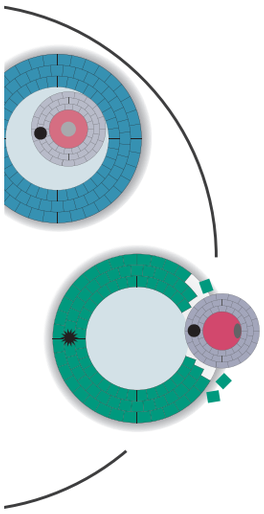

GENE EXPRESSION IN HUMAN OOCYTES AND GRANULOSA CELLS

Competence classification and effect of age, developmental stage and ART regimens

Marie Louise Grøndahl

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Competence classification and effect of age, developmental stage and ART regimens

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This Dissertation is based on nine original publications (Paper I-IX)

Paper I

Markholt S, **Grøndahl ML**, Ernst EH, Andersen CY, Ernst E, Lykke-Hartmann K.

Global gene analysis of oocytes from early stages in human folliculogenesis shows high expression of novel genes in reproduction.

Mol Hum Reprod. 2012 Feb;18(2):96-110.

Paper II

Grøndahl ML, Yding Andersen C, Bogstad J, Nielsen FC, Meinertz H, Borup R.

Gene expression profiles of single human mature oocytes in relation to age.

Hum Reprod. 2010 Apr;25(4):957-68.

Paper III

Grøndahl ML, Borup R, Vikeså J, Ernst E, Andersen CY, Lykke-Hartmann K.

The dormant and the fully competent oocyte: comparing the transcriptome of human oocytes from primordial follicles and in meta-phase II.

Mol Hum Reprod. 2013 Sep;19(9):600-17.

Paper IV

Grøndahl ML, Andersen CY, Bogstad J, Borgbo T, Boujida VH, Borup R.

Specific genes are selectively expressed between cumulus and granulosa cells from individual human pre-ovulatory follicles.

Mol Hum Reprod. 2012 Dec;18(12):572-84.

Paper V

Grøndahl ML, Nielsen ME, Dal Canto MB, Fadini R, Rasmussen IA, Westergaard LG, Kristensen SG, Yding Andersen C.

Anti-Müllerian hormone remains highly expressed in human cumulus cells during the final stages of folliculogenesis.

Reprod Biomed Online. 2011 Apr;22(4):389-98.

Paper VI

Wissing ML, Kristensen SG, Andersen CY, Mikkelsen AL, Høst T, Borup R, **Grøndahl ML**.

Identification of new ovulation-related genes in humans by comparing the transcriptome of granulosa cells before and after ovulation triggering in the same controlled ovarian stimulation cycle.

Hum Reprod. 2014 Feb; 29(5):997-1010.

Paper VII

Grøndahl ML, Borup R, Lee YB, Myrhøj V, Meinertz H, Sørensen S. Differences in gene expression of granulosa cells from women undergoing controlled ovarian hyperstimulation with either recombinant follicle-stimulating hormone or highly purified human menopausal gonadotropin.

Fertil Steril. 2009 May;91(5):1820-30.

Paper VIII

Borgbo T, Povlsen BB, Andersen CY, Borup R, Humaidan P, **Grøndahl ML**.

Comparison of gene expression profiles in granulosa and cumulus cells after ovulation induction with either human chorionic gonadotropin or a gonadotropin-releasing hormone agonist trigger.

Fertil Steril. 2013 Oct;100(4):994-1001.

Paper IX

Rehannah Borup, Lea Thuesen, Claus Yding Andersen, Anders Nyboe-Andersen, Søren Ziebe, Ole Winther, **Marie Louise Grøndahl**.

Competence classification of cumulus and granulosa cell transcriptome in embryos matched by morphology and female age.

PlosOne. 2016 Apr; 29;11(4):1-19.

Papers number II, IV and IX were previously included in the PhD thesis, 'Application of Microarray technology in research and diagnostics: differential expression catalogs and molecular signatures' by Rehannah Borup, 2015, and paper VI in the PhD thesis, 'Non-invasive markers on oocyte quality - special emphasis on PCOS' by Marie Louise Wissing, 2014.

Preface and acknowledgement

This doctoral dissertation is based on research work carried out between 2006-2016, during my employments at the fertility clinics at Copenhagen University Hospitals, Hvidovre Hospital, Rigshospitalet and Herlev Hospital as well as at the Laboratory of Reproductive Biology at Rigshospitalet.

My warmest gratitude goes to DMSci Professor Claus Yding Andersen, for being an inspirational and outstanding mentor. His unique enthusiasm, innovative ideas and support have always inspired and encouraged me to carry on the research in 'the most unique cells and the most transformational structure and function of the body' – the ovarian follicle.

Likewise, I am greatly indebted to Bioinformatician and PhD Rehannah Borup, Panum, for her tireless collaboration, and patience in explaining me the math behind the model used to analyze the array data.

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MSD Denmark is gratefully acknowledged for financial support.

Finally, I wish to thank my family especially Veronica, Magnus, Johann, Tobias and Christian for their unlimited love, help and support.

LIST OF CONTENT

ABBREVIATION

ART	assisted reproductive therapy
CC	cumulus cells
CL	corpus luteum
COC	cumulus-oocyte-complex
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
FF	follicular fluid
FSH	follicle stimulation hormone
GSEA	gene set enrichment analysis
GnRHa	gonadotrophin releasing hormone agonist
hCG	human chorionic gonadotrophin
hMG	human menopausal gonadotropin
ICSI	intra-cytoplamic sperm injection
IVF	in vitro fertilization
LH	luteinizing hormone
MGC	mural granulosa cells
MII	metaphase II
MTZ	maternal-to-zygotic transition
OHSS	ovarian hyper stimulation syndrome
OPU	oocyte pick up
OS	ovarian stimulation
PGD-A	preimplantation genetic diagnosis for aneuploidy
PN	pronuclei
polyA	polyadenylation
rFSH	recombinant follicle stimulation hormone
TE	trophectoderm
WTA	whole transcriptome amplification

Gene and protein symbols and names are listed in section ‘Gene symbols and names’.

1. INTRODUCTION

This thesis provides 1) an introduction to the research area, oogenesis and folliculogenesis, 2) research objectives 3) a description of the methods used, 4) a review of the major findings gathered from study I-IX in the context of current knowledge on gene expression and function of the human oocyte and the ovarian follicle, 5) methodological considerations 6) concluding remarks and 7) suggestions for future studies and 8) a summary.

The human oocyte is a unique cell that differs from all other cells in the human body. First of all, it is the cell that possesses the capacity to form a new offspring and secure continuation of the gene pool and from an evolutionary point of view the oocyte is the obligatory indispensable cell. Further, the oocyte is the largest cell of the body and the length of the oocyte's cell cycle is extraordinary long, potentially up to five decades under the condition that it becomes surrounded by somatic cells and forms the primordial follicle during fetal life (III). The pool of primordial follicles, oocytes enclosed in one layer of flattened pregranulosa cells, is laid down in the ovaries during fetal life and constitutes the entire reproductive potential of a female (Skinner, 2005). At its pinnacle in numbers, in week 26 of gestation, the number of primordial follicles is 4 million where after follicles start to activate and undergo atresia. At birth, the ovaries of a baby girl contain around 1 million and at menarche, 400.000 primordial follicles are present. Of these, only 400 follicles will over the reproductive lifespan succeed in developing all the way to selection and final maturation following ovulation of one oocyte in each menstrual cycle (Gougeon, 1998). Thus, recruitment of primordial follicles into the growing phase of follicular development starts before birth and continues throughout the reproductive years (Choi and Rajkovic, 2006) until menopause reflecting depletion of the primordial follicle pool (Richardson *et al*., 1987). The number of all classes of growing follicles decreases with age reflecting the number of the resting follicles in example from 26, 15 and 6 early growing follicle to 10, 5 and 1 selectable follicles in each ovary in women aged 25, 35 and 45 years of age, respectively (Gougeon, 1998). In assisted reproduction treatment, ovarian stimulation ensures growth of a number of the selectable follicles for oocyte retrieval and fertilization in vitro. Interestingly, it has been shown based on more than 400.000 treatment cycles that there is a relationship observed between the number of retrieved oocytes and live birth across all female age groups, suggesting that the ovarian reserve reflects some degree of follicle competence potentially due to better selection process (Sunkara *et al*., 2011).

The human folliculogenesis is well characterized morphologically, from a diameter of 30µm in the primordial stage over primary and secondary pre-antral stages reaching 200µm proceeding to early antral and small antral entering the selectable phase at the size of 2mm and to the pre-ovulatory follicle of 20mm (Williams and Erickson, 2000). Upon activation of the primordial follicle the diameter of the oocyte increases, from 30µm to its final diameter (120µm) before the antral stage (Williams and Erickson, 2000) (Griffin *et al*., 2006). The human folliculogenesis, from activation of the primordial follicle to ovulation, is estimated to be almost a year, of which the main part is primarily independent of gonadotrophins and dependent of locally produced growth factors, while the last 50 days (from the antral stage of 2mm) is dependent of follicle stimulating hormone (FSH) (Williams and Erickson, 2000) (Erickson and Shimasaki, 2000).

The insight in molecular mechanisms involved during human folliculogenesis and how competence of the ovarian follicle and the oocyte is gradually achieved and sustained is limited as oocytes, embryos and ovarian tissue for research in human reproduction in general, is sparse due to limited access to this precious and ethical delicate material. To generate additional insight in human oogenesis and folliculogenesis, the present thesis concentrates on genes expressed in oocytes as well as in the somatic cell compartment of the follicle during various developmental stages and treatment regimens for ovarian stimulation used in assisted reproduction.

The material included in study I-IX is donated under ethical approval and donor consent in connection to assisted reproduction therapy and fertility preservation programs.

In all studies (I-IX) the high throughput technique, DNA whole genome microarray analysis, was used to generate the transcriptome of oocytes and somatic cells, respectively. The transcriptome or the gene expression profile represents snapshot of the activity of thousands of genes at the same time and provide a global picture of the cellular activity and potential function. For confirmation of the array data, RT-PCR and/

or Western blot analysis were performed for selected genes.

1.1 Folliculogenesis

Ensuring the pool of primordial follicles as well as the transition from primordial to primary follicle are vital processes for mammalian reproduction and remain to be fully elucidated. Key functions in folliculogenesis and some molecular mechanisms involved in regulating the activation of primordial follicles have been identified, such as the phosphatidylinositol 3 kinase (PI3K) and the mammalian target of rapamycin complex 1 (mTORC1) pathways as well as i.e. KIT, KIT ligand (KITLG), phosphatase and tensin homolog (PTEN) (Zhang and Liu, 2015) and anti-Müllerian hormone (AMH) (Kano *et al.*, 2017). Identifying the pathways controlling these early events may provide basis for the fundamental mechanisms regulating follicle protection and activation and could potentially lead to new therapeutics in female reproduction as well as improvements in reproductive health in women of advanced maternal age and in women in risk of premature ovarian failure (Kawamura *et al.*, 2016) or going through chemotherapy (Donnez *et al.*, 2013) (Kano *et al.*, 2017).

A morphological sign of activation of the primordial follicle to the primary follicle is the transformation of the squamous pregranulosa cells, surrounding the oocyte, into cuboidal granulosa cells. During the following several months, follicular growth proceeds with granulosa cell proliferation and formation of theca cell layer from surrounding stromal cells (Liu *et al.*, 2015) establishing the mesenchymal-epithelial crosstalk with an array of paracrine factors acting between theca cells and granulosa cells that continues throughout folliculogenesis sustaining the development and the local two-cell-two-gonadotrophin feedback loop regulating fol-licular steroid production (Hillier *et al.*, 1994). By far most of the activated follicles undergo atresia along the early developmental path where the follicular development is predominantly independent of gonadotropin stimulation, however, emerging evidence show some responsiveness to gonadotrophins even in early stages of follicular development (Hsueh *et al.*, 2015) (Kristensen *et al.*, 2015). Only few follicles make it to the developmental stage characterized by having more than approximately 6 layers of granulosa cells proceeding to the antral stage with a fluid-filled cavity, approaching the gonadotrophin dependent phase of the folliculogenesis ensuring further differentiation of the granulosa cells and continued maturation of the oocyte (Rodgers and Irving-Rodgers, 2010). After the intercycle rise in FSH, selection of the dominant follicle takes place culminating in ovulation of one fertilizable oocyte, the meta-phase II (MII) oocyte, in each menstrual cycle.

1.2 Granulosa and cumulus cells

The granulosa cells proliferate and differentiate from one single layer of cells comprising around 30 pregranulosa cells in the primordial follicle (Westergaard *et al.*, 2007) into numerous layers of mural granulosa cells (MGC) lining the inside of the follicular wall in antral follicles and layers of cumulus granulosa cells, cumulus cells (CC), immediately surrounding the oocyte (McNatty *et al.*, 1979). It is established that the oocyte by secreting paracrine factors exerts a profound impact on this differentiation process to secure its own optimal growth and maturation (Diaz *et al.*, 2007) (Zhang *et al.*, 2010) appointing the oocyte as 'the captain' of the intra follicular communication, in review (Russell *et al.*, 2016) (Monniaux, 2016). The very specialized function of the MGC and CC is the essential close communication with the oocyte providing nutritional support and trafficking of macromolecules throughout folliculogenesis. The transport occurs through an extensive network of intercellular connections that exist between the somatic cells and the oocyte, constantly interacting through receptors (cytokines and growth factors) and gap junctions (ions, cAMP, cGMP, amino acids, and metabolites) to coordinate the functionality of the follicle. It is noteworthy that the follicular cells produce many growth factors, for instance inhibins and activins, AMH, epidermal growth factor (EGF), insulin-like growth factors, and fibroblast growth factors, all able to modulate the dialog between the oocyte and its surrounding granulosa and cumulus cells (Monniaux, 2016). Additionally, recent publications also suggest potential roles of extracellular vesicles, carrying regulatory molecules as microRNAs and proteins, in follicular growth and oogenesis (Machtinger *et al.*, 2015).

The granulosa cells upregulate sensitivity to FSH from the antral stage of folliculogenesis and subsequently some of these follicles will be recruited to

the final growth phase by the intercycle rise in FSH (Hsueh *et al*., 2015). In assisted reproductive therapy (ART), ovarian stimulation (OS) with exogenous FSH ensures that no single follicle becomes dominant and sustains growth of the cohort of recruited follicles for final maturation and oocyte retrieval, 34-36 hours after administration of the ovulation trigger, for in vitro fertilization.

During the later phase of folliculogenesis, MGC have both auto/paracrine and endocrine function switching from estradiol to progesterone production when becoming luteinized by the ovulation trigger. At the time of ovulation, the CC still communicate directly with the oocyte through gap-junctions (Makabe *et al*., 2006). In connection to oocyte retrieval following OS, MGC and CC are available and easy to isolate without affecting the planned in vitro fertilization procedure.

1.3 Ovulation

The mid-cycle surge of FSH and luteinizing hormone (LH) is the physiological trigger of ovulation, while a bolus of human chorionic gonadotrophin (hCG), the golden standard, or a bolus of gonadotrophin releasing hormone agonist (GnRHa) are used in ART cycles.

The ovulation signal essentially induces three distinct complex processes comprising 1) oocyte maturation and resumption of meiosis, 2) ovulation with release of an MII oocyte and 3) transformation, characterized as a local inflammatory process, of the avascular granulosa cells into the highly vascularized granulosa-lutein cells leading to the formation of a corpus luteum (CL) supporting possible implantation. The molecular processes behind the ovulatory cascade have only to a limited extend been studied in humans because of limited access to follicles immediately before triggering of final oocyte maturation. Some data are available from animal studies revealing dramatic timing and cell-type dependent changes in gene expression (Xu *et al*., 2011) (Christenson *et al*., 2013).

1.4 Achievement of oocyte competence

During folliculogenesis and oogenesis, the oocyte grows in size from 30µm in diameter as the oocyte is arrested in the pro-phase of the 1st meiosis in the primordial follicle to 120µm of the oocyte in the pre-ovulatory follicle (Griffin *et al*., 2006). During this growth, there is an increase in number and spatial organization of cytoplasmic organelles e.g., the number of mitochondria increases from 10.000 in the primordial oocyte to around 200.000 in the fully grown oocyte and the number of mitochondria is stable until implantation occurs (Darbandi *et al*., 2016). The transcriptional and translational activity in the oocyte is high until the mid-cycle surge of LH, where after very low level of transcription is present (Miyara *et al*., 2003). Throughout follicular development and unlike other cells, where RNAs and proteins are used within minutes or hours after formations, oocytes store large quantities of mRNA and proteins in days and weeks in an inactivated form (selective cytoplasmic polyadenylation (polyA) of the mRNAs). The complexity and careful regulation of the transcriptional activity of the oocyte dictates its ultimate acquisition of developmental competence being capable of recruiting the stored mRNA and protein in a tight scheduled manner with the clock set by the signal from first the LH surge and secondly fertilization undertaking a multitude of functions: resumption of meiosis, becoming fertilized with a single spermatozoa, decondensing the sperm head, completing meiosis, creating two pro-nuclei, and sustaining the first cleavages until the embryonic genome becomes active (Gosden, 2002). In addition to the protein coding (polyA-positive RNAs), small, long and latest circular regulatory RNAs – non protein coding and polyA-negative have emerged to be maternally expressed and of significance in the early embryonic development (Karlic *et al*., 2017) (Dang *et al*., 2016).

The age of a woman in her reproductive lifespan is the most significant parameter for her chance to conceive (Menken *et al*., 1986). In addition to the gradual decline in the number of ovarian follicles, the major cause for the decline in female reproductive capacity with increasing age appears to be a decreased developmental competence of the oocyte. This relation was established in oocyte donation programs, where women in their forties experienced pregnancy potential similar to the age of the young donors (Navot *et al*., 1991).

The processes governing oocyte development competence is usually divided into cytoplasmic maturation, covering broadly and unspecific what is laid down in the cytoplasm during oogenesis to orchestra meiosis and early embryogenesis, and nuclear maturation being the stage of meiosis and chromosomal status; the

two assumed being highly interdependent. Nuclear maturation includes resumption of meiosis envisioned (light microscopy) by germinal vesicles breakdown followed by 1st and 2nd meiosis (extrusion of 1st and 2nd polar body), the quality of which can be approached by polar body biopsy and/or by trophectoderm (TE) biopsy followed by comprehensive chromosome screening. There is a strong age depended increase in the aneuploidy rate of both oocytes and embryos created in connection to ART from 30% in women aged 30 years increased to 60% at 40 years and approaching 100% when passing 45 years (Franasiak *et al.*, 2014). Several non-disjunctional mechanisms have been suggested to cause the aneuploidy: failure in meiotic pairing, synapsis, recombination and segregation (Ottolini et al., 2015) (Wang *et al.*, 2017).

Biomarker for oocyte competence

Since the beginning of the human in vitro fertilization (IVF) era in 1978 there has been development and refinements of the protocols for OS and luteal support, the in vitro culture conditions as well as in the oocyte and embryo assessment in order to improve the efficacy of in vitro fertilization treatment. There is ongoing research to define good non-invasive oocyte and/or embryo markers to increase the present average implantation rate from of around 25% achieved for cleavage stage embryos and 40% for blastocysts with the standard morphology and cleavage markers (Alpha SiRM e ESHRE SIGE, 2011). The more detailed assessment of the embryo development by time lapse recordings may provide better selection, however, it still lacks sufficient evidence of differences in live birth, miscarriage, stillbirth or clinical pregnancy as compared to standard assessment (Armstrong *et al.*, 2015). The high rate of aneuploidy in the human oocyte is one of the main challenges in ART and the relative low implantation rate after selection based on morphology and cleavage kinetics assessment is in line with reports on limited correlation between the morphokinetics and the ploidy of the embryo (Fragouli *et al.*, 2014) (Minasi *et al.*, 2016).

During the years, several attempts have been made to identify oocyte competence markers in the follicle: size of the follicle, contents of varies substances in the follicular fluid as well as blood flow of the surrounding compartment. Retrospective studies have shown correlations to competence, however, no confirmatory prospective randomized trials have been conducted. The focus of the last decade has additionally been on gene expression, single genes as well as expression profile, of the somatic compartment of the follicle connected to competent and non-competent oocytes suggesting expression level of i.e. pentraxin 3 (*PTX3*) in CC being related to competence (Zhang *et al.*, 2005). Disappointingly, there is generally a lack of consistency in published marker genes probably reflecting the different measures for competence as well as difference in isolation technique and detailed timing of sampling from the follicles in a highly complex and transformational phase.

2. OBJECTIVES

The objectives of this thesis fall in three parts:

Oocyte transcriptome

- To describe the gene expression of the oocyte in two highly unique developmental stages and how the oocyte at its pinnacle of developmental competence is affected by age of the women. (I-III)

Follicle cell transcriptome

- To describe the spatial and temporal dynamics in the gene expression of the follicle somatic cells during the late phase of follicular development prior to ovulation and how it is affected by the stimulation regimens for OS and ovulation induction in ART cycles. (IV-VIII)

Gene expression classifier

- To classify the competence of isolated oocytes with or without ability to sustain pregnancy subsequently based on the gene expression of the connecting cumulus and granulosa cells of the follicle. (IX)

3. MATERIAL AND METHODS

In the following an overview is presented of the material and methods used. More elaborate details can be found in the individual papers.

Patients and treatment protocol

In study I, a 21-year-old woman and a 11-year-old girl donated a small fragment of ovarian cortex to research at Laboratory of Reproductive Biology, Rigshospitalet, after unilateral oophorectomy for cryopreservation of the ovary for fertility preservation before gonadotoxic treatment of malignant disease.

The women recruited for study II-IX were undergoing fertility treatment at the Danish Fertility Clinics at Hvidovre Hospital, Copenhagen University Hospital (II-V, VII), Holbæk Sygehus (VI), Regionshospitalet Skive (VIII) and Rigshospitalet, Copenhagen University Hospital (IX). Eligibility criteria included age 20-40 years, regular menstrual cycle and referral diagnosis of male factor and/or tubal disease, unexplained infertility and mild endometriosis. All women had OS with recombinant FSH (rFSH; I, IV-IX) and/or highly purified human menopausal gonadotropin (hMG; VII) dosed individually (Popovic-Todorovic *et al.*, 2004) aiming for a moderate number (8-10) of ooctytes for standard IVF or micro insemination, intra-cytoplamic sperm injection (ICSI). For final follicle and oocyte maturation ovulation trigger was administered when three follicles had reached 17mm in diameter, and oocyte pick up (OPU) was performed 34-36 hours thereafter. The ovulation trigger of choice, a bolus of hCG, was used in the majority of the studies (II, III, IV-IX) and study VIII additionally used GnRHa as a part of the experimental set-up.

The *in silico* based study III included gene expression data achieved in study I, II and IV.

Isolation of cells

The donated cells from the periovulatory follicle were isolated, rinsed, tubed, flash frozen in liquid nitrogen and stored at -80°C until RNA extraction, within 30-60 minutes after OPU to avoid in vitro influence on the gene expression. To protect the RNA during the in vitro handling RNase inhibitor was added to the medium used for handling and storage of the cells (study I-VIII).

Granulosa and cumulus cells

In study VII, MGC were isolated from sediment from the pool of follicular fluid from all follicles emptied, while in study IV, V, VI and VIII, MGC and CC were isolated from individual follicles. Isolation of the cumulus mass was mechanically removed from the cumulus-oocyte-complex (COC) by needles. As erythrocyte derived factors have been suggested to affect PCR analysis (Crinelli *et al.*, 2000), contaminating erythrocytes were removed by erythrolysis buffer or simply by avoiding isolation from follicular fluid contaminated with blood. Various techniques for isolating the MGC were used to eliminate contamination of white blood cells and theca cells.

Oocytes

Donated oocytes were isolated within 30 minutes after OPU. After dissection of the cumulus mass, the oocyte was enzymatically denuded with hyaluronidase and to avoid contaminating cumulus cells, zona pellucida was removed by short incubation in Tyrodes solution. Maturity of the oocytes was noted and only oocytes in meta-phase II proceeded to transcriptome analysis.

Laser captured primordial follicle

The vast majority of the oocytes isolated by laser capture micro-dissection microscopy were from the resting pool of primordial follicles, however few captures of oocytes from intermediate and primary follicles could not be excluded. The lack of appreciable expression of central genes expressed in the somatic cells of the follicle and ovary demonstrated that the dissection of the oocytes from the pregranulosa cells and the surrounding ovarian was successful (Study I).

Extraction of total RNA, quality and quantity

In all studies kits for extraction of RNA from minute samples were used followed by analysis of the quantity and quality of the total RNA extracted. Only samples with clear visible 18S and 28S bands and RNA Integrity Number above 8 were processed further.

The average amount of total RNA extracted from granulosa cells isolated from individual peri-ovulatory follicles by light microscopy selection varied from a mean of 27ng (VI), 95ng (IV) to 342ng (IX), respectively, while an average of 1964ng RNA per women was isolated from sediment of multiple follicles (mean number of follicles was 12) followed by anti CD45+ magnetic beads cell sorting to avoid leucocyte contamination (VII).

From the individual pieces of CC an average amount of 51ng of total RNA was isolated. From individual MII

oocytes an average of 35ng RNA was isolated, while the pools of laser captured oocytes had RNA yield as low as 2ng of total RNA.

Microarray analysis

To ensure sufficient material for the microarray analysis kits for small RNA samples whole transcriptome amplification (WTA) was used in study I-VI and VIII-IX. Starting material in study VII using granulosa cells from pools of follicles from 5 patients was sufficient without WTA.

Amplified cRNA was biotin labelled and hybridized to the Human Gene U133plus2 GeneChip array (I-V and VII) or Human Gene 1.0ST (VI, VIII and IX) (Affymetrix, CA, USA). The arrays were washed and stained with phycoerytrin conjugated streptavidin and scanned to generate flourescent images generating Cell intensity files, Cel files (Scanner and Software were all from Affymetrix).

Microarray Data analysis

The Cel files from the microarray analysis were imported into statistical software packages (i.e. the Partek Genomics Suite 6.5, R and gene set enrichment analysis (GSEA)) and robust multichip average normalized using quantile normalization and "Median Polish summarization" (Bolstad *et al.*, 2003). The modelled log-intensity of the 54,675 (I-V and VII) or 33,293 (VI, VIII and IX) probe sets was used for detection call evaluation either by implementation of the MAS5 algo-rithm (I, V and VII) or by thresholding, the expression profiles in the cell types were analyzed and genes defined as being differentially expressed when they were selected in suitable statistical tests. The gene ontology enrichment analysis was performed to define overrepresentation of functional categories and enriched pathways of the differentially expressed genes using various software (i.e. Ingenuity and dChip).

Public availability of data

All samples were MIAME (minimum information about a microarray experiment) compliant and microarray data submitted to ArrayExpress using MiameExpress at EMBL (European Molecular Biology Laboratory) which imply that all data is publicly available. All publications present unique accession number of the experiments.

Validation of microarray data

RT-PCR of central genes in biological replicates confirmed the array data (I-VI) and in study VIII Western blot analysis additionally confirmed the expression data at the level of protein detection.

In the RT-PCR analysis *GAPDH* was suitable as reference gene in the study I, II and IV-VI, while hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was chosen by NormFinder (Andersen *et al.*, 2004) followed by test of several candidate genes in study III, comparing the oocyte in it very different developmental stages. In study VI, concentration of steroid hormones in the follicular fluid (FF) further supported the gene expression data.

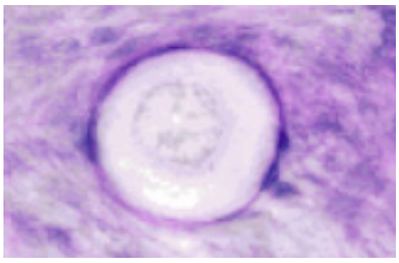
4. RESULTS, INCLUDING DISCUSSION OF EXISTING LITERATURE

4.1 The oocyte transcriptome (I-III)

Knowledge of the gene expression profiles that occur during oocyte development will provide the first glimpse at understanding the molecular basis for development of an oocyte of high quality (Pan *et al.*, 2005). Paper I-III provide data on the transcriptome present in the oocytes in the primordial follicle and in the follicle hours before ovulation using Affymetrix arrays representing the whole human genome.

In study III we compared the expression profile of the oocyte in the starting point of the oogenesis with the profile of the oocyte in the end of the oocyte development. The assumption being that genes expressed in both oocytes from the primordial follicle and in MII oocytes are of general importance for oogenesis and genes differentially and even specifically expressed in either of the developmental stages may be of special importance for oocytes in that particular stage.

In addition, the transcriptomes of oocytes, having different potentials to undergo embryogenesis, from young and reproductive aged women, were compared (II).



Human oocyte in the primordial follicle. *Diameter: 30µm*

4.1.1 The transcriptome of the oocyte in the primordial follicle (I, III)

Most transcriptomic studies of early folliculogenesis are based on whole ovary or follicles (Yoon *et al.*, 2006) (Kristensen *et al.*, 2014), not taking the multiplicity of cell types into consideration potentially loosing valuable spatial information.

We were the first to publish the transcriptome of the isolated human oocyte in its very beginning, the cell cycle quiescent state in the primordial follicle (I) and highlight overexpressed genes by comparison with MII and MGC transcriptomes (III).

Major findings (I and III)

- A total of 6,301 genes were defined to be expressed in the oocyte in the primordial follicle. The number of active genes is an importing finding demonstrating that the oocyte in the human primordial follicle isn't generally quiescent or resting but indeed transcriptionally active.
- High expression level (as in the MII oocytes) of Maternal-Effect genes i.e. zygote arrest 1 (*ZAR1*), folliculogenesis specific basic helix-loop-helix (*FIGLA*), developmental pluripotency associated 3 (*DPPA3, STELLA*) and NLR family pyrin domain containing 5 (*NLRP5, MATER*).
- 2,556 genes were selectively expressed in the oocyte of the primordial follicle as compared to MII oocytes and granulosa cells.
- A total of 729 genes were more than 10-fold higher expressed in oocytes from primordial follicles as compared to MII oocytes.
- 'PI3K/AKT/mTOR', 'androgen signaling' and 'estrogen receptor signaling' were the top enriched pathways in these 729 overexpressed genes.
- 'Cell Death, Cell cycle, Cellular growth and proliferation' was the top bio-function enriched highly represented by tumor suppressors genes as BUB1 mitotic checkpoint ser-ine/threonine kinas (*BUB1*), tumor protein P63 (*TP63*), WNT signaling pathway regulator (*APC*), catenin beta 1 (*CTNNB1*) and cyclin K (*CCNK*) involved in cell cycle regulation check and protection of the genome.
- SKI like proto-oncogene (*SKIL*), inhibitor of transforming growth factor beta signaling was selectively expressed in the oocytes from primordial follicles.
- High expression levels of genes regulating DNA methylation and demethylation, i.e. folate receptor 1 (*FOLR1*).
- High expression levels of genes without previous reference to oocyte or reproduction, i.e. transmembrane protein with EGF like and two follistatin like domains 2 (*TMEFF2*), cerebellar degeneration related protein 1 (*CDR1*), reported to be influenced by androgens and estrogens in other tissues.

In the following, main enriched functions and selected genes are discussed.

Tumor suppressors and protection of the genome

Several of the highly expressed genes in the oocyte of the primordial follicle have been reported to be tumor suppressor genes representing both caretakers (repairing damaged genes and holding the cell cycle until repaired) and gatekeepers (inducing apoptosis or senescence) which are regarded as central processes in keeping the oocyte arrested and the genome protected in the primordial follicle. The *TMEFF2* gene encodes a protein with both EGF-like and follistatin-like do-mains, was highly expressed in the oocyte from the primordial follicle and is reported to be a tumor suppressor. Interestingly, the anti-proliferative effect of TMEFF2 had been shown to be regulated by androgens (Gery *et al.*, 2002) and Activin A (Tsai *et al.*, 2010), both suggested to be involved in the activation of the primordial follicle (McLaughlin and Telfer, 2010) (Labeur et al., 2015). Latest reports on TMEFF2 in human corticotroph cells (Labeur *et al.*, 2015) and rat ovary sug-gest that TMEFF2 may act as a negative feedback on increase in cAMP (Petersen *et al.*, 2015).

To further evaluate the cell stage specific transcriptome of the oocyte in the primordial oocyte, study III was conducted. Comparing the transcriptome of oocytes from human primordial follicles with that of MII oocytes and MGC allowed us to highlight the genes

specific for the early oocyte, while the comparison to MII directly identified genes of specific importance at each of these unique developmental stages of the oocyte. Of 1,333 genes, more than 10-fold differentially expressed, 729 genes showed a higher expression in oocyte in the primordial follicle as compared to MII. *TMEFF2* expression was 135-fold higher expressed in oocyte in the primordial follicle, indicative for a special function in the early stage. Functional studies will show whether TMEFF2 is of importance in keeping the oocyte at the special quiescent cell cycle stage. Subjecting the genes overexpressed in the primordial oocyte as compared to MII to functional enrichment analysis 'Cell cycle' and 'Cell cycle network' was among the top bio-function represented tumor suppressor genes as *BUB1*, *TP63*, *APC*, *CTTNB1* and *CCNK*; all reported to be involved in cell cycle checkpoint control and DNA damage response. Of the p53 family of tumor suppressor proteins, TP63 has been suggested to control the quality and survival of the oocyte pool by involvement in the post-pachytene check-point as TP63 is kept inactive in the absence of DNA damage but causes rapid oocyte elimination (mouse experiment) in response to a few DNA double strand breaks thereby acting as the key quality control factor in maternal reproduction (Coutandin *et al*., 2016). In accordance, our data demonstrated that *TP63* was substantially expressed in human oocytes from the primordial follicle while expression in MII oocytes and somatic cells were absent or very low confirming previous studies in mice (Suh *et al*., 2006). Moreover, CCNK also reported to be involved in genome maintenance (Yu and Cortez, 2011) and *CCNK* was moderately and selectively expressed in the oocyte in the primordial follicle.

The gene *RELN* encoding a large extracellular glycoprotein, Reelin, was moderately and selectively expressed in the oocyte in the primordial follicle (III). *RELN* expression has been related to cancer in humans and suggested to be a possible tumour suppressor. The signaling pathway in the effect of *RELN* has been shown to include the PI3K/Akt pathway and RELN has recently been suggested to negatively regulate TGFB-induced signaling (Khialeeva and Carpenter, 2016). Interestingly, change in testosterone level has been shown to be involved in methylation of the Murine *Reln* Promoter CpG (cytosine-phosphate-guanine) (da Silva *et al*., 2015). Whether RELN is involved in retaining the follicle in the cell cycle dormant stage remains to be clarified. Also, genes as *DSCAM* (Down Syndrome cell adhesion molecule), *DCC* (Netrin 1 receptor) and *NTNG2* (Netrin G2) involved in cell adhesion were moderately and selectively expressed in the very immature oocyte as compared to the MII (additionally the genes were absent in CC and MGC) further reflecting an intense and unique anchoring to its neighbouring cells in the early folliculogenesis.

Interestingly, the nicotine receptor cholinergic receptor, nicotinic, alpha 3 (*CHRNA3*) was represented in the top 'cell cycle/cell death' network enriched in the oocytes from primordial follicles. This was the first report of a gene related to smoking being expressed in oocytes. Nicotine has been shown to dose-dependently impair the follicullogenesis in hamster ovaries by increasing the apoptotic cell death in granulosa cells (Bordel *et al*., 2006). Whether the negative effect of smok-ing on female fecundity and fertility involves the oocyte nicotine receptor CHRNA3 needs further investigations (III).

Signaling pathways

Knock-out animal studies have revealed signaling pathway as PI3K and MTORC1 and molecules KITL, KIT, AMH and PTEN having central roles in holding the oocyte in the resting stage in primordial follicles (Kano *et al*., 2017). In support of this notion, when subjecting the set of 729 genes being more than 10-fold higher expressed in the oocyte from the human primordial follicle as compared to MII to GSEA, the top canonical pathway enriched was the 'PI3K/AKT/mTOR', signaling pathway including i.e. SMAD family member 2 (*SMAD2*), mitogen-activated protein kinase 1 (*MAPK1*) and *MAPK8*. The gene encoding Insulin like growth factor binding protein 5 (*IGFBP5*) highly overexpressed in the oocyte in the primordial follicles may be involved in the cell cycle quiescence as secreted IGFBP5 was recently reported to mediate mTORC1-dependent feedback inhibition of insulin like growth factor 1 (IGF-1) signalling (Ding *et al*., 2016).

No clear hormonal control has yet been detected for being involved in the recruitment of the primordial follicle. However, estradiol has been shown to increase *PTEN* expression in estrogen receptor (ER) positive cells (Yang *et al*., 2011) and androgen receptor (AR) signaling suggested to inhibit *PTEN* transcription in prostate and breast cancers (Wang *et al*., 2012). It is intriguing that both the ER signaling pathway and

androgen signaling pathway were enriched in oocytes from primordial follicles and the estrogen receptors (*ESR1* and *ESR2*) as well as *AR* were expressed at a low level suggesting importance of these pathways in the oocytes from the primordial follicle potentially involved in the PTEN regulation and other pathways leading to the activation of growth in the primordial follicle. Androgens have been suggested to be involved in activation of primordial follicles in the primate ovary (Vendola *et al.*, 1999). Estrogen has been shown to be involved in the primordial follicle formation in mice (Britt *et al.*, 2004) while the function of ER in the oocyte in the human primordial follicle remains to be elucidated. The enrichment of AR and ER signaling in the transcriptome of the oocyte in the primordial follicle have been further confirmed in a recent study from our group comparing the transcriptomes of the oocytes from primordial and primary human follicles (Ernst *et al.*, 2017).

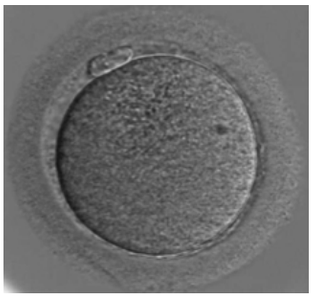
Growth factors

Certain controversies exist in the literature regarding how the transforming growth factor beta (TGFB) super family is involved in the activation of the primordial follicle (Rossi *et al.*, 2016). In agreement with the TGFB super family members being key regulators of the folliculogenesis and oogenesis (Trombly *et al.*, 2009) following activation, the transcripts of family members BMP15 and *GDF9* (growth differentiation factor 9) were absent or marginally expressed in the oocytes from the primordial follicle. Absence of significant *GDF9* transcripts in the oocyte in the primordial follicle confirms previous immune-histochemical data in human ovaries (Aaltonen *et al.*, 1999), and absence of *BPM15* expressiom in the early human follicles has been further confirmed (Kristensen *et al.*, 2014). Several of the receptors from the TGFB family, bone morphogenetic protein receptor types 1A, 1B and 2 (*BMPR1A, BMPR1B, BMPR2*), activin A receptor type 2A and 2B (*ACVR2A, ACVR2B*) were, however, expressed in the oocyte of the primordial follicle as were several of the *SMADs* involved in the intracellular signalling, thus the oocyte appears to be able to respond to members of the TGFB family. The only one of the *BMPs* expressed in the primordial oocyte was *BMP6*, while recent publication also reported expression of *BMP1,2,3,4* and *8B* in the early stages of the human follicle (Kristensen *et al.*, 2014) indicating production in the somatic compartment and possible regulators of the transition from primordial to primary stage (Knight and Glister, 2006). Notably, the transcript encoding the SKIL protein with vital roles in murine follicle development and acting as inhibitor of TGFB signaling (Xu *et al.*, 2009) was selectively expressed in the oocytes from primordial follicles.

Epigenetics and genetics

In the female germ line, initiation of imprinting occurs after birth during oocyte growth (Hiura *et al.*, 2006) (Kota and Feil, 2010). Many well-known epigenetic genes were expressed in both oocytes from primordial follicle and in MII, such as the DNA methyltransferase 1 (*DNMT1*) and histone deacetylase 1 (*HDAC1*) (III). Among the highest expressed genes in the oocyte from the primordial follicle was FOLR1 contrasting a marginal expression in MII, suggesting a function in early oogenesis. Folate-deficiency has been shown to induce DNA breakage in various cell types in vivo and in vitro as well as cause genomic hypo-methylation in humans and in cells in culture (CROTT *et al.*, 2008). Thus, the high expression of *FOLR1* may be involved in protection of the DNA as well as in sustaining appropriate levels of methylation. Moreover, *FOLR1* genotypes has been related to oocyte fertilization rate, treatment outcome and risk of pregnancy loss in women undergoing IVF treatment connecting *FOLR1* to oocyte developmental competence (Sivakumaran *et al.*, 2010). Regulation of *FOLR1* expression in other cell types has recently been shown to be under exquisite control of steroid hormones (Sivakumaran *et al.*, 2010) thus, the canonical steroid signaling pathways enriched in the oocytes from the primordial follicles may also involve regulation of the *FOLR1* expression (III).

It can be speculated, that the adverse effect of insufficient folate intake impairing fertility in animals (Mohanty and Das, 1982) and inducing adverse pregnancy outcomes in human (early spontaneous abortions and birth defect) (George *et al.*, 2002) may involve the highly expressed *FOLR1* in early oogenesis, as daily pre-conceptional supplementation of folate is recommend by WHO.



Human MII oocyte. *Diameter: 130µm*

4.1.2 The transcriptome of the MII oocyte (II and III)

High transcriptional activity in the oocytes throughout follicular development ensures storage of large quantities of mRNA in an inactivated form (selective cytoplasmic polyadenylation of the mRNAs) for later recruitment for resumption of meiosis, fertilization and early embryo development. We have by analysis of the transcriptomes from 15 MII oocytes from 15 women aged 27-39 years provided a picture, a snapshot of the mRNAs stockpiled in the human oocyte at the time of ovulation. Comparing the MII transcriptome with the transcriptome of the immature oocyte of the primordial follicle allow us to highlight overexpressed genes in the mature oocyte, genes being activated somewhere along the path of oogenesis after activation in the primordial follicle and stored for later use (III).

Major findings (II and III)

- A total of 7,470 genes were expressed in the human MII oocyte.
- Main functional categories enriched in these genes were 'cell cycle', 'cell division', 'chromosome segregation', 'electron transport chain', 'establishment of protein localization' and 'establishment of RNA localization'.
- 1,456 genes were more than 10-fold higher expressed as compared to the immature oocyte in the primordial follicle.
- Main functional categories enriched in these genes were 'DNA replication recombination and repair' and 'Cellular growth and proliferation', 'Microtubule organizing center' and the top signalling pathways was 'mitotic roles of polo-like kinases' and the ERK/MAPK pathway.
- Top Hallmark gene sets highly enriched (FDR<$1.6x10^{-14}$) were 'E2F Targets' (primarily involved in the G1/S transition), 'G2M DNA damage Checkpoint', 'P53 Pathway' and 'MTORC1 signaling' highlighting the overexpressed genes involved in cell cycle regulation.
- High expression of known oocyte genes, i.e. *GDF9*, *BMP15* and MOS proto-oncogene, serine/threonine kinase, MOS, were among the genes which were absent in the oocyte in the primordial follicle with mRNA highly accumulated during oogenesis.
- And new genes in oogenesis were discovered i.e. bi-orientation of chromosomes in cell division 1 (*BOD1*) involved in correct chromatid separation and ubiquitin like with PHD and ring finger domains 2 (*UHRF2*) involved in cell cycle regulation and coupling between the cell cycle network and the epigenetic landscape.

The enriched functions and signalling pathways are in accordance with the major orchestrated functions of meiosis, mitosis and cytokinesis that the oocyte has to sustain until the new genome become transcriptionally active between 4 and 8 cell stage of the embryonic development. The high enrichment of 'electron transport chain' likely reflect the high number of mitochondria present in the mature oocyte ensuring the energy for the process (Darbandi *et al.*, 2016).

Maternal RNAs

How oocytes store and subsequently activate mRNA for translation is not well understood. Decapping mRNA 1A, (*DCP1A*), known to be a part of the mRNA processing-bodies in somatic cell, re-localize to subcortical aggregates as the mice oocyte meiotically matures and the products of the maternal mRNAs are needed (Flemr et al., 2010). In accordance, the *DCP1A* transcript was found in our data to be 35 times higher expressed in the human MII oocytes as compared to the oocytes from primordial follicles. Additionally, *CPEB1* (cytoplasmic polyadenylation element binding protein 1) encoding a protein that modulates poly(A) tail length was highly overexpressed in human MII oocytes and may be involved in the post-transcriptional and post-translational regulation as suggested in mouse oocyte meiotic maturation and in maternal-to-zygotic transition in (MTZ) (Sha *et al.*, 2017).

BTG anti-proliferation factor 4, (*BTG4*) was selectively and highly expressed in the human MII oocyte

confirming findings in bovine and mice MII oocytes (Vallée *et al*., 2005) and interestingly BTG4 has recently been identified as a meiotic cell cycle–coupled MTZ licensing factor fostering mRNA degradation by promoting RNA de-adenylation in maturing mice oocytes (Yu *et al*., 2016).

Cell cycle regulation

Comparison between the transcriptomes of the two oocytes stages provided data on the transcripts stockpiled in the MII oocyte during oogenesis. Subjecting the 1,456 genes meeting the criteria to be more than 10-fold upregulated in the MII as compared to the early oocyte to function enrichment 'Cell Cycle' was among the top functions represented as was gene sets for 'E2F Targets' (primarily involved in the G1/S transition), 'G2M DNA damage Checkpoint', 'P53 Pathway' and 'MTORC1 signalling' further underlining the multitude of functions the transcripts are to undertake.

Thus, the enriched signalling pathways revealed the mature stage of the oocyte being prepared to development. The enriched 'mitotic roles of polo-like kinases' pathway involved the anaphase promoting complex, sev-eral cell division cycle proteins (*CDC7*, *CDC20*, *CDC25*), checkpoint kinase 2 (*CHEK2*) and the dominant cyclin B (*CCNB1*) as well as the up-regulation of ESPL1 (extra spindle pole bodies like 1, separase) and pituitary tumor-transforming 1, (PTTG1, securin) revealing genes likely to be important in promotion of meiosis, mitosis and cytokinesis upon fertilization. In addition to genes (i.e. *GDF9*, *BMP15* and *ZAR1*) previously reported as expressed in MII oocytes in other species (Vallée *et al*., 2005) several genes, not previously reported expressed in oocytes were highly expressed in the MII oocyte. In example, among the genes with the highest expression level is the BOD1 that encodes a kinetochore protein and of utmost importance for proper chromosome bi-orientation, and correct chromatid separation (Porter *et al*., 2013) suggesting a role in the second meiosis and the subsequent mitosis. Tubulin beta 8 (*TUBB8*) expression was absent in the somatic cells of the follicle, low expressed in oocyte in the primordial follicle and among the 25 highest expressed genes in the MII and has recently been revealed as an oocyte specific tubulin and cases with meiosis failure due to mutation in the coding region of *TUBB8* has been reported (Feng *et al*., 2016).

UHRF2, a candidate tumor suppressor, showed high and selective expression in the MII oocyte and interestingly UHRF2 is reported to interact with cyclins, CDKs, p53, PCNA (proliferating cell nuclear antigen), HDAC1, DNMTs and methylated DNA and suggested to contribute to the coupling between the cell cycle network and the epigenetic landscape (Mori *et al*., 2012).

Enrichment of the ERK/MAPK pathway and the selectively high expression of MOS in MII oocytes further enforces that the MOS–MAPK enzyme system is a critical regulator of the meiotic processes including the resumption of meiosis and cycle arrest upon fertilization (Dupré *et al*., 2011). The unique task of the MII oocyte in first holding and subsequently progressing in the cell cycle is reflected in the ingredients; the upregulated expression of genes decreasing and increasing cell cycle progression, respectively.

The transcriptome of the competent oocyte

Based on published CC marker genes for ploidy (Fragouli *et al*., 2012) and our transcriptome data on the CC corresponding to 12 of the 15 MII oocytes, we succeeded in categorizing 6 of these 12 MII into 'aneuploid' (3) and 'euploid' (3), while six clustered in between. Hence the number of observation in the two groups was limited and the findings need to be validated in a larger scaled study. 1,871 genes were differentially expressed ($p<0.05$) between the 'aneuploid' and 'euploid' MII of which 236 had fold-change above 2 (Borup et al., 2014). Functional enrichment analysis of the differentially expressed genes showed increase in 'microtubule-dynamics' ($p<0.001$), 'cell-viability' ($p<0.01$) and 'RNA-transactivation' ($p<0.01$) activity in 'euploid' as compared to 'aneu-ploid', while enrichment of genes related to mitochondrial function was upregulated in 'aneuploid' oocytes as compared to 'euploid' ($p<0.01$). Interestingly and in line with this finding, recent study suggests that elevated mtDNA levels in the trophectoderm biopsy, above a defined threshold, are strongly associated with blastocyst implantation failure and represent an independent biomarker of embryo viability (Ravichandran *et al*., 2017).

Among the genes with highest positive fold-change in the 'euploid' oocytes as compared to the 'aneuploid' were *LIN28A* (fold=4.6; $p<0.01$) and *LIN28B* (fold=4.5; $p<0.01$). LIN28A+B are regulators of micro-RNAs and mRNAs and involved in pluipotency. The gene with the highest down-regulation in 'euploid' as compared to 'aneuploid' oocytes was the

imprinted maternally expressed H19 (fold=-11.8; p<0.05). Moreover, *MTHFR* (methylenetetrahydrofolate re-ductase), *MBD1* (methyl-CpG binding domain protein 1) and MTRR (5-methyltetrahy-drofolate-homocysteine methyltransferase reductase), all involved in DNA-methylation, were differentially expressed (Borup *et al*., 2014).

4.1.3 The effect of age on the transcriptome of the MII oocyte (II)

The developmental competence of human early embryos declines with increasing age due in part to increase in aneuploidy. The errors that lead to aneuploidy almost always occur in the oocyte but, despite intensive investigation, the underlying molecular basis has remained elusive (Nagaoka *et al*., 2012). Mechanisms involved in the high frequency of meiotic error in human oocytes have recently been suggested to be connected to recombination not only affecting homolog segregation at meiosis I but also in the fate of sister chromatids at meiosis II (Ottolini *et al*., 2016).

In order to expand our knowledge regarding the molecular events, linked to competence and age, we compared the mature oocyte transcriptome from a group of women with an advanced reproductive age (between 37 and 39, mean: 37.8 years) and a younger patient group (≤35 years; mean: 31.1 years).

Major findings (II)

- 342 genes, representing 4.6% of the MII transcriptome, many of which appear to be of importance for the development of the fertilized oocyte, are significantly differentially expressed (>1.5-fold) between the age groups.
- Highest enriched functional networks in the list of differentially expressed genes were 'Organism development/cell cycle', 'Cell Death/Inflammatory disease/cancer', 'Cell assembly and organization/ Molecular transport, protein trafficking' and 'DNA Replication, Recombination and Repair'.
- Top Hallmark gene sets enriched (FDR<$4.5x10^{-4}$) in the genes affected by age were 'Mitotic spindle' and 'G2M checkpoint'.
- *SMAD2*, central in the 'organism development and cell cycle' network was down-regulated 2.8-fold in the old MII oocytes.
- *ESPL1*, separase, involved in sister chromatid separation was up-regulated (1.6-fold) in the older oocytes compared to the younger oocytes.
- Essential meiotic endonuclease 1 (*EME1*) contributing to chromosome stability and euploidy were 1.6-fold down regulated in the older oocytes compared to the younger.
- RPTOR independent companion of MTOR complex 2 (*RICTOR*), was 1.5-fold down regulated in the older oocytes compared to the younger.
- Genes involved in Ca^{2+} oscillations capacity (*ITPR1*, *CAMK2G*) were affected by age.

Thus, considerable difference in the gene expression profile of human MII oocytes between the younger and the older age group was found (125 genes up- and 217 down-regulated by age). Importantly, functional enrichment of 'Cell Death/Inflammatory disease/cancer' network in the differentially expressed genes is in agreement with a general age induced increase in deleterious inflammatory disease and cancer (Fulop *et al*., 2016).

In the following some genes and enriched functions in the list of genes differentially expressed between the two age groups are highlighted and discussed in relation to key mechanisms and tasks of the MII oocyte.

Cell cycle

One of the most pronounced differences was the number of transcripts annotated to be involved in the cell cycle regulation and the functional network most significantly affected by age was the network denoted 'organism development and cell cycle' with more than 75% of the pathway affected by age. The network represents several pathways in the TGFB signalling, well-known to be operative during mammalian pre-implantation development and in embryonic stem cell (Sudheer and Adjaye, 2007). SMAD2, that was down-regulated by 2.8-fold with increasing age, is centrally placed in the network, and shown to be essential for early embryonic development in mice (Li and Nomura, 1998). Down regulation of SMAD2 in aged oocytes has also been reported in mice (Pan *et al*., 2008). Inhibitory phosphorylation of CDKN1 is central in the prolonged meiotic arrest of female germ cell (Adhikari et al., 2016) and dosage of Cdk inhibitor 1C (CDKN1C), controlling cell cycle progression, has been shown to be essential for mouse embryogenesis (Zhang *et al*., 1997).

CDKN1C was more than 3-fold decreased in the oocytes from mature patients aged 37-39 years as compared to the younger patients, which may be involved in deregulation of the cell cycle by increasing age. A trend towards slower progression in the first cell cycles in embryogenesis with increasing age of the woman has recently been suggested by our team (Grøndahl *et al.*, 2017).

RICTOR, a component of the mechanistic target of rapamycin complex 2 (mTORC2) controlling cytoskeletal organization and cell survival, in oocytes, regulates folliculogenesis, and its inactivation causes premature ovarian failure in mice (Chen *et al.*, 2015). RICTOR was 1.5-fold lower expressed in the aged MII oocytes as compared to the young. Interestingly both age and reactive oxygen species have been shown to induce RICTOR depletion, and reduced survival in osteoblasts (Lai *et al.*, 2016).

DNA stability, spindle checkpoint regulation and chromosome segregation

In the fetal ovary cohesion is established during pre-meiotic DNA replication which has to be maintained up to 50 years, until meiosis is completed. Gradual loss of cohesion during these long periods of time is thought to be a major cause of the maternal age effect of oocyte aneuploidy (Hunt and Hassold, 2010). This sister chromatid cohesion resists spindle pulling forces, thereby enables the generation of tension, which stabilizes microtubule–kinetochore attachments and therefore is essential for chromosome bi-orientation. Once all chromosomes have become bi-oriented, cohesion is destroyed and sister chromatids are segregated by the meiotic or mitotic spindle. In line with age-induced increase in aneuoploidy top hallmark gene sets enriched in the genes affected by age were 'Mitotic spindle' and

The human oocyte and granulosa cell transscriptomes

Figure 1. 1-3: Genes expressed in the oocytes at the beginning[1] and end[2] of folliculogenesis, and the difference between the two stages. Genes influenced of the women's age[3] at the pinnacle of development in MII. **4**: Genes differentially expressed in granulosa cells before and after ovulation induction.

[2]MII oocyte (7,470, **1,456**)

C-MOS, BMP15, MELK, PNAC, DPPA5, TCL1A, BOD1, EGF, BTG4, CHEK2, CCNB1, ESPL1, PTTG1, HAS3, TUBB8

- Cell cycle, DNA replication, Cellular growth and proliferation, Mitotic roles of polo-like kinases, P53 Pathway and MTORC1 signalling

[3]MII oocyte and Age (**217**, **125**)

ACVR1B, UBE1, SPIN3, HAS3, G6PD, ESPL1, ESCO1, CSPP1, CEP85 SMAD2, RICTOR, EME1, PTEN, ANAPC4, GJAL7, PLAC1

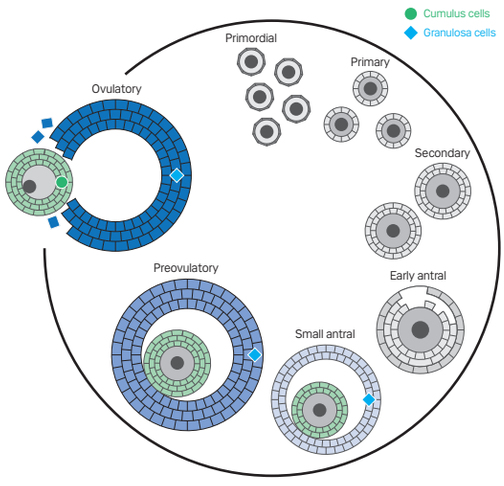
- Mitotic cell cycle, Spindle organization, DNA repair, Metabolic process

Number of genes expressed in the cell types are marked with **BLACK** print. Number of genes upregulated in the compartment or condition are marked in **RED**, while **BLUE** represent the number of down regulated/lower expressed genes. Selected genes are shown in matching colors and the top function enrichment of the differentially expressed genes are shown with bullet points.

[1]Oocyte in the Primordial follicle (6,301, **729**)

CDR1, RELN, SMARCA2, PNN, BUB1, TMEFF2, FOLR1, OPHN1, ABO, CHRNA3, DSCAM, DCC, NTNG2, P63, CCNK, PIK3C2A, EZR

- Gene expression, Cell cycle, Cellular organization and maintenance, PIK3/AKT- signaling



[4]Granulosa cells and ovulation (**614**, **572**)

LHCGR, FSHR, HSD11B2, ERS1+2, IGF2, CYP19A1, INHA, PCNA, CCNA, TOP2A

- Cyclins and Cell cycle regulation, Mitotic roles of polo-like kinases, Estrogen mediated S-phase entry

CD24, AREG, EREG, CLDN1, PTX3, NTS, HSD11B1, FN1, F2R, TGFB1, OXTR, ADAMST1

- Role of tissue factor in cancer, Coagulation system, Acute Phase response signaling

'G2M checkpoint'. Genes coding for proteins involved in spindle checkpoint regulation, DNA stability and chromosome segregation were influenced by age.

Overexpression of *Espl1* induces premature separation of chromatids, lagging chromosomes, and anaphase bridges (Zhang *et al*., 2008). *ESPL1* was up-regulated (1.6-fold) in the older oocytes compared to the younger oocytes. The gene, *ESCO1* (encoding Establishment of cohesion homolog 1), essential to the establishment of sister chromatid cohesion (Zhang *et al*., 2016) was also higher expressed by age, further indicating an altered composition of genes transcripts involved in chromosome segregation. The spindle check-point ensures accurate chromosome segregation by delaying anaphase in response to misaligned sister chromatids during meiosis and mitosis. Upon checkpoint activation, MAD2 binds directly to CDC20 and inhibits the anaphase-promoting complex (Xia *et al*., 2004). A protein, the Mad2l1BP (mitotic arrest deficient 2 like 1 binding protein), counteracts the function of MAD2 and is required for the silencing of the spindle checkpoint (Xia *et al*., 2004). This *MAD2L1* binding protein gene was in our study observed to be up-regulated (1.6-fold) by age in the MII oocytes. Interestingly the *MAD2L1* expression in 2PN (2 pronuclei) zygotes has recently been correlated to viability (Yanez *et al*., 2016).

EME1 contributes to chromosome stability and euploidy in human cells (Hiyama *et al*., 2006). Small decreases in gene dosage of EME1 promote re-replication and polyploid cells in addition to chromosome aberrations as DNA breaks (Hiyama *et al*., 2006). EME1 expression was down-regulated (1.6-fold) in older oocytes compared to younger, which may increase the rate of cleavage stage embryos with polyploid blastomeres.

CSPP1 (centrosome and spindle pole associated protein 1) encodes a protein playing a role in cell-cycle progression, spindle organization and cytokinesis was higher expressed in the older oocytes. Interestingly, single nucleotide polymorphism in *CSPP1* has recently been reported to have strong association to fertility in cows (Ortega *et al*., 2017).

In a large multicentre cohort study on the effect of women age on standard oocyte and embryo assessment data from more than 100,000 oocytes and 42,000 embryos we found that the rate of 3PN zygotes (in both IVF and ICSI) was significantly increased by increasing age, which may reflect the ultimate incompetent spindle failing to extrude the second polar body (Grøndahl *et al*., 2017). In contrast, age did not impose a significant impact on neither blastomere size, symmetry or fragmentation confirming that the morphological evaluation of the cleavage stage embryo fail to reflect the chromosomal status of the embryo (Fragouli *et al*., 2014).

Thus, several transcripts involved in actually getting the chromosomes positioned and separated correctly are differentially expressed between the two age groups.

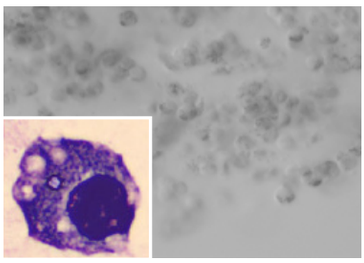
Oocyte activation

Upon sperm–oocyte binding and fusion, a sperm-specific phospholipase C triggers an Inositol(1,4,5)P3-mediated increase in intra-cellular Ca^{2+}, which activates calci-um/calmodulin-dependent protein kinase 2 (CAMK2) leading to recruitment of the maternal mRNAs, initiating DNA repair and resumption of the cell cycle (Martin *et al*., 2017). ITPR1 (inositol 1,4,5-triphosphate receptor, type 1) is important for initiating the Ca^{2+} oscillations that lead to the activation of the oocyte upon fertilization. Decreased *ITPR1* expression level in non-viable human 2PN zygotes as compared to viable zygotes has recently been reported (Yanez *et al*., 2016). Notably, we observed that moderately expressed *ITPR1* (7-fold increase in comparison to the primordial stage) in the human MII oocytes was decreased almost 2-fold in the oocytes from the mature patient group pointing towards altered Ca^{2+} oscillations capacity. The transcripts of calcium/calmodulin-dependent protein kinase 2 gamma (*CAMK2G*) was significantly stockpiled (>6-fold) during oogenesis and was higher expressed (1.7-fold) in the mature patients MII oocytes as compared to young patients. In mice CAMK2G is involved in promoting the cell cycle resumption in MII (Medvedev *et al*., 2014). Whether oocytes from reproductive aged women have an impaired Ca^{2+} homeostasis needs further clarification.

Impaired Ca^{2+} homeostasis has been connected to post ovulatory aging of mice oocytes displaying characteristic hallmarks of oxidative stress suggested to resemble that of increased maternal age (Martin et al., 2017). To further support this hypothesis, expression of *Smad2* and *Has3* in murine MII were reported to decrease and increase, respectively, with increasing post ovulatory age (Zhang *et al*., 2013), corresponding to the changes in human MII oocytes with increasing age of the woman.

The oocyte transcriptome from dormancy in the primordial follicle to metaphase II in the preovulatory follicle

- Study I, II and III, the transcriptome comparisons, listed for the first time the unique gene expressions associated with human oocytes from primordial follicles and MII oocytes. Genes, which are likely to be of significance for essential biological questions related to female reproduction, and candidates for downstream analysis in functional studies. Figure 1[1,2] highlights selected genes from these lists.
- The age of the woman does impose a significant impact on the MII oocyte transcriptome. Figure 1[3] highlights key genes affected by age.



Human granulosa cells from the ovulatory follicle.
Diameter:10-15µm

4.2. Cumulus and Granulosa cell transcriptome (IV-VIII)

The somatic compartments of the human follicle sustain throughout folliculogenesis signalling substances, nutritional support and trafficking of macromolecules to the oocyte and constitutes the auto/paracrine and endocrine ovarian functions ensuring the local two-cell-two-gonadotrophin feedback loop regulating steroid production as well as ovulation of MII oocyte competent for fertilization and embryo development (Eppig *et al*., 2002).

Study IV, V and VI provide insight into the spatial and temporal dynamics in gene expression in the somatic compartment of the human follicle including the hallmark of ovulation. In study VII, VIII we have made observations as how the somatic cell transcriptome is influenced by procedures of ART.

4.2.1 Cumulus and granulosa cell tran-scriptomes: differences and similarities (IV, V)

The somatic compartment of the follicle starting as one population of pregranulosa and upon activation becoming granulosa cells proliferating and subsequently differentiating at the time of antrum formation to become two highly specialized cell types. Mural granulosa cells (MGC) mainly engaged in estradiol and growth factor production both with autocrine, paracrine and endocrine effects ensuring the progress in folliculogenesis. And cumulus granulosa cells (CC) primarily ensuring nutrients and signals to the oocyte. Substantial temporal change in granulosa cell gene expression along growth of the follicles exist in both poly- (Munakata *et al*., 2016) and mono-ovulatory (Hatzirodos *et al*., 2014) species including women (Borgbo *et al*., 2016). In study IV and V, a bolus of 10.000IU hCG brings a supra-physiological activation of the LH receptor inducing a huge change in task of the cell compartments. MGC are induced to prepare for ovulation and form the CL, while CC mediates the resumption of meiosis and prepare for interaction with the coming environment, the oviduct and the spermatozoa. Despite the supra-physiologic induction potentially erasing details in difference, we find substantial differences between the transcriptomes of the two correspondent cell types. We speculate that this reflects a substantial difference also in the follicular phase before the ovulation induction.

Our data highlight genes differentially and selectively expressed contributing to elucidate specific functions of the two somatic cell types just prior to ovulation.

Major findings (Study IV and V)

- In MGC 11,739 genes fulfilled the criteria for being expressed.
- In CC 11,090 genes fulfilled the criteria for being expressed.
- Unsupervised hierarchical cluster analysis revealed that the samples clustered within each of the two cell types demonstrating general difference in the transcriptome.
- The pair wise (within individual follicle) comparison between MGC and CC defined the following:

A. 1,562 genes being differentially expressed representing the enriched functions of angiogenesis, steroid hormone receptor activity, inflammatory response and growth factor binding.
B. 85 genes being exclusively present in MGC, representing the enriched functions of mitochondrion

and hydrolase activity. In example, arylsulfatase G (*ARSG*), aldehyde dehydrogenase 5 family member A1 (*ALDH5A1*), carbonic anhy drase 5B (*CA5B*).

C. 61 genes being exclusively present in CC, representing the enriched functions of extracellular region, cell communication and channel activity. In example, *AMH*, ryanodine receptor 2 (*RYR2*), calcium voltagegated channel subu-nit alpha1 *C (CACNA1C)*.

- Genes coding for extracellular matrix and its modulators were highly differentially expressed, Tenascin C (*TNC*) were dominating in CC and marginally present in MGC, Fibrinogen gamma chain (*FGG*) was highly and selectively expressed in MGC. The matrix metallopeptidases *MMP16* and *MMP10* were selectively expressed in CC and MGC respectively.
- *AMH* expression and synthesis was compartmentalized with higher expression in CC than in MGC in both small antral and in pre-ovulatory follicles and furthermore *AMH* was significantly higher expressed in compacted CC compared to those that had expanded.

In the following individual genes and key enriched functions in the lists of differentially expressed genes are discussed.

Steroidogenesis and receptors

Central genes in steroidogenesis, i.e. Steroidogenic acute regulatory protein (*STAR*) were among the highest expressed genes in both cell types, though with differences in the first and rate-limiting step in the synthesis of the steroid hormone, cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*), being significantly higher expressed in MGC confirming and extending that MGC is the main follicular producer of steroids close to ovulation. *CYP19A* (aromatase) was expressed at a lower level and showed 6-fold higher expression in CC as compared to MGG, which may reflect differences in response to the ovulation trigger inducing reduction in *CYP19A* expression (VI). Progesterone production in COC has been connected to oocyte developmental competence (Bar-Ami and Gitay-Goren, 2000) and progesterone has been reported to have positive chemotaxis for spermatozoa (Lishko *et al*., 2011). The observed expression of *CYP19A* in CC indicates production of estradiol with possible local effect inducing increased activity in the oviduct epithelial cilia as recently shown (Zhu *et al*., 2016). *FSHR* was absent in both compartments confirming finding in non-human primate follicles (Xu *et al*., 2011) (Jeppesen *et al*., 2012) while *LHR* were expressed at the same level indicating an ongoing responsiveness to LH in both cell types at this very late stage of folliculogenesis. The estrogen receptor 1 (*ESR1*) was 18-fold higher expressed in MGC compared to CC, while the androgen receptor (*AR*) was higher expressed in CC, both confirming a study comparing pooled samples of CC and MGC collected at OPU (Kõks *et al*., 2010). A potential role of AR signaling in the CC in the nearest proximity to oocyte has also been suggested in a porcine study (Hickey *et al*., 2004).

Cell-cell communication

The special close communication between the cells of the follicle involving GAP junctions as well as adhering and junctional proteins ensure optimal and fast signaling within and between the cell types. Gap junction protein alpha 1 (*GJA1*, also known as connexion 43 (*CX43*)) and *GJA7* (*CX45*) were present in all cell types, MGC, CC and the connecting MII oocyte (II) confirming previous studies (Wang *et al*., 2009), while *GJA5 (CX40)* was selectively expressed in the CC, indicating a specific function in this compartment. Whether the function includes heteromeric gap junction and functional suppressive interactions with GJA1 as descried in other tissues (Beyer et al., 2013) remains to be elucidated.

Among the selectively and highly upregulated genes in CC as compared to MGC were several central regulators of intracellular cell signaling involving Ca^{2+} as *RYR2, CACNA1* previously described to regulate Ca^{2+} homeostasis in another syncytium, the syncytium of myocardial cells (Jurkat-Rott and Lehmann-Horn, 2004). The calmodulin-binding functional category being highly significantly present in the CC further underline that Ca^{2+} regulation has a special role in the communications between the cells in the COC. It is established that the mid-cycle surge of gonadotropins induce resumption of meiosis through gap junction signaling (Toranzo *et al*., 2007) while the role in and which key ions and molecules passing through the functional syncytium of the mature COC remains unclear.

Extracellular matrix formation and remodelling

Extracellular matrix (ECM) has many different roles including effects on cell adhesion, cell shape, migration, division and differentiation and continuous remo-

delling of the ECM is a central mechanism in folliculogenesis and CL formation (Thorne *et al.*, 2015).

Both CC and MGC expressed highly and differentially extracellular matrix and matrix modulator genes preparing for the specialize function for ovulation and fertilization, respectively. In example fibrinogen gamma chain (*FGG*) was highly expressed in the MGCs only. Extrahepatic production of FGG in the human granulosa cells has been describes previously and the role suggested to be as well initiation of fibrinolysis preparing for ovulation as ensuring clotting upon ovulation (Parrott *et al.*, 1993). However, *FGG* expression wasn't significantly changed by the ovulation induction (VI) suggesting a role in the follicle before ovulation as well.

Surprisingly, pepsinogen was selectively and highly expressed in the MGC, suggesting a specific role in these cells. The role of this extra-intestinal expression of pepsinogen in the ovulatory process and whether there is a pepsin-like activity needs further investigation.

In CC, *TNC* (Tenascin-C) expression was dominating and up 50-fold higher expressed in CC confirming findings in mice pre-ovulatory follicle with CC showing selective expression (Hernandez-Gonzalez *et al.*, 2006). TNC has many extracellular binding partners, including matrix components and soluble factors and TNC exhibits an extraordinarily wide range of functions (Midwood *et al.*, 2016), including a potential role in the mature COC, the late maturational process of the oocyte or the expanded cumulus cells preparing for meeting the oviduct and the spermatozoa.

Anti Müllerian Hormone (AMH) in the late folliculogenesis

AMH, a glycoprotein of the TGFB superfamily, is primarily produced in the granulosa cells of developing, non-atretic follicles and one of its roles have been suggested to inhibit primordial follicle activation, slowing the rate at which the ovarian reserve is depleted (Pankhurst, 2017). However, some controversy exists in the literature as other studies show that AMH stimulates human primordial follicle activation (Schmidt *et al.*, 2005) and primate pre-antral follicle growth in vitro. The total follicular content of AMH peaks in follicles with a diameter of around 8mm (Jeppesen *et al.*, 2012) just around follicular selection. AMH appears to down-regulate *CYP19* expression and estradiol production and AMH has been suggested to act as a gate-keeper to prevent small antral follicles from producing estradiol which may interfere with the fine-tuned interplay between the selected follicle and the pituitary (Jeppesen *et al.*, 2012) (Dewailly *et al.*, 2014). Our data confirm and extends data from sheep (Campbell *et al.*, 2012). The compartmentalized expression and synthesis of AMH and its receptor suggests that AMH may exert intra-follicular functions even in human large antral and pre-ovulatory follicles and may be related to follicular health.

4.2.2 Granulosa cell transcriptome; identification of new ovulation-related genes (Study VI)

The comparison of transcriptome of MGC from follicles before and after ovulations induction isolated from the same woman in an actual treatment cycle provided us with a unique list of genes related to the intense change in function in the human ovulatory follicle.

Unsupervised principal component analysis of the transcriptomes revealed that the samples clustered within each of the two time-frames and demonstrated significant difference in the transcriptome in the MGC before and after hCG triggering.

Major findings (Study VI)

- 1,186 unique genes were found to be differentially expressed above 2-fold with 572 genes being upregulated and 614 genes downregulated in the MGC 36 hours after ovulation triggering as compared to before the administration.
- Subjecting these lists to gene function enrichment 'cell cycle', 'cancer', 'inflammation' and 'coagulation' were among the functions enriched.
- Of the 5 top Hallmark gene sets highly enriched (FDR<4.5×10^{-24}) were besides 3 dealing with cell cycle regulation 'TNFA signaling via NFKB' and 'Epithelial mesenchymal transition'.
- Expression of cell cycle related genes i.e. *CDC20, CCNB1*, *CCNB2*, *CCNA2* and topoisomerase II alpha (*TOP2A*) were highly downregulated 36h after hCG.
- Genes related to cancer (i.e CD24 protein (*CD24*), claudin 11 (*CLDN11*), inflammation (i.e. prostaglandin E synthase (*PTGES*), interleukin 6 signal transducer (*IL6ST*), solute carrier organic anion transporter family member 2A1 (*SLCO2A1*) and coagulation (i.e. coagulation factor III, tissue factor

(*F3*) and coagulation factor II thrombin receptor (*F2R*), were highly upregulated after hCG.

Exit from cell cycle and change in steroidogenesis

The LH surge in natural cycle as well as the hCG bolus in ART cycle promote the final MGC differentiation: the conversion from a proliferating and estradiol producing compartment to the progesterone producing CL. The 'cell cycle' enriched function represented down regulated proliferation marker genes as *TOP2A* and *CDC20* and upregulated cell cycle inhibitor genes as *CDKN1A* presenting candidate genes involved in the hCG induced inhibition of mitosis prior to ovulation (Fru *et al*., 2007).

The unique pairwise comparison of both the MGC transcriptome and concentrations of steroids in follicles from the same women collected just prior to and 36 hours after ovulation reflected and confirmed the shift from estrogenic to progestogenic follicles after hCG. The decrease in expression of *FSHR* and *LHR* was expected (Xu *et al*., 2011) (Jeppesen *et al*., 2012) and probably reflects receptor down-regulation (Hirsh *et al*., 2005).

Ovulation and cancer-related genes

More than 400 of the differentially expressed genes were connected to cancer (72 of these have been related to ovarian cancer) in the biofunctional enrichment analysis underlining the invasive character of the CL formation and suggesting that some forms of ovarian cancer could originate from ovulatory molecular processes that have gone astray, supported by the fact that ovarian epithelial tumors developed and maintained a milieu rich in pro-inflammatory chemokines and cytokines, reviewed in (Macciò and Madeddu, 2012).

A gene related to cancer invasiveness, *CD24* (Kang *et al*., 2013), showed massive expression following hCG exposure and was a candidate in the *in silico* up-stream regulator analysis. This was a new and unexpected finding and enforces an association between CD24, vascularization and angiogenesis characterizing CL formation. Recently CD24 has been shown to mediate epithelial-mesenchymal transition (EMT) in a hepato-cellular carcinoma model (Wan *et al*., 2016). The process of luteinization is considered to be an EMT (Thorne *et al*., 2015); to elucidate whether CD24 is central in mediating this transition calls for further investigations.

The EGF-family members are central mediators in the induction of the maturation of the oocyte and the ovulatory cascade. The EGF-family members are induced by both LH surge and hCG trigger and propagate the stimulus in a autocrine and paracrine manner in the ovarian follicle (Conti et al., 2006). In support of this notion, our data showed that EGF-like ligands *AREG* (amphiregulin), *EREG* (epiregulin) and *TGFB1* were highly upregulated after hCG and the *in silico* analysis suggested EGF and TGFB1 as upstream activating regulators for the observed response.

TGFB1 has been proposed as a local regulator of microvascular angiogenesis (Maroni and Davis, 2011) and has also been proposed as a facilitator of CL formation in pigs (Sriperumbudur *et al*., 2010). Interestingly, ovarian epithelial cancer cell lines have shown to be refractory to TGFB1 signalling through loss of expression of TGFB1/SMAD4 target F-box protein 32 gene (FBXO32) (Chou *et al*., 2010). FBXO32 was among the highly upregulated genes described here for the first time as being involved in human ovulation. Hence, TGFB1 and its target genes could serve as a control mechanism of the invasive process of CL formation.

Inflammatory response and Extracellular matrix

In line with ovulation being compared to an acute inflammatory reaction in the ovary (Espey, 2006) we demonstrated an upregulation of series of genes involved in inflammation such as immune cell trafficking, interleukin-signalling, tumor necrosis factor a (TNFA) activation and prostaglandin synthesis. *PTGSE* (>2-fold) and *PTGS2* (>13-fold) was highly up-regulated and one of the highest up-regulated (>27-fold) and expressed genes was *SLO2A1*, a PG transporter. Interestingly, blockage of the SLO2A1 has recently been shown to inhibit ovulation in a mice model suggesting a key role of this transporter in mediating ovulation and that its inhibitors may be viewed as potential candidates for non-hormonal contraception (Yerushalmi *et al*., 2016). An increased expression of coagulation factors *F3* and *F5* promoting coagulation in the microvasculature, extended previous findings in mice of increased F3 expression shortly after hCG triggering (Carletti and Christenson, 2009). The coagulation factors might prevent micro-bleeding during ovulation.

Many of the genes highly upregulated 36 hours after administration of the hCG trigger encodes proteins involved in extracellular matrix formation (i.e. hyaluronan synthase 2 (*HAS2*), *PTX3*, fibronectin leucine

rich transmembrane protein 2 (*FLRT2*), collagen type V alpha 2 chain (*COL5A2*), neural cell adhesion molecule 1 (*NCAM1*), fibronectin 1 (*FN1*), laminin subunit alpha 2 (*LAMA2*), laminin subunit beta 1 (*LAMB1*)) and modula-tion (i.e. ADAM metallopeptidase with thrombospondin type 1 motif 1 and 9 (*ADAMST1*, *ADAMST9)*, *MMP19*).

FN1, encoding a glycoprotein present in a soluble dimeric form and in multimeric form in extracellular matrix and reported to be involved in cell adhesion, growth and wound healing (Marzotto *et al*., 2016), was highly expressed in the MGC after hCG and more than 7-fold higher than before triggering. This confirms report on increased FN1 expression level in bovine granulosa cells 21 hours after LH administration (Christenson *et al*., 2013) and data from murine folliculogenesis showing FN1 primarily in the FF of pre-ovulatory follicles (Berkholtz *et al*., 2006).

The gene encoding neurotensin (*NTS*), a secreted tridecapeptide, were more than 15-fold higher expressed after the hCG bolus, while inconsistent and very low expression in CC as compared to the corresponding MGC (IV). Neither of the two NTS receptors (*NTSR1* and *2*) were expressed in the CC, MGC or the corresponding oocyte (II) indicating an effect elsewhere. NTS expression are in tumors related to expression of matrix metallopeptidases and invasiveness (Younes *et al*., 2014). Whether these matrix modulating effects of NTS are involved in the formation of CL remains to be elucidated. Interestingly, recent murine and bovine studies report that Ntsr1/ NTSR1 is expressed in spermatozoa and NTS present in the female reproductive tract capable of inducing sperm capacitation (Hiradate *et al*., 2014) (UMEZU *et al*., 2016). The authors suggested that NTS act as a promoter of sperm capacitation and acrosome reaction. Our observation on hCG inducing high NTS expression in MGC may suggest that follicular fluid NTS content provides the female reproductive tract with TNS for activation of the spermatozoa, although this is still speculative.

4.2.3 Cumulus and granulosa cell transcriptomes and ART; effect of OS and ovulation trigger regimens (Study VII and VIII)

Clinical trial comparing regimens for OS, with comparable number of oocyte and clinical outcome, have shown difference in the endocrine profiles during follicle stimulation as well as composition of the FF suggesting difference in the activity of the somatic compartment of the follicle (Smitz *et al*., 2007). We were the first to demonstrate that two standard regimens for OS (rFSH vs hMG) do impose a different footprint on the gene expression profile of the MGC in the pre-ovulatory follicle.

Triggering of final oocyte maturation following OS can be performed with either a bolus of hCG or a bolus of gonadotrophin releasing hormone agonist (GnRHa) in patients co-treated with a GnRH antagonist for IVF and ICSI. Initial trial comparing the two methods for triggering revealed increase in oocyte maturation potential (MII rate) in the GnRHa group, while the luteal phase after GnRHa triggering were highly insufficient (Humaidan *et al*., 2005). GnRHa trigger for final oocyte maturation is now used routinely to reduce the risk of ovarian hyper stimulation syndrome (OHSS) and after establishment of modified luteal phase with either low dose of hCG, hLH or GnRHa adequate reproductive out-comes have been ensured (in review (Dosouto *et al*., 2017)). The detailed comparisons between the two triggering protocols showed differences in the peri-ovulatory, luteal endocrine as well as FF growth factor profile (Humaidan *et al*., 2005) (Andersen *et al*., 2006). In order to further understand the observed effect during the peri-ovulatory and early luteal phase, we performed a prospective randomized trial investigating whether the mode of triggering was reflected in the transcriptome of the somatic cells of the follicle.

Major findings (Study VII and VIII)

Ovarian stimulation

- The different hormone composition of the two drugs used for OS had a significant impact on the gene expression profile of MGC. 85 genes were differentially expressed in MGC between OS with rFSH and hMG. 'Lipid me-tabolism' was highly enriched in these genes representing central genes in cholesterol and steroid biosynthesis i.e. lanosterol synthase (*LSS*) and ATP citrate lyase (*ACLY*).
- Expression level of LH/hCG receptor gene (*LHR*) and genes involved in biosynthesis of cholesterol and steroids were expressed in lower levels in the hMG treated cells. S100-calcium-binding-protein-P (*S100P*) (anti-apoptosis protein) was expressed in higher levels in hMG than in rFSH.

Ovulation trigger

- In CC 391 genes were more than 1.5-fold differentially expressed between the two protocols for triggering.
- Top network enriched in this list of gene was 'Lipid metabolism and Small Molecule Biochemistry' involving i.e. *CYP11A1*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 and 2 (*HSD3B1+2*) and *LHR*.
- In MGC 252 genes were more than 1.5-fold differentially expressed between the two protocols for triggering.
- Top network enriched in this list of gene was 'Cardiovascular Development and function and Cellular movement' involving i.e. semaphorin 3A (*SEMA3A*), angiopoietin 1 (*ANGPT1*) and *FN1*.
- 30% of the genes in MGC influenced by the choice of trigger overlapped with the genes suggested to be involved in the ovulatory cascade (VI).
- *ANGPT1* was significantly lower expressed in MGC after GnRHa trigger as compared to hCG.

rFSH versus hMG for OS

A multi-center study showed a higher rate of morphologically top quality embryos day 3, higher implantation rate and birth rate in hMG (containing both FSH and hMG/LH) treated cycles as compared to rFSH (Platteau *et al*., 2008) (Ziebe *et al*., 2007). The same authors later reported no difference neither in the morphology score at the blastocyst stage nor in clinical outcome now extended to 1-year cumulative birth rate as well (Devroey *et al*., 2012) in line with the conclusion of latest Cochrane review (van Wely *et al*., 2011). However, both studies reported differences between rFSH and hMG in the endocrine profile during and at the end of stimulation also after adjusting for number of follicles (Smitz *et al*., 2007) (Devroey *et al*., 2012). In MGC from the pre-ovulatory follicle, we showed that 85 genes were differentially expressed between the two stimulations. Notably, this list of genes showed high enrichment of 'cellular lipid metabolism' and 'regulation of intracellular signal transduction' represented by central genes known to play an essential role in folliculogenesis, i.e. differences in the expression level of the *LHR* and *LSS*. We suggest that the lower level of LH receptors in hMG is due to a combined effect of ligand (primarily hCG) induced down-regulation and a FSH induced up-regulation by rFSH. Whether the effects observed is due to the presence of hCG/LH in hMG or the difference in FSH profile in the two hormone preparations remain to be determined.

GnRHa versus hCG for ovulation trigger

The GnRHa induces an endogenous flare of FSH and LH which is significantly different in regard to luteal endocrinology when compared to the long-acting LH like activity of a bolus of hCG (Dosouto *et al*., 2017). In addition to differences in half life, the action of LH and hCG on the same receptor results in quantitatively and qualitatively different intracellular signaling (Casarini *et al*., 2012).

The observed fold changes of the differentially expressed genes per se were relatively subtle (from the cut off 1.5 to 3.3), however while submitting the genes to functional enrichment analysis, a highly significant overlap with known functions and very high network scores emerged, indicating a substantial functional divergence induced by the type of ovulation trigger. Reassuring however, in both CC and MGC the up-stream regulator analysis of the observed transcriptome difference suggested LH as a significant regulator.

GnRHa triggering has been reported to induce significantly more MII oocytes than hCG trigger, though this finding is based on results from a limited number of studies (Oktay *et al*., 2010) (Humaidan *et al*., 2005). However, the developmental competence of the MII oocytes appears to be comparable between the two groups since fertilization rate, embryo development in vitro as well as the implantation rate of subsequent transferable embryos are comparable (Erb *et al*., 2010) (Griesinger et al., 2007). Hence, the observed difference in CC transcriptome, including differences in expression level of several central gene in steroidogenesis (*CYP11A1*, *HSD3B1+2*, *LHR*) seem not to be of high importance neither for successful final maturation of the oocyte nor the CC compartment preparing for meeting the spermatozoa in the process of fertilization.

In the MGC compartment, 72 of the 252 genes with significantly more than 1.5-fold difference between the two protocols for triggering, overlapped with the genes suggested to be involved in the ovulatory cascade (VI). Gene function enrichment of this overlap represents the main ovulation related functions as 'movement of cell', 'extracellular matrix', 'wound healing', and 'tissue development' (FDR<8.07E-7). The majority of the overlapping genes showed higher expression in the MGC from the GnRH-agonist triggered as compared

to the hCG triggered. In example, extracellular matrix formation genes as *FN1*, *LAMA3* and vinculin (*VCL*) were higher expressed, coagulation factor *F5* was higher expressed as was the PG transporter, *SLO2A1*. This suggests that GnRHa induces a strong initial ovulatory response.

The genes involved in the 'cell movement' enrichment were mainly reported to be involved in angiogenesis. *ANGPT1* expression was lower in the GnRH-agonist triggered MGC as compared to hCG triggering. Furthermore, an increased expression was found in *SEMA3A* that has been reported to inhibit angiogenesis in tumors potentially by antagonizing the effect of vascular endothelial growth factor A (VEGFA) (Casazza and Mazzone, 2014). Interestingly, recent publications have shown that women in risk of developing OHSS have a higher ANGPT1 content in FF which further confirmed OHSS being an dysregulation of angiogenic factors (Scotti *et al.*, 2016). It can be speculated that the reduced expression of *ANGPT1* is involved in the significant reduced risk of developing OHSS after GnRHa as compared to hCG triggering (Dosouto *et al.*, 2017). Additionally, a reduced expression in Chemokine CXC motif receptor 4 (*CXCR4*), was found in the MGC from the GnRHa triggered women. CXCR4 implicated in angiogenesis among other functions, has been shown to be induced by hCG in MGC in human (VI) mice, cow and horse (Sayasith and Sirois, 2014).

Although fundamental mechanistic questions still remain to be answered, our data indicate that a reduction in angiogenesis may hold a part of the explanation for GnRHa reducing the risk of OHSS development. Whether reduction in angiogenesis additionally is involved in the CLs failing to sustain proper luteal phase after GnRHa triggering, needs further investigations.

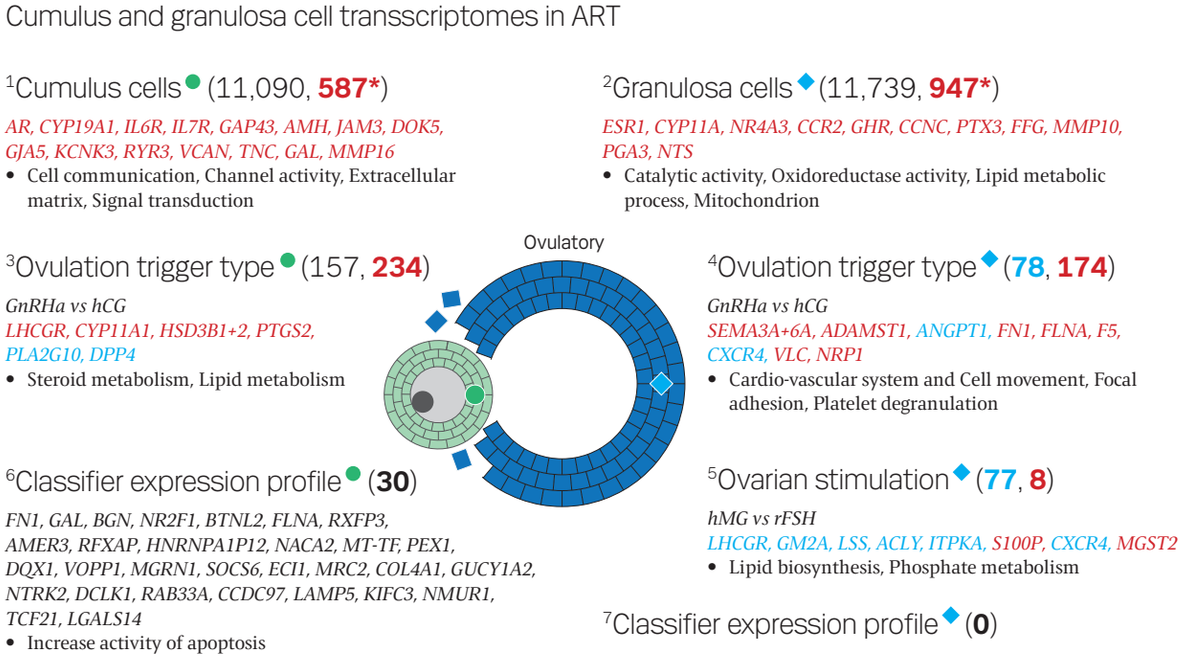


Figure 2. 1-2: Genes differentially expressed between cumulus cells[1] and granulosa cells[2] in the ovulatory follicle. **3-4:** Genes differentially expressed in cumulus[3] and granulosa[4] cells, respectively, between the two types of ovulation trigger used in ART cycles, GnRHa and hCG. **5:** Genes differentially expressed in granulosa cells between ovarian stimulation with hMG and rFSH. **6-7:** 30 genes in the cumulus[6] cell compartment showed promising predictive value for birth of the enclosed oocyte, while no gene signature in the granulosa[7] cells could be found to correlate to competence.

Number of genes expressed in the cell types are marked with **BLACK** print. Number of genes upregulated in the compartment or condition are marked in **RED**, while **BLUE** represent the number of down regulated/lower expressed genes. Selected genes are shown in matching colors and the top function enrichment of the differentially expressed genes are shown with bullet points. *These numbers have been generated based on the list of transcripts published in paper VI. The divergence in number of genes (1,562 (VI) vs 1,534 (587+947)) reflects updated databases on coding and non-coding genes.

Cumulus and Granulosa cell transcriptome, temporal and spatial differences

- Study IV-VIII provide data on significant difference in gene expression between MGC and CC as well as temporal dynamics in gene expression in these somatic compartments during late folliculogenesis. Figure 2[1,2] highlights gene highly expressed in MGC or CC and Figure 1[4] highlights genes in MGC related to the ovulatory cascade.
- The procedures of ART do have a significant impact on the follicle somatic cell transcriptome. Figure 2[3,4,5] highlights genes in MGC affected by the choice of OS regimens and genes in CC and MGC affected by the ovulation trigger type.

4.3 Gene expression classifier for oocyte competence (Study IX)

During the last decade, numerous studies have searched for competence genes relating gene expression in MGC and CC to the developmental competence of the corresponding oocyte and implantation potential of the subsequent embryo. Their approach has primarily been on single genes. Taking the complexity of the pre-ovulatory follicle into account, the aim of identifying single marker genes seems to be less robust than markers based on the scenario of multi interacting genes. Therefore, we focused on differences in the MGC and CC transcriptomes between oocytes from competent and non-competent follicles aiming to develop classifier profiles in the two cellular compartments to select the embryos with the best potential to result in birth of a healthy child. Approaching the scenario for selecting a competent embryo for transfer in a cohort of available embryos, a case control design was chosen matching women's age and embryo morphology.

Major findings (Study IX)

- A signature of only 30 discrete genes expressed in CC was predictive of live birth: *FN1*, *MTTF*, *FLNA*, *COL4A1*, *GAL*, *MGRN1*, *BGN*, *NR2F1*, *UNC93B3*, *CCDC97*, *HNRNPA1P12*, *VOPP1*, *GUCY1A2*, *ECI1*, *SOCS6*, *MRC2*, *DCLK1*, *PEX1*, *NMUR1*, *AMER3*, *RAB33A*, *KIFC3*, *DQX1*, *RXFP3*, *TCF21*, *RFXAP*, *BTNL2*, *LGALS14*, *NTRK2*, *LAMP5*.
- This live birth prediction model had an accuracy of 81%, a sensitivity of 0.83, a specificity of 0.80, a positive predictive value of 0.77, and a negative predictive value of 0.86.
- When applied on 3 external data sets with the end-point outcome measure of blastocyst formation, the signature resulted in 62%, 75% and 88% accuracy, respectively.
- The genes in the classifier are primarily connected to apoptosis and involvement in formation of extracellular matrix.
- In contrast, no robust MGC gene expression classifier signature could classify live birth with accuracy above random chance level.

Genes in the CC classifier

Of the 30 genes in the classifier, 10 (nuclear receptor subfamily 2, group F, member 1 (*NR2F1*), collagen type IV alpha 1 chain (*COL4A1*), galanin and GMAP prepropeptide (*GAL*), doublecortin like kinase 1 (*DCLK1*), mannose receptor C type 2 (*MRC2*), filamin A (*FLNA*), suppressor of cytokine signaling 6 (*SOCS6*), member RAS oncogene family (*RAB33A*), *FN1*, biglycan (*BGN*)), showed an overlap with genes that were differentially expressed between corresponding CC and MGC in the pre-ovulatory follicle (IV). Interestingly, of these genes all, except *COL4A1*, were 2-10-fold higher expressed in CC as compared to MGC indicating a specific function of the gene products in the CC compartment in the ovulatory follicle, while *COL4A1* was 2.8-fold higher expressed in MGC (IV). Notably, FN1, BGN and COL4A1 are all constituents of extracellular matrix and cell to cell adhesion involved in cumulus cell expansion in response to final maturation of the follicle (Oksjoki *et al.*, 1999; Adriaenssens et al., 2009; Thys *et al.*, 2009).

The signature genes showed modest level of expression changes between the competent and non-competent follicles and no overlap with previously suggested marker genes (Assou et al., 2008; Hamel *et al.*, 2010; Assidi *et al.*, 2011; Devjak *et al.*, 2012; Iager *et al.*, 2013) (Burnik Papler *et al.*, 2015). Subjecting the 30 annotated genes in the classifier signature to Pathway analysis, 'increased activity of apoptosis' in the CC surrounding oocytes with competence to develop, implant and succeed in birth of a healthy baby was suggested. Studies using classical apoptotic markers (i.e. BCL2, apoptosis regulator (*BCL2*) and BCL2 associated X, apoptosis regulator (*BAX*) expression) techniques to detect early and late stages of apoptosis, respectively, have shown a correlation between acquired developmental compe-

tence and a certain level of apoptosis (Corn et al., 2005; Yuan *et al.*, 2005; Filali *et al.*, 2009; Janowski *et al.*, 2012). An increase in apoptotic activity in CC connected to mature oocytes in MII as compared to GV stage has been suggested based on transcriptome comparison (Feuerstein *et al.*, 2012). Recent studies (Ebner *et al.*, 2014; Lourenço *et al.*, 2014) show that increasing age of the women correlates to increased degree of apoptosis in CC. We have by the case control design aimed at minimizing a potential age induced bias in the results. The genes in the classifier which significantly enriches 'increase activity of apoptosis' in competent CC as compared to incompetent, are all reported in the literature to protect against apoptosis. Whether the level of apoptosis in CC is related to competence remains to be answered, however, the expression level of central genes in apoptosis regulation, *P53, BAX, BCL2* were not related to competence in our data.

Lack of classifier genes in MGC

Whereas we observed a varying performance of the different cumulus classifiers depending on the choice of algorithm applied for normalization and classification, all tested combinations of MGC classifiers showed very poor performance in discriminating oocytes leading to live birth from oocytes which failed to establish pregnancy. These data indicate that that gene expression in MGC does not reflect competence of the connecting oocyte.

The lack of consistency in published marker genes probably reflect the different measures for competence, varying from oocyte maturational stage and the cleavage stage embryo morphology to positive hCG, ongoing pregnancy and birth as well as difference in isolation technique and detailed timing of sampling from the follicles in a highly complex and transformational phase. Furthermore, the majority of studies did not control for potential confounders as women age, regimens for OS and embryo morphology.

Gene expression classifier for oocyte competence

- The study demonstrated that it is possible to develop a classifier based on 30 genes from transcriptome data from CC that predict the chance of pregnancy and birth in an IVF setting with 81%. This classifier proved its value by prediction of the ability of embryos to reach the blastocyst stage in vitro from independent data sets. Therefore, we believe that this classifier has the potential to be developed into a clinical useful tool if confirmed in a large scaled study. Figure 2[6] highlights the classifier genes.
- Gene expression in MGC failed to reflect competence of the connecting oocyte.

5. METHODOLOGICAL DISCUSSION

Cell populations

The relative strict compartmentalization of the cell types analyzed ensured low risk of contamination. Blood contamination was negligible throughout the studies as no samples expressed the leucocyte marker, protein tyrosine phosphatase, receptor type C (*PTPRC*, also known as *CD45*). Minor contamination of the MGC from the neighbouring theca cell compartment, evaluated by *CYP17* expression, was present in the samples based on sediments of multiple FF (VIII) and in study VI in the samples collected before ovulation, potentially inducing bias in the analysis. Isolation of floating granulosa cells avoiding aggregates with visible blood contamination and vessels structures seems to be the most effective technique to isolate MGC from FF (IV, VII, IX).

Small amount of RNA

Due to low starting quantities of RNA in the small samples following individual follicles and oocytes, Whole Transcriptome Amplification (WTA) was necessary to fulfill the requirement of the Affymetrix platforms used (I-VII, IX). For the newer generations of whole transcriptome arrays (Human Gene 1.0 ST), potential amplification biases were minimized by the use of amplification protocols specialized for minimal RNA samples based on the linear isothermal amplification of double-stranded cDNA initiated at the 3' end as well as randomly throughout the whole transcriptome in the sample, showed to provide a uniform and accurate representation of the transcriptome (Faherty *et al.*, 2015). Working with such low starting material was challenging and 10% of the samples did not succeed in sufficient amount of RNA to proceed to hybridization despite the use of RNA extraction protocols for minute samples and WTA.

Furthermore, amplification differences between

studies applying the 3′-probe bases arrays (Human Genome U133 plus 2.0) and subsequent the whole transcript arrays where the probes are designed to cover the entire length of the gene are most likely. Consequently, direct comparison of data obtained from the two generations of arrays was not performed.

Number of samples and fold change of biological relevance

As the technique were quite new we failed to find previous relevant studies on follicle transcriptome upon which a power calculation could be based. The number of observations in the studies reflects both golden standard for the transcriptome studies as well as access to the biological samples. The minimal number (n=3) of observations were used in study I and VIII. At the time of study VIII (2009) 3 observations in each group in the prospective randomized trial were accepted as data was substantiated by each observation being on pools of cells from 5 patients. In the later studies on individual follicles, numbers of observations were substantially increased. The descriptive study of the oocyte in the primordial follicle was based on 3 pools of 330-620 captured oocytes from a 21-year-old woman (1 pool), and from an 11-year-old girl (2 pools).

The fold change cut-offs were set based on prior publication, using 1.5- and 2.0-fold-change, and considered relevant based on our biological understanding of gene expression and its biological significance in the environment and microenvironment of the follicle. To acknowledge the potential limitation of the *in silico* study design (III) a fold change as high as 10 was chosen.

Biomarker

The 30 gene CC classifier showed a promising performance on external data on blastocyst development. Though, a prospective randomized clinical trial comparing IVF/ICSI treatment outcome after embryo selection with the standard morphokinetic embryo as-sessment with standard morphokinetic embryo assessments plus CC classification is needed to evaluate the clinical potential and true predictive value of the classifier.

Women

This work is based on transcriptome data on oocytes and somatic cells donated by women undergoing ovarian stimulation due to female and/or male infertility. Therefore, the findings may not fully reflect the follicle transcriptome in the population of fertile women. The design of the studies aimed at avoiding selection bias. However, it cannot be excluded that the oocytes from the primordial follicles, isolated from ovarian tissue from women having removed their ovaries before chemotherapy, could be affected by the condition of the primary malignant disease.

6. MAJOR THESIS CONCLUSIONS

- The analysis of and comparison between the unique transcriptomes of the human oocyte in its two hallmarks of development, in the primordial follicle an in metaphase II, identified known and new genes associated with oocyte development and reproduction, genes centred on cell cycle regulation, DNA protection and epigentics.
- Tumor suppressor genes were dominating in the highly enriched ‘cell cycle’ network in the oocytes from the primordial follicle potentially of importance for holding and protecting the oocyte pool. The dominating enriched signaling pathway was ‘PI3K/AKT/mTOR‘ signaling in line with observation in animal studies. Interestingly, androgen and estrogen receptor signaling were enriched and it is intriguing that several of the new and highly expressed genes as *CDR1*, *FOLR1* and *TMEFF2* have been reported to be controlled by steroid hormones in other tissues. Genes encoding P63 and CCNK, both suggested to be involved in genome maintenance, were highly and selectively expressed in the oocyte of the primordial follicle.
- Top functional enrichment in the mRNAs stockpiled in the MII oocytes during oogenesis was also ‘cell cycle’ though now with genes involved in mitosis progression in synergy with the enriched signaling with proliferative functional hallmark genes in ‘G2M Checkpoint’, ‘P53 Pathway’ and ‘MTORC1 signaling‘. In addition to known genes maternal expressed genes as *DCP1A*, *CPEB1*, *BTG4*, *CCNB1 ESPL1*, *PTTG1*, *TUBB8* and *BOD1* were highly enriched in the MII.
- Age does significantly influence the MII transcriptome, a change representing hallmark genes of ‘mitotic spindle’ and ‘G2M checkpoint’ including altered expression of genes central for meiosis and

mitosis and development as *SMAD2, ESPL1*, *EME1* and *RICTOR*. Furthermore, genes involved in calcium oscillation, known to be of utmost importance for oocyte activation, were influenced by age.

- The described changes in MGC transcriptome induced by the ovulation trigger signal supports and extends the anticipation of ovulation having switches being turned on or off in the MGC compartment over a relative short time course. Functions as 'cell cycle', 'cancer', 'inflammation' and 'coagulation' were enriched in the differentially expressed genes. Genes central for mitosis as *CCNB1*, *CCNB2, CCNA2* and *TOP2A* were highly downregulated while genes related to cancer, *CD24, CLDN11*, inflammation, *PTGES, IL6ST, SLCO2A1*, and coagulation (*F3, F2R*), were highly upregulated after ovulation induction.
- MGC and CC transcriptomes in the pre-ovulatory follicle revealed two distinct and highly specialized cell types and differentially expressed gene enriched functions as angiogenesis, steroid hormone receptor activity, inflammatory response and growth factor binding. Genes exclusively present in MGC, represented the enriched functions of mitochondrion and hydrolase activity as *ARSG, ALDH5A1* and *CA5B*. Genes exclusively present in CC, represented extracellular region, cell communication and channel activity, i.e. *TNC, AMH, RYR2, CACNA1C*.
- *AMH* expression and synthesis showed compartmentalisation with higher expression in CC than in MGC in both small antral and in pre-ovulatory follicles. *AMH* expression was inversely correlated to maturity of the COC.
- OS regimens, as type of FSH and ovulation trigger do significantly pose a footprint in the transcriptome of MGC and CC. Genes differentially expressed in MGC between OS with rFSH and hMG included *LHR* as well as other central genes in cholesterol and steroid biosynthesis, *LSS ACLY*.
- The MGC and CC transcriptomes were affected by the protocol for ovulation induction. The top networks enriched in the differentially expressed MGC genes between the two golden stand inducers for ovulation induction, GnRHa and hCG, were 'Lipid metabolism' involving central genes in steroidogenesis, *CYP11A1*, *HSD3B1 +2* and *LHR* as well as 'Cardiovascular Development and function and Cellular movement' involving genes as *SEMA3A*, *ANGPT1* and *FN1*, potentially explaining the difference in CL formation and incidence of OHSS between the two protocol.
- A CC gene expression classifier comprising 30 genes predicting live birth in an IVF setting was robustly defined, while a robust MGC classifier signature that could classify live birth with accuracy above random chance level could not be identified. The CC classifier showed promising augmented predictive performance in an external data set with predictive value ranging 62-88% in accuracy over 3 external datasets; however, the clinical prognostic value needs to be proven in a prospective randomized clinical trial.

Overall, the lists of genes being selectively or highly differentially expressed a) in either of the two developmental hallmarks of the human oocyte b) in the two somatic cell types for the pre-ovulatory follicle and c) in MGC before and after ovulation trigger provide an important source for future downstream analysis to identify genes of significance in human oogenesis and folliculogenesis.

7. PERSPECTIVES AND FUTURE STUDIES

Transcriptome analysis of the different cell compartments of the ovarian follicle provide insight into which genes are engaged in various developmental, physiological as well as pathological stages and how these may be influenced by treatment regimens involved in ART and i.e. chemotherapy. An insight that adds to the understanding of the folliculogenesis and competence achievement and ultimately potentially suggests modes of how to protect the ovarian reserve in general and in women having increased risk of entering menopause in an early age.

In addition, being a source for future down-stream analysis to identify genes of significance in human oogenesis and folliculogenesis in general, the lists of genes presented in this thesis can be used to identify candidate genes in the search for genetic variants involved in cases with compromised fertility. A recent case, a couple referred to our clinic with 'unexplained infertility' had in 4 treatment cycles (3 IVF and 1 IVM)

60 immature oocytes (no resumption of the meiosis) retrieved indicating a general maturation arrest. Gene variants in the exome analysis of the woman showed no overlap with genes listed to 'infertility' or 'maturation arrest' in Ingenuity Variant Analysis databases. When comparing with our lists we found an overlap of 7 genes being highly overexpressed in MII as compared to MGC and the oocyte in the primordial follicle, suggesting a specific function in the oocyte in the pre-ovulatory follicle. Functional characterization of the identified variants in animal model would show if these variants may be involved in the resumption of meiosis.

We will continue our research on the cellular mechanisms involved in the default/programmed aging of the ovarian follicles by investigating if age influences the DNA methylation profile of the different cell types of the follicle: the oocyte, MGC and CC. In collaboration with the group that first published on parallel DNA methylome and transcriptome analysis on single mouse oocytes (Veselovska *et al*., 2015), we aim for comparing the DNA methylome and transcriptome in oocytes between women in their mid-twenties and late thirties. Furthermore, we will compare the 'DNA methylation Age' between leucocytes and MGC and CC, respectively to investigate if the follicle cells age as other cells of the body or show an accelerated age pattern. The 'DNA methylation Age' model refer to 353 CpG sites across tissue found to predict the age with 2-3 years accuracy (Horvath, 2013).

As our latest study (Ernst *et al*., 2017), future studies on transcriptomics and genomics will be on the NGS platform. A recently emerged low cost technique using droplet microfluidics, capturing and processing thousands of individual cells for whole-transcriptome or genomic analysis in a massively parallel manner with minimal reagent, will indeed be a powerful tool for mapping cellular heterogeneity in tissues (Zilionis *et al*., 2016). This technique gives us the opportunity to firstly describe the cellular heterogeneity in granulosa and cumulus cells and secondly investigate if cellular heterogeneity in these cells relates to e.g. the competence of the connecting oocyte, the infertility cause and ovarian reserve.

The genomic era with emerging analytic and therapeutic tools, some with substantial promise and others carrying high degree of uncertainty, will bring breakthroughs to our understanding of reproductive biology, and enhance the toolbox available for the ART laboratory to improve embryo selection and clinical outcomes. In regard to competence marker and in line with practice in many clinics in US and Europe, our clinic will in 2018 introduce pre-implantation genetic testing for aneuploidy (PGT-A) on trophectoderm (TE) biopsy for women/couples with recurrent implantation failure as well as recurrent pregnancy loss. The biopsies contain 5-8 TE cells and a NGS platform is used to evaluate the chromosome numbers. Some controversies exist on the accuracy of the technique as well as how results suggesting mosaicism should be interpreted and handled (Capalbo and Rienzi, 2017). Recent studies have reported on further analysis of the biopsied TE cells, i.e. the quantity of mitochondrial DNA was found to correlate with implantation potential which may provide an additional tool for selection (Ravichandran *et al*., 2017). Given the promising technique for analysis on DNA and RNA in parallel in tiny samples, a study performing the transcriptome analysis alongside the aneuploidy analysis would allow us to search for gene expression patterns in the TE correlated to pregnancy and birth. Genetic screening or testing prior to conception is already approaching the field of ART, as some sperm donor banks offers matching of donors and recipients based on exome analysis to reduce the likelihood for genetic diseases. Future additional genetic testing post conception on TE biopsy for pre-implantation risk reduction will indeed demand further legal and ethical discussion and guidelines for their use.

8.a SUMMARY (in English)

The reproductive potential of a woman depends on the quality of her oocytes. The developmental competence of the human oocyte is achieved through a lengthy development of the ovarian follicle comprising the oocyte and the surrounding somatic cell, the cumulus granulosa (CC) and mural granulosa cells (MGC).

This thesis aims to provide additional insight in human oogenesis and folliculogenesis, by analysis of genes expressed in oocytes as well as in the somatic cell compartments of the follicle during various developmental stages, treatment regimens and women age, and if genes expressed in the somatic cells of the follicle hold an oocyte biomarker potential in assisted reproductive therapy (ART).

By comparing the **unique transcriptomes of the human oocyte in its two hallmarks** of development, in its dormant immature stage in the primordial follicle and in the mature stage (MII), we identified known and new genes associated with oocyte development. In the oocytes from the primordial follicle, tumor suppressor genes were dominating in the highly enriched 'cell cycle' network potentially of importance for holding and protecting the oocyte pool (i.e. *P63* and *CCNK*). In the MII oocytes, top functional enrichment in the mRNAs stockpiled during oogenesis, was also 'cell cycle'; here genes involved in mitosis progression in synergy with the enriched signaling with proliferation hallmark genes. Besides known genes maternally expressed, genes as *DCP1A*, *CPEB1*, *BTG4*, *CCNB1 ESPL1*, *PTTG1*, *TUBB8* and *BOD1* were highly enriched in the MII.

Women's age is the dominant prognostic factor of oocyte quality. We showed that age of the women significantly changed the MII transcriptome, a change representing hallmark genes of 'mitotic spindle' and 'G2M check-point' including altered expression of genes central for meiosis and mitosis and development as *SMAD2*, *ESPL1*, *EME1* and *RICTOR*, potentially being involved in the molecular mechanism increasing the number of aneuploid oocyte by increasing age.

The **ovulation** trigger signal induced significant changes in the MGC transcriptome supporting and extending the anticipation of ovulation having switches being turned on or off over a short time course. Functions as 'cell cycle' (*i.e. CCNB1*, *CCNB2*, *CCNA2 TOP2A*), 'cancer' (*i.e. CD24, CLDN11*), 'inflammation' (*i.e PTGES, IL6ST, SLCO2A1*) and 'coagulation' (*F3*, *F2R*) were enriched in the differentially expressed genes.

Comparison of corresponding **MGC and CC transcriptomes** in the pre-ovulatory follicle revealed two distinct and highly specialized cell types. Differentially expressed genes represented enriched functions as angiogenesis, steroid hormone receptor activity, inflammatory response in MGC, and growth factor binding and cell communication in CC.

We showed that **ART regimens**, as types of follicle stimulating hormone and ovulation trigger do pose a significant footprint on the transcriptome of MGC and CC, potentially providing genes and mechanisms involved in known clinical difference as corpus luteum formation and incidence of the most severe side effect in ART, the ovarian hyper stimulation syndrome.

In the search for a **biomarker in ART**, a 30 gene CC classifier predicting live birth in an IVF setting was robustly defined, while no MGC classifier signature above random chance level was present. The CC classifier showed promising augmented predictive performance in 3 external data sets with predictive value ranging 62-88% in accuracy; however, the clinical prognostic value needs to be proven in prospective randomized clinical trial.

Finally, the lists of genes being selectively or highly differentially expressed in either of the two developmental hallmarks of the human oocyte, in the two somatic cell types for the pre-ovulatory follicle, and in MGC before and after ovulation trigger provide an important **source for candidate genes** which warrants future downstream analysis to identify genes of significance in human oogenesis and folliculogenesis.

8.b SAMMENDRAG (in Danish)

En kvindes chance for opnåelse af graviditet afhænger af kvaliteten af hendes oocytter (ubefrugtede æg). Oocytterne opnår deres kompetence gennem en op til 6 måneders lang udviklingsproces i ovariets (æggestokkens) follikler (ægblærer). Folliklen består af oocytten og de omkringliggende celler, cumulusceller (CC; cellerne, der har en tæt kommunikation med oocytten) og granulosacellerne (murale granulosaceller, MGC; cellerne, der producerer vækstfaktorer og hormoner, bla. østrogen). For at bidrage til forståelsen af follikeludviklingen hos kvinder analyserede vi genekspressionsprofilen i de forskellige celletyper i forskellige udviklingsstadier, hormonbehandlinger og aldersgrupper. Desuden undersøgte vi om genekspressionen i CC og MGC har biomarkørpotentiale i assisteret reproduktions terapi (ART).

Ved at sammenligne genekspressionen i de tidligste **umodne og de fuldt modne oocytter** (i metaphase II, MII oocytter) kunne vi danne lister af gener, hvis aktivitet er speciel og dermed antageligvis vigtigt i det respektive udviklingsstadie. Listerne indeholdt gener, der tidligere har været forbundet med reproduktion, og nye gener i reproduktion og dannelsen af oocytter. I den helt umodne oocyt dominerede 'cellecyklus'-gener repræsenterende gener, der potentielt er involveret i at bevare og beskytte ægget, eksempelvis *P63* og *CCNK*, der udelukkende var aktive i den umodne oocyt. I den modne oocyt er alle de gener udtrykt, der skal sørge for, at oocytten kan blive befrugtet og klare de første dages

udvikling. Ligeledes i dette stadium var gener involveret i 'cellecyklus' dominerende, her var det imidlertid gener, der er involveret i mitose og celledelinger (eks. *DCP1A*, *CPEB1*, *BTG4*, *CCNB1 ESPL1*, *PTTG1*, *TUBB8* og *BOD1*).

Kvindens alder er den mest prognostiske faktor for oocyttens kvalitet. Vi viste, at kvindens alder har signifikant effekt på oocyttens genekspression involverende centrale gener i funktionerne 'mitotic spindle' og 'G2M checkpoint', gener som *SMAD2*, *ESPL1*, *EME1* og *RICTOR*. Disse gener kan være involveret i den molekylære mekanisme bag den stigende forekomst af kromosom antalsfejl med kvinders stigende alder.

I forbindelse med induktion af **ovulation** ændredes genekspressionen i MGC radikalt, hvilket støtter og udbygger antagelsen, at processen indebærer, at funktioner slukkes og andre tændes over kort tid. Differentielt udtrykte gener repræsenterede mitose og celledelinger (eks. *CCNB1*, *CCNB2, CCNA2, TOP2A*) eller var relateret til cancer (eks. *CD24, CLDN11*), inflammation (eks. *PTGES*, *IL6ST*, *SLCO2A1*) og koagulation (eks. *F3, F2R*).

Sammenligning af MGC og CC fra den modne follikel umiddelbart før ægløsningen viste, at der er tale om to distinkte og specialiserede celletyper. Valg af type af hormonstimulation af æggestokkene ved ART såvel som, hvordan ægløsningens induceres, sætter et signifikant aftryk på genekspressionen i både **MGC og CC**. Dette kan være baggrunden for den kendte forskel i dannelsen af det gule legeme og forekomsten af den alvorlige bivirkning ved **ART**, overstimulations-syndrom, mellem de to måder til induktion af ægløsningen. Undersøgelse af genekspressionen i CC og MGC fra follikler, hvor det tilhørende æg blev befrugtet og lagt tilbage i livmoderen, og hvor der enten opnåedes graviditet og fødsel eller ikke opnåedes graviditet, viste, at der i CC, cellerne, der ligger tæt på ægget, var 30 gener, hvis ekspressions-mønster kunne prædiktere fødsel, mens dette ikke var muligt at finde blandt generne udtrykt i MGC. Ved test af de 30 CC **biomarkør-gener** i 3 eksterne datasæt viste disse biomarkører en lovende prædiktiv værdi varierende fra 62-88%. For at kunne bedømme om biomarkøren i CC genekspressionen er klinisk anvendelig i ART, skal der større prospektive randomiserede undersøgelser til.

Samlet set udgør listerne af gener, der er selektivt eller meget forskelligt udtrykt i de umodne eller modne oocytter, i de to celletyper CC og MGC i den modne follikel, og i MGC før og efter induktionen af ægløsningen, vigtige **kilder til kandidat gener** til fremtidige funktionelle studier med henblik på at identificere gener med betydning for menneskets oocyt-og follikeldannelse.

9. GENE SYMBOLS AND NAMES

Human gene symbols and names in this list follow the HUGO Gene nomenclature and in the text, additional species (mouse/rat) specific nomenclature is used. The protein symbols are in all species the same as the human gene symbol, all upper case but not italicized.

If a gene has been known with another gene symbol and/or name, this is marked in ( ) after the gene name.

ACLY	ATP citrate lyase
ACVR2A,B	activin A receptor type 2A and 2B
ADAMST1,9	ADAM metallopeptidase with thrombospondin type 1 motif 1 and 9
ALDH5A1	aldehyde dehydrogenase 5 family-member A1
AMER3	APC membrane recruitment protein 3
AMH	anti-Müllerian hormone
ANGPT1	angiopoietin 1
APC	APC, WNT signaling pathway regulator
AR	androgen receptor
AREG	amphiregulin
ARSG	arylsulfatase G,
BAX	BCL2 associated X
BCL2	apoptosis regulator
BGN	biglycan
*BMP_*	bone morphogenetic protein 1,2,3,4, 6,8B and 15
BMPR1, 2	bone morphogenetic protein receptor types 1A, 1B and 2
BTNL2	butyrophilin like 2
BOD1	biorientation of chromosomes in cell division1
BTG4	anti-proliferation factor 4
BUB1	mitotic checkpoint serine/threonine kinas
CA5B	carbonic anhydrase 5B
CACNA1C	calcium voltage-gated channel subunit alpha1 C
CAMK2G	calcium/calmodulin-dependent protein kinase 2 gamma

CCNA, B	cyclin A2, B1, B2
CCNK	cyclin K
CD24	CD24 protein
CDC7, 20, 25	cell division cycle proteins, CDC7, *CDC20*, *CDC25*
CCDC97	coiled-coil domain containing 97
CDKN1A, C	Cdk inhibitor 1A and C
CDR1	cerebellar degeneration related protein 1
CHEK2	checkpoint kinase 2
CHRNA3	nicotinic, alpha 3
CLDN11	claudin 11
COL4A1	collagen type IV alpha 1 chain
COL5A2	collagen type V alpha 2 chain
CPEB1	cytoplasmic polyadenylation element binding protein 1
CSPP1	centrosome and spindle pole associated protein 1
CTNNB1	catenin beta 1
CXCR4	chemokine CXC motif receptor 4
CYP11A1	cytochrome P450 family 11 subfamily A member 1
CYP19A	cytochrome P450 family 11 subfamily A member 19 (aromatase)
DCC	DCC netrin 1 receptor
DCLK1	doublecortin like kinase 1
DCP1A	decapping mRNA 1A, *CPEB*
DNMT1	DNA methyltransferase 1
DPPA3	developmental pluripotency associated 3 (*STELLA*)
DSCAM	DS cell adhesion molecule
DQX1	DEAQbox RNAdependent ATPase 1
EGF	epidermal growth factor
ECI1	enoyl-CoA delta isomerase 1 (DCI)
EME1	Essential meiotic endonuclease 1
ESR1,2	estrogen receptors 1 and 2
EREG	epiregulin
ESCO1	encoding Establishment of cohesion homolog 1
ESPL1	extra spindle pole bodies like 1 (Separase)
F2R	coagulation factor II thrombin receptor
F3, F5	tissue factor 3 and 5
FBXO32	F-box protein 32
FGG	fibrinogen gamma chain
FIGLA	folliculogenesis specific basic helix-loop-helix
FLNA	filamin A
FLRT2	fibronectin leucine rich transmembrane protein 2
FN1	fibronectin 1
FOLR1	folate receptor 1
GAL	galanin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF9	growth differentiation factor 9
GJA1	Gap junction protein alpha 1 (CX43)
GJA5	Gap junction protein alpha 5 (CX40)
GJA7	Gap junction protein alpha 7 (CX45)
GUCT1A2	guanylate cyclase 1 soluble subunit alpha 2
H19	H19, imprinted maternally expressed transcript
HAS2	hyaluronan synthase 2
HDAC1	histone deacetylase 1
HNRNPA1P12	heterogeneous nuclear ribonucleoprotein A1 pseudogene 12
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSD3B1, 2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 and 2
IGF1	insulin like growth factor 1
IGFBP5	insulin like growth factor binding protein 5
IL6ST	interleukin 6 signal transducer
ITPR1	inositol 1,4,5-triphosphate receptor, type 1
KIFC3	kinesin family member C3
KITLG	KIT ligand
LAMA2, 3	aminin subunit alpha 2 and 3
LAMB1	laminin subunit beta 1
LAMP3	lysosomal associated membrane protein family member 5
LGALS14	galectin 14
LHR	LH/hCG receptor
LIN28A, B	lin-28 homolog A and B
LSS	lanosterol synthase
MAD2L	mitotic arrest deficient 2 like 1
MAPK1, 8	mitogen-activated protein kinase 1, 8
MBD1	methylCpG binding domain protein 1
MGRN1	mahogunin ring finger 1
MMP10, 16	matrix metallopeptidases *10 and 16*
MOS	MOS proto-oncogene, serine/threo-

	nine kinase
MRC2	mannose receptor C type 2
MTHFR	methylenetetrahydrofolate reductase
MTORC1	mammalian target of rapamycin complex 1
MTRR	5-methyltetrahy-drofolate-homocysteine methyltransferase reductase
MTTF	mitochondrial transcription termination factor
NCAM1	neural cell adhesion molecule 1
NMUR1	neuromedin U receptor 1
NLRP5	NLR family pyrin domain containing 5 (*MATER*)
NR2F1	nuclear receptor subfamily 2 group member 1
NTNG2	netrin G2
NTRK2	neurotroph receptor tyrosine kinase 2
NTS	neurotensin
NTSR1,2	neurotensin receptor 1 and 2
PCNA	proliferating cell nuclear antigen
PEX1	peroxisomal biogenesis factor 1
PI3K	phosphatidylinositol 3 kinase
PTEN	phosphatase and tensin homolog
PTGES	prostaglandin E synthase
PTPRC	protein tyrosine phosphatase, receptor type C (CD45)
PTTG1	pituitary tumor-transforming 1 (securin)
PTX3	pentraxin 3 and anti-Müllerian hormone
RAB33A	member RAS oncogene family
RELN	reelin
RFXAP	regulatory factor X associated protein
RICTOR RPTOR	independent companion of MTOR complex 2
RYR2	ryanodine receptor 2
RXFP3	relaxin/insulin like family peptide receptor 3
S100P	S100-calcium-binding-protein-P
SEMA3A	semaphorin 3A
SKIL	SKI like proto-oncogene
SLCO2A1	carrier organic anion transporter family member
SMAD2, 4	SMAD family member 2 and 4
SOCS6	suppressor of cytokine signaling 6
STAR	steroidogenic acute regulatory protein
TCF21	transcription factor 21
TGFB1	transforming growth factor beta 1
TMEFF2	EGF like and two follistatin like domains 2
TNC	tenascin C
TNFA	tumor necrosis factor a
TOP2A	topoisomerase II alpha
TP63	tumor protein p63
TUBB8	tubulin beta 8 class VIII
UHRF2	ubiquitin like with PHD and ring finger domains 2
UNC93B3	unc-93 homolog B3
VEGFA	vascular endothelial growth factor A
VCL	vinculin
VOPP1	vesicular, overexpressed in cancer, prosurvival protein 1
ZAR1	zygote arrest 1

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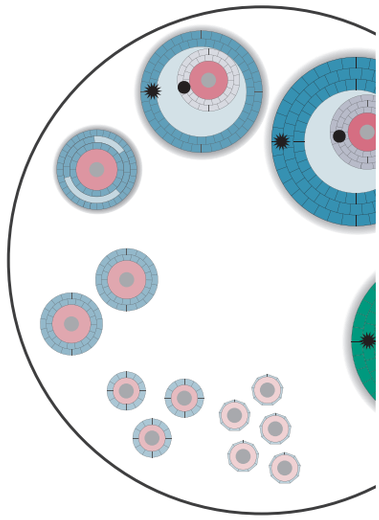

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