetud valkudest neliteist võimaldasid eristada patsiente nende viljatuse põhjuse, munasarja stimulatsiooni või IVF protseduuri tulemuslikkuse alusel. Potentsiaalset kliinilist väärtust omavad IL-12 kontsentratsioon, mis seostus embrüo kvaliteediga, ning MIP-1β, IL-8 ja IL-18, mille tasemed follikulaarvedelikus erinesid oluliselt IVF-i tulemusena rasestunud ja mitterasestunud patsientide vahel. Lisaks leiti geeniekspressiooni andmete põhjal, et mitmed uuritud tsütokiinidest omavad võimalikku olulist rolli granuloosrakkude omavahelises interaktsioonis.

# *CURRICULUM VITAE*

**Personal data**

Name: Agne Velthut-Meikas (born Velthut)
Date and place of birth: 30.09.1982, Tallinn, Estonia
E-mail address: agnevelthut@gmail.com

**Education**

2010 – ... Tallinn University of Technology, PhD studies in chemistry and gene technology
2005 – 2007 University of Tartu, Faculty of Medicine, MSc in biomedicine
2005 – 2007 – visiting student at the Institute of Biotechnology, Helsinki University, Finland
2001 – 2005 University of Tartu, Faculty of Biology and Geography, BSc in molecular diagnostics, gene technology
1998 – 2001 Tallinn English College, secondary education

**Language competence**

Estonian – native speaker
English – fluent
Finnish – average
Russian – average
French – average

**Special courses**

2013 – EMBO (European Molecular Biology Organization) Practical Course on Analysis of High-Throughput Sequencing Data, Hinxton, England.
2012 – EMBO course on miRNA analysis, Heidelberg, Germany.
2011 – EMBO (European Molecular Biology Organization) course on mass spectrometry and proteomics, University of Southern Denmark, Odense, Denmark.
2007 – Real-time PCR advanced course, Applied Biosystems, Manchester, England.
2006 – Certificate for the completion of course on laboratory animal science, organized according to the European Union standards (Decree 85/90, Article 26, Council of Europe), at University of Helsinki, Finland.

**Professional employment**

2010 – ... Competence Centre on Reproductive Medicine and Biology; Researcher
16/08/2010 – 19/12/2011 Tallinn University of Technology, Faculty of Science, Department of Gene Technology, Chair of Genomics and Proteomics; Researcher
01/03/2009 – 01/09/2010 University of Tartu, Faculty of Medicine, Department of Obstetrics and Gynecology, Chair of Obstetrics and Gynaecology; Researcher
01/09/2008 – 31/12/2008 University of Tartu, Faculty of Medicine, Department of Obstetrics and Gynecology; Specialist

2007 – 2010 Nova Vita Clinic; biologist

**Supervised dissertations**
2014 – Ilmatar Rooda, bachelor's thesis, „The gene expression profile of human granulosa cell-lines KGN and COV434 compared to primary luteinized granulosa cells"
2011 – Anu Ruusmann, bachelor's thesis, „Detection of metallothioneins from HEP3B cell lysate"

**International conferences**
2014 – BFS (Baltic Fertility Society) annual meeting, Vilnius, Lithuania. Oral presentation.
2013 – ESHRE (European Society of Human Reproduction and Embryology) annual conference, London, UK. Poster presentation.
2013 – SSR (Society for the Study of Reproduction) annual conference, Montreal, Canada. Poster presentation.
2012 – ESHRE annual conference, Istanbul, Turkey.
2011 – ESHRE annual conference, Stockholm, Sweden. Poster presentation.
2010 – ESHRE Campus Workshop „Array technologies to apprehend developmental competence and endometrial receptivity: limits and possibilities". Brussels, Belgium.
2009 – ESHRE annual conference, Amsterdam, The Netherlands. Poster presentation.
2009 – ESHRE "Workshop on mammalian folliculogenesis and oogenesis: from basic science to clinic". Potsdam, Germany.
2008 – ESHRE annual conference, Barcelona, Spain.
2007 – EMBO/FEBS/ISF (European Molecular Biology Organization/Federation of European Biochemical Societies/Israel Science Foundation) Workshop on Systems Dynamics of Intracellular Communication – Overcoming Distance in Signalling Networks, Jerusalem, Israel. Poster presentation.
2006 – FEBS Special Meeting on Molecular Signalling, Cavtat, Croatia. Poster presentation.

**Publications**
1. Saare M, Rekker K, Laisk-Podar T, Sõritsa D, Roost AM, Simm J, **Velthut-Meikas A**, Samuel K, Metsalu T, Karro H, Sõritsa A, Salumets A, Peters M. High-throughput sequencing approach uncovers the miRNome of peritoneal endometriotic lesions and adjacent healthy tissues. PLoS One. 2014, In press.
2. **Velthut-Meikas A**, Simm J, Tuuri T, Tapanainen JS, Metsis M, Salumets A. Profiling of microRNAs in human preovulatory granulosa cells by deep sequencing reveals novel miRNAs in FSH receptor and aromatase genes. Mol Endocrinol. 2013, Jul;27(7):1128-41.

3. **Velthut A**, Zilmer M, Zilmer K, Kaart T, Karro H, Salumets A. Elevated blood plasma antioxidant status is favourable for achieving IVF/ICSI pregnancy. Reprod Biomed Online. 2013 Apr;26(4):345-52.
4. Sarapik A, **Velthut A**, Haller-Kikkatalo K, Faure GC, Béné MC, de Carvalho Bittencourt M, Massin F, Uibo R, Salumets A. Follicular proinflammatory cytokines and chemokines as markers of IVF success. Clin Dev Immunol. Volume 2012 (2012), Article ID 606459.
5. Oitmaa E, Peters M, Vaidla K, Andreson R, Mägi R, Slavin G, **Velthut A**, Tõnisson N, Reimand T, Remm M, Schneider M, Ounap K, Salumets A, Metspalu A. Molecular diagnosis of Down syndrome using quantitative APEX-2 microarrays. Prenat Diagn. 2010 Dec;30(12-13):1170-7
6. Kõks S, **Velthut A**, Sarapik A, Altmäe S, Reinmaa E, Schalkwyk LC, Fernandes C, Lad HV, Soomets U, Jaakma U, Salumets A. The differential transcriptome and ontology profiles of floating and cumulus granulosa cells in stimulated human antral follicles. Mol Hum Reprod. 2010 Apr;16(4):229-240.
7. **Velthut A,** Peters M, Roovere T, Kilusk G, Horelli-Kuitunen N, Sois M, Salumets A. Fetal trisomy 13 and 21 mosaicism diagnosed at amniocentesis: a case report. Prenat Diagn. 2009 Oct;29(10):995-7.
8. Altmäe S, Haller K, Peters M, Saare M, Hovatta O, Stavreus-Evers A, **Velthut A**, Karro H, Metspalu A, Salumets A. Aromatase gene (CYP19A1) allele variants are essential to controlled ovarian hyperstimulation outcome. Reprod Biomed Online. 2009 May;18(5):651-7.
9. Paveliev M, Lume M, **Velthut A**, Phillips M, Arumäe U, Saarma M. Neurotrophic factors switch between two signaling pathways that trigger axonal growth. Journal of Cell Science, 2007;120:2507-16

# ELULOOKIRJELDUS

**Isikuandmed**
Ees- ja perekonnanimi: Agne Velthut-Meikas (sünd. Velthut)
Sünniaeg ja -koht: 30.09.1982, Tallinn
Kodakondsus: Eesti
E-posti aadress: agnevelthut@gmail.com

**Hariduskäik**
2010 – ... Tallinna Tehnikaülikool, keemia ja geenitehnoloogia doktorantuur
2005 – 2007 Tartu Ülikool, Arstiteaduskond, biomeditsiini teadusmagister, MSc
2005 – 2007 – külalisüliõpilane Biotehnoloogia Instituudis, Helsingi Ülikool, Soome
2001 – 2005 Tartu Ülikool, Bioloogia-geograafiateaduskond, geenitehnoloogia õppekava, molekulaardiagnostika eriala bakalaureusekraad, BSc
1998 – 2001 Tallinna Inglise Kolledž, keskharidus

**Keelteoskus**
Eesti keel – emakeel
Inglise keel – kõrgtase
Soome keel – kesktase
Vene keel – kesktase
Prantsuse keel – kesktase

**Täiendusõpe**
2013 – EMBO (*European Molecular Biology Organization*) suuremahulise RNA sekveneerimise andmeanalüüsi praktiline kursus, Hinxton, Inglismaa.
2012 – EMBO miRNA analüüsi kursus, Heidelberg, Saksamaa.
2011 – EMBO mass-spektromeetria ja proteoomika praktiline kursus, Lõuna-Taani Ülikool, Odense, Taani.
2007 – Reaalaja-PCR-i tehnoloogia süvendatud kursus, Applied Biosystems, Manchester, Inglismaa.
2006 – Katseloomateaduse sertifikaat, mis vastab Euroopa Liidus kehtivatele standarditele (määrus 85/90, artikkel 26, Euroopa Nõukogu), Helsingi Ülikool, Soome.

**Teenistuskäik**
2010 – ... Reproduktiivmeditsiini TAK; teadur
16.08.2010 – 19.12.2011 Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Geenitehnoloogia instituut, Genoomika ja proteoomika õppetool; teadur

01.03.2009 – 01.09.2010 Tartu Ülikool, Arstiteaduskond, Naistekliinik, Sünnitusabi ja günekoloogia õppetool; teadur
01.09.2008 – 31.12.2008 Tartu Ülikool, Arstiteaduskond, Naistekliinik; spetsialist
2007 – 2010 Nova Vita Kliinik; bioloog

**Juhendatud lõputööd**
2014 – Ilmatar Rooda, bakalaureusetöö, „Inimese granuloosa rakuliinide KGN ja COV434 geenikspressiooni profiili võrdlus primaarsete luteiniseerunud granuloosa rakkudega“
2011 – Anu Ruusmann, bakalaureusetöö, „Metallotioniinide detekteerimine HEP3B rakulüsaadist“

**Konverentsid**
2014 – BFS (*Baltic Fertility Society*) aastakonverents, Vilnius, Leedu. Suuline ettekanne.
2013 – ESHRE (*European Society of Human Reproduction and Embryology*) aastakonverents, London, Inglismaa. Posterettekanne.
2013 – SSR (*Society for the Study of Reproduction*) aastakonverents, Montreal, Kanada. Posterettekanne.
2012 – ESHRE aastakonverents, Istanbul, Türgi.
2011 – ESHRE aastakonverents, Stockholm, Rootsi. Posterettekanne.
2010 – ESHRE töötuba teemal *„Array technologies to apprehend developmental competence and endometrial receptivity: limits and possibilities*“. Brüssel, Belgia.
2009 – ESHRE aastakonverents, Amsterdam, Holland. Posterettekanne.
2009 – ESHRE töötuba teemal “*Mammalian folliculogenesis and oogenesis: from basic science to clinic*”. Potsdam, Saksamaa.
2008 – ESHRE aastakonverents, Barcelona, Hispaania.
2007 – EMBO/FEBS/ISF (*European Molecular Biology Organization/Federation of European Biochemical Societies/Israel Science Foundation*) töötuba teemal “*Systems Dynamics of Intracellular Communication – Overcoming Distance in Signalling Networks*”, Jeruusalemm, Iisrael. Posterettekanne.
2006 – FEBS eriseminar teemal “*Molecular Signalling*”, Cavtat, Horvaatia. Posterettekanne.

**Publikatsioonid**
1. Saare M, Rekker K, Laisk-Podar T, Sõritsa D, Roost AM, Simm J, **Velthut-Meikas A**, Samuel K, Metsalu T, Karro H, Sõritsa A, Salumets A, Peters M. High-throughput sequencing approach uncovers the miRNome of peritoneal endometriotic lesions and adjacent healthy tissues. PLoS One. 2014, In press.

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9. Paveliev M, Lume M, **Velthut A**, Phillips M, Arumäe U, Saarma M. Neurotrophic factors switch between two signaling pathways that trigger axonal growth. Journal of Cell Science, 2007;120:2507-16

**DISSERTATIONS DEFENDED AT**
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